18th Meeting of the Society for Natural Immunity (NK2019)

30th Sept. to 03rd Oct.
2019 Luxembourg

ABSTRACT BOOK

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Society for Natural Immunity

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18th Meeting of the Society for Natural Immunity

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Programme at a glance

Monday, 30.09.2019

14:00 - 19:00	Registration open
17:00 - 17:30	Welcome
17:30 - 18:30	Keynote Lecture
19:00	Welcome Cocktail

Tuesday, 01.10.2019

08:30 - 10:00	**SESSION 1** – NK Cell Receptors and Education
10.00 - 10.30	Coffee Break
10:30 - 12:00	**SESSION 2** – NK Cell Development and Differentiation
12:00 - 13:30	Lunch
13:30 - 15:00	**SESSION 3** – Innate Lymphoid Cells
15:00 - 15:30	Coffee Break
15:30 - 17:00	**SESSION 4** – NK cells and Metabolism
17:00 - 19:00	Free Time
19:00	Poster Session 1

Wednesday, 02.10.2019

08:30 - 10:00	**SESSION 5** – NK cells and Anti-Tumour Immunity
10.00 - 10.30	Coffee Break
10:30 - 12:00	**SESSION 6** – NK cell Memory
12:00 - 13:30	Lunch
13:30 - 15:00	**SESSION 7** – NK cell Immunotherapy and Immunomodulation
15:00 - 17:00	Poster Session 2
17:00 - 19:30	Social Event
19:30	Gala Dinner

Thursday, 03.10.2019

08:30 - 10:00	**SESSION 8** – Tissue-associated NK cell Subsets
10.00 - 10.30	Coffee Break
10:30 - 12:00	**SESSION 9** – NK Cells and infection
12:00 - 13:30	Lunch
13:30 - 15:00	**SESSION 10** – NK cells in clinics
15:00 - 15:30	Concluding remarks and invitation to the next NK meeting

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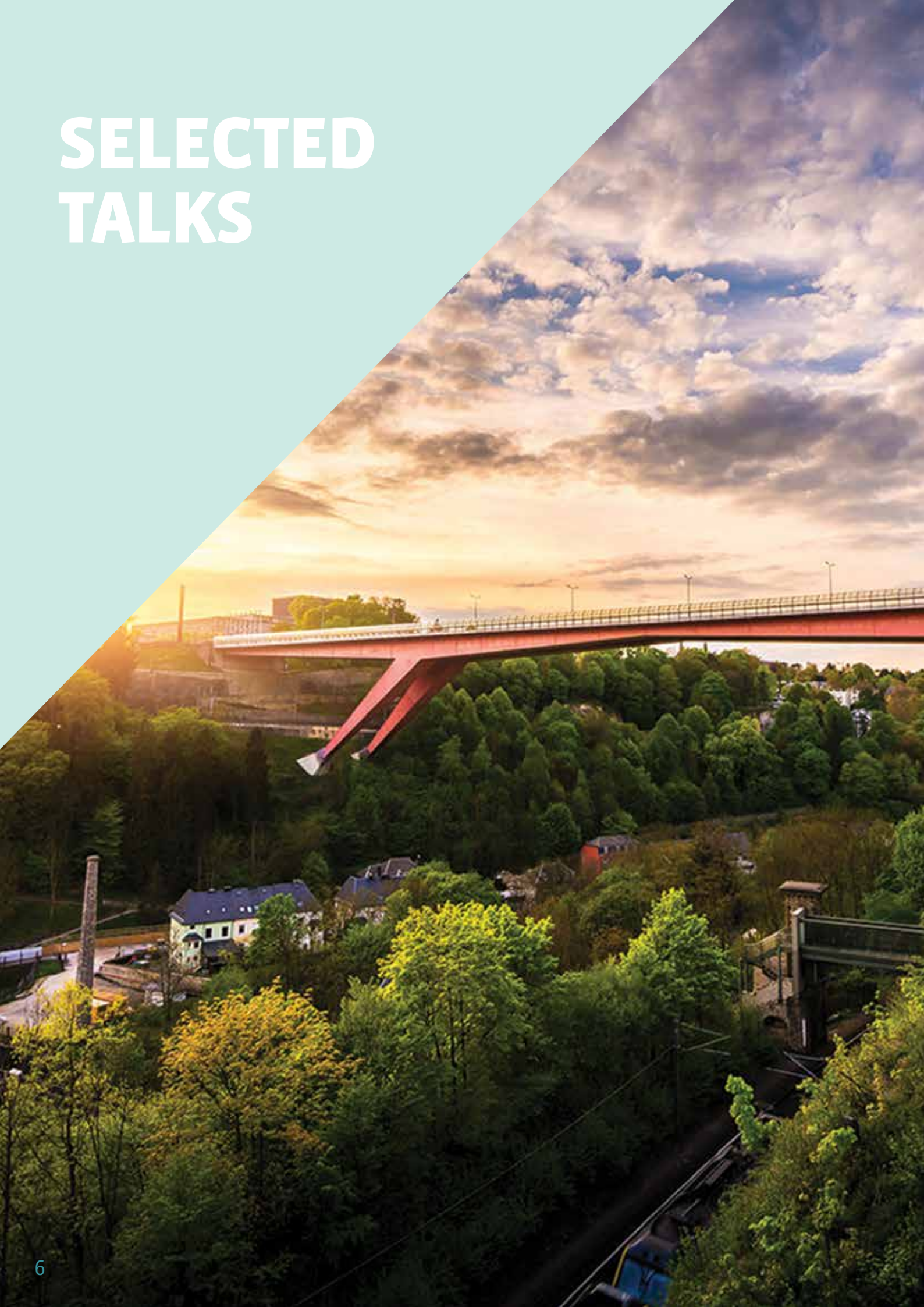

ACTIVATION RECEPTOR-DEPENDENT IFNγ PRODUCTION BY NK CELLS IS CONTROLLED BY TRANSCRIPTION, TRANSLATION AND THE PROTEASOME

PIERSMA Sytse [1], PAK-WITTEL Melissa [1], LIN Andrea [1], PLOUGASTEL-DOUGLAS Beatrice [1], WAYNE Yokoyama [1]

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Natural killer (NK) cells can recognize target cells such as virus infected and tumor cells through integration of activation and inhibitory receptors. Recognition by NK cells can lead to direct lysis of the target cell and production of the signature cytokine IFNγ. Yet it is unclear whether stimulation through activation receptors alone is sufficient for IFNγ production. For example, NK cells produce IFNγ in response to stimulation with plate-bound anti-activation receptors antibodies, which has been reported to trigger via Fc receptors as well. However, stimulation with soluble antibody alone does not result in IFNγ production. These observations suggest that soluble antibody does not provide a strong enough signal or additional signals are required for IFNγ production. Here, we describe that NK activation receptor-engagement requires additional signals for optimal IFNγ production, which could be provided by IFNγ or IL- 12. Stimulation of NK cells with soluble antibodies directed against NK1.1, Ly49H, Ly49D and NKp46 in combination with the aforementioned cytokines resulted in IFNγ production similar to stimulation with plate-bound antibody, indicating that a range of activation receptors require additional signals for IFNγ production. The requirement for multiple signals extended to stimulation with primary m157Tg target cells, which triggers the activation receptor Ly49H, suggesting that NK cells do require multiple signals for IFNγ production even in the context of target cell recognition. Pretreating NK cells with cytokines allowed IFNγ protein production upon subsequent stimulation with m157Tg target cells, indicating that NK cells do not need to encounter both signals simultaneously. Using qPCR and RNA flow cytometry for Ifng mRNA, we found that cytokines, not activating ligands, act on NK cells to express Ifng transcripts. Ly49H engagement was required for IFNγ translation. Results using inhibitors suggest that the Proteasome-Ubiquitin-IKK-TPL2-MNK1 axis was required during activation receptor engagement. Thus, this study indicates that activation receptor-dependent IFNγ production is regulated at the transcriptional and translational levels and by the proteasome.

EPIGENETIC DYSREGULATION OF EBV(+) EXTRANODAL NK/T CELL LYMPHOMA DEFINES DISTINCT SUBSETS OF DISEASE

MUNDY-BOSSE Bethany [1], WEIGEL Christoph [1], WU Yue Zhong [1], YOUSSEF Youssef [1], BECEIRO CASAS Susana [1], POLLEY Nicholas [1], ERNST Gabrielle [1], YOUNG Karen [1], MCCONNELL Kathleen [1], NALIN Ansel [1], WU Kevin [2], YU Shan-Chi , SALEEM Atif [3], SUAREZ Carlos [3], MOLINA-KIRSCH Hernan [4], LOUGHRAN Thomas [5], WEINSTOCK David [6], PLASS Christoph [7], NATKUNAM Yaso [3], CALIGIURI Michael [8], MISHRA Anjali [9], PORCU Pierluigi [9], BAIOCCHI Robert [1], BRAMMER Jonathan [1], FREUD Aharon [10], OAKES Christopher [1]

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Extranodal natural killer/T cell lymphoma (ENKTL) is an aggressive, rare disease, and one of only a few malignancies known to be of natural killer (NK) cell origin. This malignancy is universally Epstein-Barr virus (EBV)-positive and most frequently affects those of Eastern and Central/South American heritage. The NK cell of origin that becomes infected with EBV and specifically how these malignant cells relate to the distinct stages that define normal NK cell development has not been fully described. Our group previously established a model of human NK cell development where early NK cell precursors progress through sequential stages of maturation within secondary lymphoid tissues (SLT) and become terminally differentiated in the peripheral blood (PB). In this study we characterized malignant ENKTL cells within this NK cell developmental framework.

Development and specification of immune cells are controlled by epigenetic processes. As EBV is known to impact DNA methylation patterns, we first determined the normal methylation profiles of NK cell developmental intermediates (NKDIs), and then compared them to ENKTL tumors (n=31 ENKTL) using Illumina MethylationEPIC arrays. We identified two molecular ENKTL subtypes corresponding to intermediate “Stage 4-like” and late “Stage 5-like” NKDIs. Flow cytometric analysis of fresh ENKTL samples (n=6) supported that ENKTLs were developmentally arrested, with all the malignant NK cell populations expressed CD56 and CD94 but lacked more mature markers such as KIRs and CD57, while 4/6 lacked NKp80, similar to a SLT-associated Stage 4A NK cell. Investigation beyond normal developmental epigenetic signatures, we uncovered an unprecedented degree of epigenetic dysregulation across all ENKTL samples. Specifically, greater than 50% of CpG islands were hypermethylated in the majority of ENKTL cases, representing the most hypermethylated tumor type currently known relative to available pan-cancer data (TCGA). RNA-sequencing revealed downregulation or complete silencing of 68% of genes with hypermethylated promoters in ENKTL.

To investigate pre-clinical therapeutic approaches directed at the epigenetic developmental blockade, we created a novel ENKTL patient-derived xenograph (PDX) model. Targeting of the hypermethylation defect with the demethylating agent 5-azacytidine (5-aza) in ENKTL PDX mice caused profound cytoreduction ($P<0.001$), phenotypic and molecular differentiation of the ENKTL cells, and prolongation of host survival ($P<0.02$). In addition, 5-aza caused robust upregulation of immunogenic cancer-testis antigens and tumor-suppressor genes, including CDKN1A. Together, these studies suggest DNA methylation may play a key role in driving ENKTL pathogenesis, and targeting this extreme hypermethylation may lead to novel therapeutic approaches for the treatment of ENKTL.

TARGETING CIS IN NK CELLES IMPROVES THEIR ANTI-TUMOR FUNCTIONS WITHOUT IMPAIRING HOMEOSTASIS

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Cytokine inducible SH2-containing protein (CIS) is a negative regulator of T and NK cell signalling pathways [1,2]. Natural killer (NK) cell activation is controlled by a balance of activating and inhibitory signals and cytokines. Encoded by Cish gene, CIS was previously identified as a negative regulator of IL-15 signalling in NK cells under inflammatory conditions. In conditional Cish deficient mice (Cish fl/fl Ncr1iCre), homeostatic numbers of NK cells were maintained without altering maturation or cycling. Despite this, Cish fl/fl Ncr1 iCre NK cells displayed lower activation thresholds and mice were more resistant to tumour metastasis. However CIS-ablation (in all tissues) promoted terminal differentiation of NK cells and increased turnover but did not manifest in NK cell accumulation. We conclude that increased anti-tumour function observed in Cish-deficient mice is not caused by alterations in cell numbers, but is intrinsic to NK cells. This effect is due to the rewiring of several activation pathways and lowering of their activation threshold.

[1] Palmer, D.C.*, Guittard G.C*., Z. Franco, J.G. Crompton, R.L. Eil, S.J. Patel, Y. Ji, N. Van Panhuys, C.A. Klebanoff, M. Sukumar, D. Clever, A. Chichura, R. Roychoudhuri, R. Varma, E. Wang, L. Gattinoni, F.M. Marincola, L. Balagopalan, L.E. Samelson, and N.P. Restifo. 2015. Cish actively silences TCR signaling in CD8+ T cells to maintain tumor tolerance. J Exp Med 212:2095-2113.

[2] Delconte, R.B., T.B. Kolesnik, L.F. Dagley, J. Rautela, W. Shi, E.M. Putz, K. Stannard, J.G. Zhang, C. Teh, M. Firth, T. Ushiki, C.E. Andoniou, M.A. Degli-Esposti, P.P. Sharp, C.E. Sanvitale, G. Infusini, N.P. Liau, E.M. Linossi, C.J. Burns, S. Carotta, D.H. Gray, C. Seillet, D.S. Hutchinson, G.T. Belz, A.I. Webb, W.S. Alexander, S.S. Li, A.N. Bullock, J.J. Babon, M.J. Smyth, S.E. Nicholson, and N.D. Huntington. 2016. CIS is a potent checkpoint in NK cellmediated tumor immunity. Nat Immunol 17:816-824.

A TRI-SPECIFIC KILLER ENGAGER (TRIKE) AGAINST MESOTHELIN TARGETS NK CELLS TOWARDS LUNG CANCER

KENNEDY Philippa [1], HALLSTROM Caroline [1], KODAL Behiye [1], ETTESTAD Brianna [1], TODHUNTER Deborah [1], HINDERLIE Peter [1], BENDZICK Laura [1], VALLERA Daniel [1], MILLER Jeffrey [1], FELICES Martin [1]

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NK cells are important effectors in the treatment of hematological malignancies, but have so far been less effective in the treatment of solid tumors. Lung cancer cells are generally refractory to NK cell killing, but we wanted to determine whether a small molecule that redirects lysis of NK cells against the common tumor antigen, mesothelin, could enhance NK cell killing in the lung cancer setting. Mesothelin is a surface protein that is overexpressed in a number of cancers, including the aggressive cancer of the lung lining: mesothelioma.

A comparison of peripheral blood NK cells from healthy donors and newly-diagnosed cancer patients revealed that lung cancer patients maintained expression of CD16 (the Fc receptor) at the cell surface with no differences in the major subsets of NK cells. We therefore designed a tri-specific killer engager (TriKE) consisting of a single domain antibody (sdAb) against CD16 and a single chain variable fragment (scFv) against mesothelin. The sdAb and scFv were linked together by recombinant IL-15. When tested on peripheral blood NK cells from healthy donors, this drug was capable of enhancing NK cell proliferation in vitro. In addition, when peripheral blood NK cells were cultured with 9 different lung cancer lines, the TriKE increased degranulation (>60% in some cases) and IFNγ production (>30% in some cases), specifically against the cancer cells.

NK cells from the peripheral blood of lung cancer patients also proliferated in response to the drug alone. Moreover, when treated with the TriKE, patients' cells increased degranulation (>60%) and IFNγ production (>40%, which was significantly more than healthy donor responses) in response to lung cancer cells.

Checkpoint blocking antibodies are the current standard of care for lung cancer patients and our further investigations are focusing on the combination of this mesothelin-targeted TriKE with checkpoint blockade, both in vitro and in vivo.

HIGH-FAT DIET CAUSES RAPID MICROBIOTA-DEPENDENT INTESTINAL ILC3 LOSS AND IMPAIRS TOLERANCE AND IMMUNITY

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Innate lymphoid cells (ILCs) are increasingly appreciated to play a critical role in tissue homeostasis, immunity, and tolerance. At steady state, intestinal ILC3 are the main source of IL-22, ensuring epithelial barrier integrity and defense against pathogenic bacteria. In addition, ILC3 support intestinal tolerance to commensal bacteria and dietary antigens. ILC3 can also sense changes in dietary nutrients, such as vitamin A and aryl hydrocarbon receptor ligands (AHR-L) that affect their development, function and ultimately intestinal health. ILC3 have been described to be able to acquire high amounts of extracellular fat but the effect of dietary fat on ILC3 homeostasis is unknown. Here we show that ILC3 are severely depleted from the small and large intestine of obese mice fed high-fat diet (HFD) but not in leptin or leptin receptor-deficient obese. Notably, consumption of HFD for only 24 hours is sufficient to trigger ILC3 cell death. Total loss of ILC3 is reached after one week of HFD without significant weight gain. Strikingly, we found that this short-term consumption of HFD increases host susceptibility to Citrobacter rodentium infection and impairs tolerance to dietary antigens. Unexpectedly, in germ-free (GF) mice fed HFD, we found that ILC3 were maintained. However, ILC3 were depleted when HFD-fed GF mice were inoculated with either living, heat-killed bacteria or with lipopolysaccharides. Furthermore, HFD-fed TLR4 deficient mice are protected from intestinal ILC3 loss. Gene expression profiling of ILC3 from short term HFD-fed mice revealed the activation of multiple TNFα target genes involved in cell activation, survival, and exhaustion. TNFα blockade is sufficient to prevent HFD-induced ILC3 loss. Collectively, our findings show that short term consumption of HFD, rather than obesity potentiates rapid loss of ILC3 in presence of microbiota that leads to early impairment of gut integrity, tolerance, and protection against pathogens.

GLUTATHIONE MODULATES NATURAL KILLER CELL FUNCTION

GUERRA Luana [1], GRUSDAT-POZDEEV Melanie [1], BONETTI Lynn [1], KURNIAWAN Henry [1], FRANCHINA Davide G. [1], SORIANO BAGUET Leticia [1], DOSTERT Catherine [1], BINSFELD Carole [1], FARINELLE Sophie [1], BRENNER Dirk [1]

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Natural killer (NK) cells are cytotoxic cells which belong to the innate immune system and recognize cells in “distress”, e.g. infected or malignant cells. NK cells can directly induce cell death of their target cells through the production of lytic effector molecules. Furthermore, due to their ability to produce cytokines, NK cells can also play an important role as modulators of the immune response, interacting not only with the innate branch, but also with the adaptive immune system. Thus, a tightly regulated function of these effector cells is crucial for immune homeostasis to prevent autoimmunity and inflammation. One of the molecules that impacts NK cell function in vitro is glutathione, a key intracellular antioxidant. NK cells from human immunodeficiency virus (HIV)-positive patients contained lower GSH concentrations in comparison to healthy controls. However, GSH’s role in vivo remained elusive. By a genetic approach we ablated GSH-synthesis in NK cells. Our data indicate that, although the absence of glutathione does not impact NK cell development in the bone marrow, it seems to modulate the maturation of these cells in the periphery. Thus, we show an implication of this antioxidant pathway in the outcome of viral infections and tumor growth, suggesting that GSH regulates not only NK cell activity but also the interaction with other immune cells. Our ongoing studies are aimed on the dissection of the role of glutathione in NK cells at a mechanistic level potentially translate these findings for future clinical applications, such as in the context of cancer immunotherapy.

DISTINCT METABOLIC PROFILES IN DIFFERENTIALLY EDUCATED NK CELL POPULATIONS

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Natural killer (NK) cells are an important part of the innate immune system involved in the control of intracellular pathogens. These cells are heterogeneous in their expression of inhibitory surface receptors involved in their functional education. Signalling through these receptors promotes increased cytotoxic potential in NK cells leading to functionally discrete educated and uneducated populations. The mechanisms involved in this process are still not fully understood but recent work showing that metabolism and calcium signalling are important in NK cell function indicate a possible link to NK cell education. The aim of this project was to investigate whether the education state of human NK cells was linked to their metabolic profile and calcium signalling.

Uptake of the metabolic substrates 2-NBDG, a fluorescent glucose analogue, and BODIPY FL C16, a fluorescent palmitate, were measured using flow cytometry. Both BODIPY and 2-NBDG had increased uptake in educated compared to uneducated NK cells. Comparison of NK cells educated via KIR or NKG2A showed that NKG2A educated NK cells were the main contributor to this difference. Metabolic blockade of oxidative phosphorylation significantly decreased the degranulation of NK cells with a greater decrease seen in KIR educated compared to NKG2A educated NK cells while only small differences were observed following blockade of glycolysis. Increased calcium flux was seen in educated NK cells compared to uneducated but it is yet unknown whether this difference is directly linked to metabolic changes.

These results indicate that metabolism plays a role in the functional differences seen between educated and uneducated NK cells and suggest that NKG2A-educated NK cells remain more functionally competent than KIReducated when oxidative phosphorylation is restricted. Understanding metabolic programming in NK cell education may unveil future targets to manipulate NK cell function for use in a clinical setting.

KLRG1 AS A NOVEL CHECKPOINT INHIBITOR TARGET

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ABSTRACT BODY:
Immunotherapy drugs targeting immune cell inhibitory receptors on T cells have been successfully developed. In contrast, significantly less work has been done on targeting natural killer (NK) cells. NK cells are intriguing targets for immunotherapy for a number of different reasons, including their cytotoxic abilities and relatively lower autoimmunity risks. Although great strides have been made in immunotherapy research, many forms of cancer escape current immunotherapy drugs. Thus, novel therapeutic targets need to be identified for the development of new treatments. We examined a potential target, Killer Cell Lectin Like Receptor G1 (KLRG1), a well conserved C-type lectin inhibitory receptor expressed on NK cells and T cells, which contains an Immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain. Upon binding to its ligands, N-cadherin and E-cadherin, Src homology region 2 domain-containing phosphatase-2 (SHP-2) and Src homology 2 (SH2) domain containing inositol polyphosphate 5-phosphatase 1 (SHIP-1) are recruited to the ITIM in the cytoplasmic tail. Engagement of KLRG1 inhibits IFN-gamma and TNF-alpha production and high KLRG1 expression has been correlated with low proliferative capacity. To determine the role of KLRG1 as a potential NK cell checkpoint, we developed a series of mice deficient for KLRG1, including mice conditionally deficient for KLRG1 in the NK cell lineage. Using several NK sensitive cancer models, our data suggest that KLRG1 is a NK cell checkpoint target candidate. Funding Sources: This work is supported by National Institutes of Health Research Grants AI46709 and AI122217 and a T32 Respiratory Research Training Program HL134625.

RADIOTHERAPY INDUCES RESISTANCE TO NK CELL CYTOTOXICITY

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Natural Killer (NK) cells contribute to immune surveillance against cancer, but the impact of commonly used treatments on their activity is poorly understood. Though radiotherapy, one of the most broadly used treatments for solid tumours, is known to influence many aspects of anti-tumour immunity, little is still known about its effect on the interaction between NK cells and cancer cells. To investigate this, we compared the response of peripheral blood human NK cells against melanoma (Colo829 and A375) and prostate cancer (DU145) cell lines treated with different doses of x-ray irradiation. Across these cell lines, we found that treatment with three doses of 8Gy on consecutive days reduced NK cell cytotoxicity dramatically by 74 ± 22%. Likewise, the killing of cancer cells by NK cells after exposure to a single high dose of radiation (8Gy-24Gy) was significantly reduced 72 hours post-treatment (69 ± 25%). Resistance to NK cell-mediated cytotoxicity was maintained for up to two weeks after irradiation while cell cycle was arrested, but susceptibility was restored after cell division. Surprisingly, NK cell conjugation, degranulation, and detachment were unaffected by irradiation of target cells as determined by flow cytometry-based assays. Live cell imaging of NK cell-cancer cell interactions in fabricated microwells further established that cancer cell irradiation had no effect on the timing of conjugate formation, detachment, or killing, but did cause a 67 ± 11% reduction in the probability of a conjugate resulting in a kill. Importantly, irradiated cancer cells were found to require a 2-4-fold greater concentration of purified perforin protein in order to induce lysis compared to untreated cells. Thus, these findings demonstrate that radiotherapy induces a profound reduction in the susceptibility of cancer cells to NK cell cytotoxicity, likely caused by an induced resistance to perforin.

THEMIS2 REGULATES INNATE AND ADAPTIVE LYMPHOCYTE RESPONSES DURING VIRAL INFECTION

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Both innate and adaptive immunity are required for optimal protection against viral pathogens. Natural Killer (NK) cells and CD8+ T cells are cytotoxic innate and adaptive lymphocytes, respectively, that share many transcriptional programs and adaptive features, including antigen specificity, clonal expansion, and immune memory. Recently, our lab has shown that memory NK cells in the context of mouse cytomegalovirus (MCMV) infection display a number of unique transcriptional and epigenetic profiles compared to naïve NK cells. Furthermore, we found similar transcriptional and epigenetic trajectories in MCMV memory CD8+ T cells, revealing a common 'memory' signature between cytotoxic lymphocytes. Within this signature, we identified Themis2, a member of the newly described Themis family of proteins that is involved in B cell positive selection, as a potential shared regulator. In both cell types, the expression of Themis2 peaked at day 7 post infection and was sustained throughout differentiation into memory cells. In NK cells, type I interferon signals poised the Themis2 locus for transcription by increasing the permissive histone modification H3K4me3 at the promoter region and induced its expression. Interestingly, deficiency in Themis2 affected antiviral NK and CD8+ T cells responses differently. During MCMV infection, NK cells lacking Themis2 were defective in clonal expansion and memory formation, whereas Themis2-deficient CD8+ T cells expanded normally but generated a larger central memory pool across lymphoid and non-lymphoid tissues. These data suggest that despite being upregulated by both NK and CD8+ T cells during viral infection, Themis2 confers differential functions in both innate and adaptive lymphocytes.

ALTERED-SELF MHC I MOLECULES IN MCMV EVASION OF NK CELLS AND EVOLUTION OF VIRUS-SPECIFIC LY49 RECEPTORS

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Cytomegaloviruses (CMVs) efficiently downregulate MHC I molecules from the surface of infected cells to avoid recognition and killing by cytotoxic T cells. However, the lack of MHC I on the cell surface renders the infected cell susceptible to NK cell killing upon missing-self recognition. To counter this, mouse CMV (MCMV) rescues some MHC I molecules in order to engage inhibitory Ly49 receptors. We have identified a new viral protein, MATp1, a product of the most abundant viral transcript (MAT), that collaborates with another MCMV-encoded protein m04 to rescue certain MHC I molecules and bring them to the cell surface. These rescued, altered-self MHC I molecules show increased affinity to inhibitory Ly49 receptors resulting in inhibition of NK cells despite substantially reduced MHC I surface levels. This enables the virus to evade recognition by licensed NK cells that are sensitive to fluctuations in surface levels of MHC I molecules. During evolution, this novel viral immune evasion mechanism has probably prompted the development of activating NK cell receptors that are specific for MATp1and m04 modified altered-self MHC I molecules. In fact, we show that this complex is specifically recognized by activating Ly49D2, L and P receptors. This work solves a long-standing conundrum of how MCMV avoids recognition by licensed NK cells, demonstrates how inhibitory NK cell receptors can be fooled not just by virus encoded MHC I like molecules but also by complexes of viral and self-molecules and proposes how this evasion mechanism could have forced the evolution of virus-specific activating MHC I-restricted Ly49 receptors.

EFFICIENT SCALE-UP AND PRE-CLINICAL EVALUATION OF NKG2C+ ADAPTIVE NK CELL EXPANSION FOR THERAPY AGAINST HIGH-RISK AML/MDS

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Natural killer (NK) cells hold great promise as a source for allogeneic cell therapy against haematological malignancies, including acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS). NK cell recognition of allogeneic tumours is strictly regulated by inhibitory killer cell immunoglobulin-like receptors (KIR) that bind to groups of HLA class I alleles. However, KIR expression on NK cells is highly diverse due to genetic variation and stochastic expression in individual cells. Hence, the number of efficacious allogeneic NK cells within a product isolated and expanded from random donors can be highly variable and even negligible. Our group has defined a repertoire of NK cells that is uniquely and specifically found in individuals with prior exposure to cytomegalovirus (CMV). We have previously described a 14-day protocol to enrich for adaptive NKG2C+CD57+ NK cells from CMV sero-positive donors with a homogenous expression of one single self-HLA specific KIR (self KIR). Here, we present new data on the GMP-transfer and clinical scale-up of this protocol, providing a route to off-the-shelf adaptive NK cell therapy for refractory high-risk AML/MDS. By screening >200 healthy donors, we established the prerequisites for robust expansion of adaptive NK cells from peripheral blood of CMV+ donors and found that donors with >20% pre-existing adaptive NK cells showed efficient expansion of adaptive NK cells. Apheresis products from a pool of pre-screened third-party donors are being collected for GMP freezing and use in an off-the-shelf setting intended for HLA mismatched patients to maximize alloreactivity by “missing” self. The GMP compatible protocol led to a robust expansion of clinical doses of self-KIR+ adaptive NK cells, with an average frequency of >60% self-KIR+ cells in the end product. Notably, the expanded adaptive NK cells were negative for the HLA-E binding inhibitory receptor NKG2A, which is a major checkpoint for T- and NK-cell based therapies. Flow cytometry-based assays revealed high functionality of the expanded cells which correlated with highly efficient killing of mismatched PHA blasts, tumour cell lines and MDS blasts. These pre-clinical data demonstrate the feasibility of off-the-shelf therapy with a nonengineered and yet highly specific NK cell population, representing the first route to clinical testing of missing selfrecognition.

A DNA VACCINE TARGETING KIR2DS2 ACTIVATES NK CELLS AND INHIBITS TUMOUR GROWTH

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BACKGROUND
Natural killer cells are an attractive opportunity for cancer immunotherapy. We recently discovered that the NK cell receptor KIR2DS2 is a peptide selective receptor that recognises highly conserved viral peptides in the context of MHC class I. Our aim is to use this finding to develop a vaccine that targets NK cells for immunotherapeutic benefit.

RESULTS
A DNA construct containing HLA-C*0102 and the viral peptide IVDLMCHATF was used to immunize the KIRtransgenic mouse, which expresses a complete human KIR "B" haplotype. Mice were given two immunizations of DNA, without adjuvant, one week apart with the vaccine or a control vaccine in which the IVDLMCHATF peptide was mutated to IVDLMCHAAA (control) to abrogate binding to HLA-C. Immunized mice expressed significantly greater levels of KLRG1 on both hepatic ($p<0.05$) and splenic ($p<0.001$) NK cells as compared to control vaccinated mice. KLRG1 was preferentially expressed on KIR2DS2+ vs KIR2DS2- NK cells in vaccinated, but not control mice ($p<0.05$), and especially on KIR2DS2+CD11b+CD27+ vs KIR2DS2-CD11b+CD27+ splenic NK cells ($p<0.001$). Consistent with this activation KIR2DS2+, but not KIR2DS2-, NK cells from vaccinated mice expressed greater levels of CD107a when cultured with 721.221 cells expressing HLA-C*0102+ IVDLMCHATF as compared to the 721.221:HLA-C*0102 control.
In vivo, DNA vaccination attenuated the growth of B16 melanoma cells in the KIR-Tg mouse as compared to control vaccination ($p<0.001$). We next tested these cells in a model of human liver cancer. The human hepatoma cell line Huh-7 transfected with HLA-C*0102 was injected subcutaneously into the NOD/SCID/g-/- (NSG) mouse. NK cells from vaccinated or control vaccinated KIR-Tg mice were adoptive transferred into these NSG mice. Mice receiving NK cells from the vaccinated mice had significantly attenuated tumour growth compared to those receiving NK cells from control vaccinated mice ($p<0.001$).

CONCLUSION
We present the first DNA vaccine that specifically targets KIR on NK cells. This vaccine activates NK cells and induces anti-tumour responses in two distinct tumour models. A vaccine strategy targeting NK cells represents a novel approach to immunotherapy for cancer.

INTERLEUKIN-33 IS A NOVEL ACTIVATOR OF A SUBSET OF POLYFUNCTIONAL NK CELLS WITH ANTI-TUMOR ACTIVITY

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Natural Killer cells have the potential to eliminate tumors through their ability to recognize and kill cancer cells but also through their production of cytokines important to elicit and support an effective anti-tumor immune response. However, NK cells infiltrating tumors show impaired functions and their response to classical stimuli seems altered. Understanding mechanisms suppressing NK cells activity in tumor microenvironment and identifying new candidates for NK cell activation is of great interest to develop new therapeutic strategies.

In this context, we are evaluating the potential of Interleukin-33 (IL-33) as a novel activator of NK cells, in physiological and pathological context of breast cancer (BC). IL-33, is an alarmin released from the nucleus of non-immune cells upon cellular stress or damage to bind the receptor ST2 and promote both type-1 and type-2 immune responses. Consistently, paradoxical roles of IL-33 were reported, promoting either anti-tumoral or pro-tumoral immune response. These observations prompted us to investigate IL-33/ST2 signaling in NK cells from healthy donors (HDs) and BC patients. We unraveled a new pathway for NK cells activation where IL-12 up-regulates ST2 in a STAT-4-dependent signaling pathway on a subset of human blood NK cells which are CD56dim CD16+ CD57-/+. Following IL-33 stimulation, ST2+ NK cells secrete high levels of pro-inflammatory cytokines (IFN-γ, TNF-α, XCL1, MIP1α and MIP1β), increase their cytotoxic activity, and retain high proliferative ability. In parallel, in the context of human BC, we observed that a subpopulation of tumor-infiltrating NK cells express ST2 at steady state. Interestingly, approximately 20% of tumor-infiltrating NK cells and blood NK cells from BC patients respond to IL-33 when combined with IL-12, supporting a good potential for the use of IL-33 to re-activate NK cells' anti-tumor immune response. We are currently evaluating the role of endogenous and exogenous IL-33 on tumor progression in vivo using a mouse model of triple negative breast cancer in WT mice vs il33-KO mice.

ST2+ NK cells represent an unprecedentedly characterized intermediate subtype between canonical CD56bright and CD56dim NK cells showing phenotypic markers and functions of both subtypes. As different epigenetic programs define NK cells differentiation from CD56bright to CD56dim, our hypothesis is that ST2+ NK cells possess a unique chromatin landscape allowing ST2 expression and therefore NK cells' ability to respond to IL-33. Ongoing ATAC-seq and RNA-seq analysis will help us to decipher the main characteristics of ST2+ versus ST2- human NK cells.

A LAGGING SENSATION PREVENTS OVEREXUBERANT IMMUNE REGULATION BY SPLENIC NK CELLS

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Natural killer (NK) cells exert immunoregulatory functions that shape adaptive immunity in the context of infection, autoimmunity, and cancer. This activity of NK cells can limit fatal immunopathology by facilitating immune exhaustion and consequent viral persistence of difficult-to-control viruses. Mechanistically, suppression of adaptive immunity by NK cells involves perforin-dependent culling of a fraction of virus-specific CD4 T cells within the first few days of infection. Since delivery of perforin to target cells is cell-contact dependent, we reasoned that NK cells normally localized in the vascularized splenic red pulp would need to re-localize into the T-cell rich white pulp to exert immunoregulatory functions. In fact, we affirm previous work by observing a transient localization of many splenic NK cells into the white pulp during the first three days of infection. Using mixed bone marrow chimeric mice in which the innate compartment (including NK cells) lacks CXCR3, a receptor implicated in white pulp trafficking of NK cells, we provide evidence that suppression of virus-specific T cell responses by NK cells is dependent on CXCR3-mediated trafficking. Thus, if white-pulp localized NK cells are responsible for suppression of T-cells, differential transcriptomic profiling of white- and red-pulp NK cells should reveal genes enriched in the white pulp that are thereby implicated in immunoregulatory activity. In fact, RNA-seq analyses of NK cells in each splenic region (demarcated by intravascular staining) revealed marked up-regulation of the CD4 homolog Lag3 in white-pulp NK cells. LAG-3 is a known inhibitory receptor for major histocompatibility complex II (MHC-II) on CD4 T cells whose function on NK cells is poorly defined. We confirm progressive up-regulation and peak expression of LAG-3 on the surface of NK cells by day 3 of infection. Using anti-LAG-3 blocking antibodies and Ncr1Cre x Lag3-flox mice, we demonstrate that LAG-3 constrains NK-cell cytolytic functions in vitro. Therefore, we hypothesize that LAG-3 up-regulation on NK cells that localize to MHC-II-rich white pulp limits killing of CD4 T cells to prevent excessive pruning of antiviral immune responses. In support of this hypothesis, virus-specific CD4 and CD8 T-cell responses were markedly suppressed in spleens but not livers of Lag3- Ncr1 mice relative to littermate controls. Overall, our data suggest that LAG-3 provides negative feedback inhibition of NK-cell immunoregulatory functions to prevent immunodeficiency. Targeting of NK-cell LAG-3 is likely to provide a translatable mechanism for therapeutically modulating the immunoregulatory functions of NK cells to enhance adaptive immunity in vaccination and disease.

HETEROGENEITY OF NK CELLS AND INNATE LYMPHOID CELLS IN THE TISSUE MICROENVIRONMENT OF HUMAN TUMORS

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The presence and function of innate lymphoid cells (ILC), including natural killer (NK) cells, within human tumors has been poorly characterized. Here, we have assessed the heterogeneity of NK and ILC populations by single-cell RNA sequencing (scRNAseq) of hundreds of individual NK cells and ILCs within human head and neck squamous cell carcinoma (HNSCC), matched lymph node metastases, matched peripheral blood, and blood from healthy donors. Fresh tumor specimens and blood were obtained from 8 patients undergoing surgical resection of HNSCC. The tumor samples were digested mechanically to obtain single-cell suspensions, which were stained with antibodies and sorted as single cells into 96-well plates by flow cytometry. Libraries were prepared using the Smart-Seq2 protocol. Samples were sequenced and analyzed using the Seurat R package. Unsupervised clustering revealed heterogeneous clusters of NK cells and ILC subsets in HNSCC primary tumor tissue and matching lymph node metastasis. NK cells and ILCs from blood showed a gene expression signature different than those from the tumors. Within the tumors, we observed significant heterogeneity, with distinct subsets showing profiles consistent with that of conventional NK cells, ILC1-like cells, ILC2-like cells, and ILC3-like cell. We further observed diverse subpopulations within the ILC1-like cell clusters. The presence of these different cell subsets within primary HNSCC tumors was confirmed by flow cytometry. Further, plasticity between the subsets is supported by in vitro and in vivo experimental data. Given the ability of ILCs to polarize the immune responses through the secretion of cytokines and the ability of certain ILCs to kill target cells, we hypothesize that the differences observed in ILC populations may result in different immune responses, influencing clinical outcomes following therapy.

INTERPLAY BETWEEN ZIKA VIRUS AND DECIDUAL NATURAL KILLER CELLS AT THE HUMAN MATERNAL-FETAL INTERFACE

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BACKGROUND

Zika virus (ZIKV) is a mosquito-borne flavivirus which belongs to the large flaviviridae family. The recent ZIKV outbreak revealed unprecedented severe adverse pregnancy outcomes including microcephaly and disease associated to placental dysfunctions. We recently provided evidence for ZIKV productive infection in the first trimester decidua basalis, the main maternal-fetal interface, and in fetal placenta[1]. We also demonstrated that several cell types such as decidual fibroblasts and macrophages, fetal trophoblasts and Hofbauer cells as well as mesenchymal stem cells of the Warton jelly are targeted by ZIKV infection.

The hallmark of the decidua basalis is the presence of unique subset of decidual Natural Killer (dNK) cells, which account for 70% of decidual leukocytes in early pregnancy. In healthy pregnancy, these cells are devoid of cytotoxicity but they produce several soluble factors/mediators that are thought to contribute to fetal tolerance and placental development. Our pioneer work demonstrated that dNK cells endowed with functional plasticity, that allow them to acquire cytotoxic function and limit HCMV dissemination to the fetal compartment[2]. Nevertheless, prolonged activation of dNK cells and/or alteration of the cytokine balance may lead to placental damage and development of congenital syndrome (CS), as observed during ZIKV infection. The aim of our study is to understand the role dNK cell in the control and/or the dissemination of ZIKV at the maternal-fetal interface. Understanding the mechanisms that regulate the dNK response may contribute to the development of therapeutic strategies.

METHODOLOGY & RESULTS

We show here that dNK cells can control ZIKV replication in the decidual stroma. Using double-chamber co-cultures, we demonstrate that the inhibition of ZIKV replication is mediated through the release of soluble mediators. However, changes in the local secretome following ZIKV infection may also modify dNK cell functions and impact their ability to effectively supervise the course of pregnancy. We are currently deciphering the cellular and molecular mechanisms underlying dNK cell response to ZIKV infection and the consequences on trophoblast cell function.

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RELEVANT ROLE FOR PD-1 IMMUNE CHECKPOINT EXPRESSED ON NK CELLS DERIVED FROM OVARIAN CANCER PATIENTS

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BACKGROUND:
Epithelial ovarian cancer (EOC), the most lethal of all gynecological malignancies, is characterized by a unique tumor microenvironment (TME) that paves the way to the formation of metastasis and mediates therapy resistance through the deregulation of immune surveillance. A characteristic feature of the ovarian cancer TME is the ascites/peritoneal fluid (PF), a malignancy-associated effusion, which enables the peritoneal dissemination of tumor cells and the formation of metastasis. Since frequently standard therapies for EOC appear completely ineffective, new therapeutic strategies are needed. Harnessing the body's natural immune defenses against cancer in the form of immune-checkpoint blockade is an innovative treatment strategy. In this context, PD-1 is an immune-checkpoint known to limit T cells function against PD-L+ tumor cells, thus contributing to immune escape mechanisms. Recently we showed that the PD-1 receptor could be expressed on a discrete cell subset of peripheral blood (PB) NK cells from healthy donors (HD).

RESULTS:
By analyzing a large cohort of EOC patients, we found that the majority of these patients showed a subset of NK cells expressing PD-1 in the PB. Notably, this PD-1+ NK cell subset was dramatically increased in the TME. Different from HD, in which PD-1 is exclusively co-expressed with KIRs, in EOC patients PD-1 is preferentially detected on NKG2A+ NK cells, which represents a large fraction of the TME-associated NK cells (over 60%). The PD- 1+ (NKG2A+ and/or KIR+) NK cell subset is characterized by a strong impairment towards EOC cells expressing both PD-Ls and HLA-I molecules. Notably, our analysis reveals a strong correlation between the PD-1+ NK cell subset in TME and the disease severity. Importantly, the compromised anti-tumor activity against PD-Ls+HLA-I+ EOC cells can be restored by mAb-mediated disruption of all these inhibitory interactions.

CONCLUSIONS:
We demonstrated the existence of a consistent PD-1+ NK cell subset in the TME of EOC patients suggesting a possible induction/expansion of this subset in tumor environment. These cells can mainly co-express NKG2A but also KIR inhibitory receptors, resulting in a complete impairment in terms of anti-tumor activity against PDLs+ HLA-I+ EOC cells. An innovative treatment based on the combined blockade of both NKG2A (and/or KIR) and PD- 1 immune-checkpoints could represent a turning point to efficiently improve the anti-tumor immunity.

EBOLA VIRUS GLYCOPROTEIN STIMULATES IL-18 DEPENDENT NATURAL KILLER CELL RESPONSES

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NK cells are activated by innate cytokines and viral ligands to kill virus-infected cells; these functions are enhanced during secondary immune responses and after vaccination by synergy with effector T cells and virus-specific antibodies. In human Ebola virus infection, clinical outcome is strongly associated with the initial, innate cytokine response but the role of NK cells in this process has not been examined. Ebola vaccine development has focussed on the viral glycoprotein, the only protein exposed on the surface of the mature virus particle, essential for viral entry into host cells and highly immunogenic. The 2-dose heterologous Adenovirus type 26.ZEBOV (Ad26.ZEBOV) and modified vaccinia Ankara-BN-Filo (MVA-BN-Filo) vaccine regimen (EBOVAC consortium, EU Innovative Medicines Initiative) has been demonstrated to be well tolerated and immunogenic in Phase 1 and 2 studies, and is currently being evaluated in Phase 3 trials. Here, we analysed NK cell phenotype and function in response to Ad26.ZEBOV followed by MVA-BN-Filo with a 28 day or 56 day interval or the reverse directionality with a 14, 28 or 56 day interval in healthy adult volunteers (18 to 50 years of age) enrolled in clinical study EBL1001 (Oxford, U.K.), and in response to recombinant Ebola glycoprotein stimulation before and after vaccination. We show enhanced NK cell proliferation and activation after vaccination compared with baseline as measured ex vivo. Ebola glycoprotein-induced activation of NK cells was dependent on accessory cells and TLR-4-dependent innate cytokine secretion (predominantly from primary CD14+ monocytes) and enriched within less differentiated NK cell subsets. Optimal NK cell responses were dependent on IL-18 and IL-12, whilst IFN-γ secretion was restricted by high concentrations of IL-10. This study suggests that NK cells could potentially be important effectors in early Ad26.ZEBOV, MVA-BN-Filo vaccine regimeninduced immune responses.

PREDICTIVE VALUE OF TUMOR-ASSOCIATED AND CIRCULATING NK CELLS FOR NEOADJUVANT THERAPY RESPONSE IN PRIMARY HER2-POSITIVE BREAST CANCER PATIENTS

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In breast cancer experimental models, NK cells have been reported to contribute to the efficacy of HER2-specific antibodies and to promote tumor-specific adaptive immunity. We investigated the value of distinct NK cell-related variables for predicting pathological complete response (pCR) in primary HER2+ breast cancer patients undergoing anti-HER2 antibody-based neoadjuvant treatment.

Tumor-infiltrating NK cell numbers (TI-NK) were assessed by double immunohistochemistry (CD56+CD3-) in pretreatment tumor biopsies from two cohorts of patients with HER2-positive breast cancer [discovery (n = 42) and validation (n = 71)]. Tumor-infiltrating lymphocytes (TIL) were scored according to international guidelines. TIL and TINK cells were detected in 96% and 68% of core biopsies. Median TIL score and TI-NK cell numbers were 20% and 2 TI-NK cells/50xHPF. In both cohorts, TILs and TI-NK cells were significantly associated with pCR, independently of clinicopathologic factors, and TI-NK cells appeared as well associated with prolonged disease-free survival. Defining a threshold of ≥3 NK cells/50x-HPF, TI-NK cells stratified patients with pCR rates of 100% and 77% in the discovery and validation cohorts, respectively. NK cell–related gene set expression correlated with that of activated dendritic cell and CD8 T cell signatures in breast carcinomas overexpressing HER2 from the Cancer Genome Atlas (n = 190), supporting the value of NK cells as surrogates of effective antitumor immunity.

On the other hand, immunophenotypic analysis of circulating NK cells in prospectively recruited patients (n=66), evidenced an inverse correlation between baseline CD57+ NK cells and pCR, independently of age, conventional clinicopathological factors and CD16A 158F/V genotype. This association was also uncoupled from the presence of HCMV-induced NKG2C+ adaptive NK cells. Circulating CD57+ NK cells displayed lower CXCR3 expression and CD16A-induced IL-2-dependent proliferation as compared to the CD57- population, yet showing comparable trastuzumab-induced in vitro degranulation against HER2+ breast cancer cells. Remarkably, CD57+ NK cells were reduced in breast tumor-associated infiltrates as compared to paired peripheral blood samples, suggesting their deficient homing, proliferation and/or survival in the tumor niche. Indeed, patients with high circulating CD57+ NK cell numbers lacked tumor-infiltrating NK cells; perhaps explaining CD57+NK cell association with resistance to anti-HER2 antibody-based treatment.

Overall, baseline tumor-infiltrating and circulating CD57+ NK cell numbers predicted pCR to anti-HER2 antibodybased neoadjuvant treatment in primary breast cancer patients, pointing to the putative influence of tumor-infiltrating and the differentiation profile of circulating NK cells on the efficacy of anti-HER2+ antibodies.

SELECTED POSTER ABSTRACTS

SESSION 1
NK Receptors and Education

1

A CRISPR APPROACH TO MANIPULATING NK CELL RECEPTOR LIGAND EXPRESSION IN LEUKEMIC CELLS

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NK cells are equipped with a battery of cell surface receptors that bind to cognate ligands on potential target cells. The balance between activating and inhibitory signals originating from these interactions determine whether the NK cell activates its cytotoxic machinery or leaves the target cell untouched. The interplay among the many activating receptors as well as the contribution of each individual receptor involved in NK cell cytotoxicity is still not fully understood and has been challenging to study. Previous studies used transfected, otherwise ligand-deficient, insect cells with human NK cell receptor ligands in order to elucidate the function of different receptors. Here, we took the opposite approach and set out to knock out ligand by ligand in the prototypic NK cell target cell line, K562. Initially, we performed a screen with blocking antibodies to determine the receptors that are important for NK cell killing of K562. In line with previous reports, these experiments identified pivotal roles for the activating receptors NKp30 and DNAM-1 in NK cell recognition of K562. We generated a Cas9-expressing K562 cell line and transduced cells with gRNAs targeting ligands in different combinations. As expected, knock-out of multiple activating ligands made the cells less sensitive to NK cell cytotoxicity. For example, knocking out the combination of a DNAM-1 ligand and B7-H6 strongly reduced the K562 cell susceptibility to NK cells. However, dKO-K562 cells were readily killed at higher effector/target ratios in a process that was NKG2D dependent. The combined knock-out of ligands thus allow skewing of NK-K562 interactions towards different receptor-ligand pairs, which can be valuable tools in studies aiming at defining novel ligands to NK cell receptors.

2

A DYNAMICS OF INHIBITORY KIRS RECEPTORS AFTER ALLOGENEIC HSCT

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NK cells are part of the innate immune response and play a key role in relapse control of leukaemia in patients´ after allogeneic haematopoietic stem cells transplantation (HSCT). These cells express a large repertoire of receptors, named inhibitors and activators that mediate their function. The main group of these receptors is called KIR (Killer-cell immunoglobulin-like receptor) which consists of activating as well as inhibitory receptors. Inhibitory KIRs interact specifically with human leukocyte antigen (HLA) class-I molecules on target cells. Leukemic cells often express high level of HLA class I molecules and can inhibit NK cell activity. The inhibition of NK cells can be decreased by lower expression of HLA class I or inhibitory KIRs.

In our study we followed dynamics of an expression of inhibitory KIRs after allogeneic allo-HSCT.

An expression of inhibitory KIRs was evaluated on the surface of NK cells from 35 donors and then in transplanted patients after 1-2-3 months (M1, M2, M3) after HSCT. Blood samples were stained with CD45-Horizon (BD Bioscience, USA), CD3-Pacific Blue, CD16-PerCP, CD56-PeCy7 (all Exbio, Czechia), KIR2DL1-PE, KIR2DL2/2DL3- APC, KIR2DL3-FITC or KIR3DL1/DL2 PE, KIR3DL3-APC, KIR3DL5-FITC (all Miltenyi, Germany). The samples were measured using BD FACSCanto II, analysis were performed in FlowJo software (FlowJo, LLC, USA). For standardization of biological differences, we determined level before HSTC as 100% and then we calculated percentage from this value.

The study showed changes in expression of KIRs receptors after HSCT with differences within patients. The main change was detected in KIR2DL1, whose expression was distinctly downregulated in more than 80% (median of percentage from original value was M1= 29%, M2+3=44%). This receptor binds ligands from HLA-C group. The HLAC also interacts with KIR2DL2 and 2DL3 receptors which were also mostly decreased. KIR2DL2 was lower in 80% in first and second month (median M1=40%, M2=37%) and then with slow expression recovery (in third month decreased in 60% with median 64%). On the other hand, KIRs reacting with HLA-A and B were without clear trend of expression with exception of KIR3DL2 where more than 60% showed increased expression (median M1=360%, M2=148%, M3=187%).

Our study confirm dynamic of expression of inhibitory KIRs receptors with association of their ligands. Deeper analysis of the inhibitory KIRs expression level and their importance for patients´ outcome has to be done.

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3

A GENOME-WIDE CRISPR/CAS9 SCREENING APPROACH FOR THE IDENTIFICATION OF NEW LIGANDS FOR NK-CELL RECEPTORS

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INTRODUCTION:
Natural killer (NK) cells are characterized by their ability to lyse virus-infected and tumor cells without prior immune cell priming. Therefore, their activity is firmly regulated by the expression of activating and inhibitory receptors on their surface. Although cellular ligands for most NK-cell receptors have been characterized, the identity of cell-surface ligands for some NK-cell receptors remains still elusive.

OBJECTIVE:
The aim of this project was to establish a CRISPR/Cas9 library-based screening approach for the identification of new cellular ligands for NK-cell receptors, with a special focus on ligand identification for Natural Cytotoxicity Receptors (NCRs).

MATERIALS AND METHODS:
K562 cells were transduced with lentivirus-like particles containing a genome-wide CRISPR/Cas9 library with ~80.000 different sgRNA. Seven days post-transduction, lentivirally-transduced cells were selected according to their level of NKp44-ligand expression using NKp44-IgG1 fusion constructs and magnetic enrichment. DNA from selected cells was isolated and sgRNA abundance was assessed using Next Generation Sequencing (NGS).

RESULTS:
A variety of human tumor cell lines were screened for their expression level of ligands for the NCRs, NKp30, NKp44 and NKp46. Since K562 cells displayed high levels of NKp44-ligand (NKp44-L) expression these cells were selected for use in a genome-wide CRISPR/Cas9 screening approach. After transduction with the CRISPR/Cas9 library, K562 cells were separated according to their level of NKp44-L expression by NKp44 Fc-construct and magnetic bead staining and subsequent magnetic enrichment. NKp44-Lbright and NKp44-Llow K562 cells differed regarding their sgRNA distribution. Enriched sgRNAs within the NKp44-Llow included transcription factors, e.g. two zinc finger proteins, proteins involved in RNA processing, e.g. REXO2, as well as proteins involved in posttranslational modifications, e.g. GCNTP7, that are potentially associated with regulation and expression of NKp44-L. In addition, sgRNAs targeting the surface protein CCR10 were enriched in the NKp44-Llow compared to the NKp44- Lbright population, indicating CCR10 protein as a potential NKp44-L.

CONCLUSION:
The genome-wide CRISPR/Cas9 screening approach provides a promising tool for the identification of new NK-cell receptor ligands.

4

ALTERNATIVE SPLICING OF HUMAN KIR RESULTS IN STRUCTURAL AND FUNCTIONAL VARIETY OF RECEPTORS

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The killer-cell Ig-like receptors (KIR) form a multigene entity involved in modulating immune responses through interactions with MHC class I molecules. The complexity of the KIR cluster is reflected by, for instance, abundant levels of allelic polymorphism, gene copy number variation, and stochastic expression profiles. In the current transcriptome study involving human families we demonstrate that KIR receptors are subjected to differential levels of alternative splicing, which may result in the partial or complete skipping of exons, or the partial inclusion of introns. This post-transcriptional process can generate multiple isoforms from a single KIR gene, which diversifies the characteristics of the encoded proteins. For example, alternative splicing can modify ligand interactions, cellular localization, signaling properties, and the number of extracellular domains of the receptor. Abundant alternative splicing was observed for KIR2DL4 transcripts, and to a lesser extent in transcripts of lineage III KIR genes. A part of these alternative splicing events might be non-functional, and subjected to the nonsense-mediated decay pathway. However, the high frequency, segregation, and conservation of certain splice events indicate a functional role of alternatively spliced KIR transcripts. Here we show that alternative splicing of KIR2DL4 9A transcripts, which normally encode a truncated receptor that is not expressed, restores the open reading frame, and results in transcripts encoding an expressed receptor. In contrast, the identical splice event truncates the KIR2DL4 10A transcripts. This recovery and truncation of KIR2DL4 9A and 10A receptors determine whether or not the receptor is expressed, and is substantiated by transfection studies. The observations indicate that alternative splicing has implications in individuals that are 9A homozygous, and suggest specialized functions of KIR2DL4 isoforms.

5

CHARACTERIZING G PROTEIN-COUPLED RECEPTOR GPR56 IN PRIMARY NK CELL SUBSETS AND ITS ROLE IN NK CELL MIGRATION

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G protein-coupled receptors (GPCRs) represent the largest family of surface receptors and are responsible for key physiological functions including cell growth, neurotransmission, hormone release and cell migration. Global transcriptional and epigenetic mapping of human NK cells have revealed a gradual increase in some GPCRs during NK cell differentiation. However, the role of GPCRs in regulating NK cell function remains elusive. The G proteincoupled receptor GPR56 is an adhesion GPCR found on diverse cell types including neural progenitor cells, melanoma cells and lymphocytes such as effector memory T cells, gamma-delta T cells and NK cells. A role for GPR56 in migration has been reported for both, neural progenitor cells and melanoma cells, but so far there are no reports of its migratory role in NK cells. Using RNA-seq and high-resolution flow cytometry, we found that GPR56 mRNA and protein expression increased as cells differentiated into a more terminally differentiated NK cell both in mRNA level and protein level. Furthermore, GRP56 expression correlated with the expression of self-specific inhibitory killer cell immunoglobulin-like receptors (KIR), suggesting that GPR56 was affected by weak agonistic input during educating cell-cell interaction. Indeed, activation of NK cells by cytokines or target cells led to a rapid downregulation of GPR56. Preliminary trans-well experiments suggest a subset-specific and dynamic regulation of GPR56 expression during random and migration. Together, these data provide insights into the role of GPR56 in the functional diversification of human NK cell subsets and may play a role in their ability to home to different tissues.

6

CONSERVATION, EXTENSIVE HETEROZYGOSITY, AND CONVERGENCE OF SIGNALING POTENTIAL ALL INDICATE A CRITICAL ROLE FOR KIR3DL3 IN HIGHER PRIMATES

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Human natural killer (NK) cell functions are modulated by polymorphic killer cell immunoglobulin-like receptors (KIR). Among 13 KIR genes, which vary by presence and copy number, KIR3DL3 is ubiquitously present in every individual across diverse populations. No ligand or function is known for KIR3DL3, but limited knowledge of expression suggests involvement in reproduction, likely during placentation. With 157 human alleles, KIR3DL3 is also highly polymorphic and we show heterozygosity exceeds that of HLA-B in many populations. The external domains of catarrhine primate KIR3DL3 evolved as a conserved lineage distinct from other KIR. Accordingly, and in contrast to other KIR, we show the focus of natural selection does not correspond exclusively to known ligand binding sites. Instead, a strong signal for diversifying selection occurs in the D1 Ig domain at a site involved in receptor aggregation, which we show is polymorphic in humans worldwide, suggesting differential ability for receptor aggregation. Meanwhile in the cytoplasmic tail, the first of two inhibitory tyrosine motifs (ITIM) is conserved, whereas independent genomic events have mutated the second ITIM of KIR3DL3 alleles in all great apes. Together, these findings suggest that KIR3DL3 binds a conserved ligand, and a function requiring both receptor aggregation and inhibitory signal attenuation. In this model KIR3DL3 resembles other NK cell inhibitory receptors having only one ITIM, which interact with bivalent downstream signaling proteins through dimerization. Due to the extensive conservation across species, selection, and other unusual properties, we are employing evolutionary and molecular analyses to elucidate the ligand and function of KIR3DL3.

7

DETECTION OF ALLOANTIBODIES RAISED AGAINST NK CELL ANTIGENS IN PATIENTS AFTER SOLID ORGAN TRANSPLANTATION

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INTRODUCTION: Chronic antibody-mediated rejection (ABMR) is known to play a key role in graft survival, dysfunction or rejection and is still a major obstacle in transplantation success. Recent studies have shown the importance of non- HLA antigens in the formation of donor-specific antibodies (DSA) in graft rejection. A pilot study revealed the presence of allospecific antibodies against antigens encoded in the NK cell gene complex (NKC) and leukocyte receptor gene complex (LRC) in 23% from a total of 92 plasma samples from liver and kidney recipients.
OBJECTIVE: To investigate the frequency and relevance of alloantibody formation against NK cell antigens and their role in transplantation outcome in patients after solid organ transplantation.
MATERIALS AND METHODS: The extracellular domain gene sequences of NKG2C, KIR2DL2, KIR2DS2, KIR2DS1, KIR2DL1 and LILRB3 followed by a V5/His-tag were cloned into a lentiviral vector. HEK293 cells were transduced with the vector encoding for the different vectors for the mass production of soluble proteins. The recombinant proteins were then coupled to differentially colored-multiplex beads which were used to screen patient plasma for the presence of antibodies using FACSCanto II flow cytometer.
RESULTS: Overall, antigen-allospecific antibodies against various NKC- and LRC-encoded receptors such as NKG2C, KIR2DL2/DS2, KIR2DS1/DL1 and LILRB3 were found in 20% of Kidney recipient patents (n=15), 19% of liver recipient patients (n=42) and 17% of lung recipient patients (n=41). Table 1 describes in more detail the absolute numbers for each post-transplantation sample that tested positive for at least one NK cell antigen-specific antibody.

Number of Patients per Antigen
KIR2DS1/DL1 KIR2DS2/DL2 NKG2C LILRB3 Overall
Kidney (n=15) 1 1 1 1 3 (1)
Liver (n=42) 2 3 3 2 8 (1)
Lung (n=41) 0 5 5 3 7 (5)
Table 1: Overview of recipient-patients screen for alloantibody formation against NK cell antigens. The values shown here refer to the absolute number of patients tested positive for the presence of specific-antigen alloantibody formation defined by each column. The overall values refer to the total number of patients tested positive for at least one type or more (in brackets) types of antibody.

CONCLUSION: The data indicate a high degree of potential mismatch in NK cell diversity between donor and recipient in the case of solid organ transplantation. Further analysis is being performed to evaluate the functional consequences and clinical relevance of these antibodies in transplantation outcome.
Supported by DFG grant SFB738/A5
The last two authors contributed equally to this study

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DIFFERENTIAL MHC-E DEPENDENT NK CELL EDUCATION IN RESPONSE TO SIVAGM AND SIVMAC INFECTIONS

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Since the discovery of HIV, tremendous progress has been made in preventing and treating HIV infection. However, a vaccine and a cure are still missing. Highly-efficient antiretroviral treatment does not eradicate HIV from the body. HIV persists in cellular and anatomical reservoirs, such as B cell follicles in secondary lymphoid organs (SLO). Our laboratory aims to discover mechanisms that could contribute to the eradication or durable control of HIV reservoirs. Our original approach consists in learning from the efficient control of HIV/AIDS that exists in HIV-infected individuals who control HIV replication, either spontaneously or after an antiretroviral treatment (post-treatment controllers) and in some animal models of spontaneous protection against AIDS. African green monkeys (AGM) are asymptomatic carriers of simian immunodeficieny virus (SIVagm). Strikingly, B cell follicles of African green monkeys (AGM) infected by SIVagm are virus-free. We recently reported that the efficient control of SIVagm in B cell follicles is mediated by NK cells. We aimed here to identify the mechanism of the strong NK cell-mediated viral suppression in SLO of SIVagminfected AGMs.

We show that CD4+follicular helper T cells (TFH) cells in lymph nodes increased in response to SIV in pathogenic infection (SIVmac infection in macaques) and 90% of the TFH expressed MHC-E. In contrast, TFH cell frequency remained normal in SIVagm infection and that of MHC-E+TFH decreased. We identified SIVmac and SIVagm peptides binding to MHC-E. The SIVmac peptide strongly inhibited MHC-E dependent NK cell lytic activity from non-infected animals in autologous cultures with SIV-infected CD4+ cells, in contrast to the SIVagm peptide. After SIV infection, NK cells from macaques further decreased their MHC-E dependent lytic activity, while the one from AGM was increased. These results indicate a MHC-E dependent NK-cell lysis of SIV-infected cells. They unraveled that the NK cell activity was SIV peptide-specific and furthermore suggest an education of NK cells in AGM during SIVagm infection distinct from that during pathogenic SIVmac infection. In the future, these findings could offer novel insights into how adaptive natural killer cells are induced, a finding that might be useful also in the context of global efforts toward HIV cure.

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DIFFERENTIAL NK CELL RECOGNITION OF ARTIFICIAL IMMUNE SYNAPSES SHAPED AS DOTS OR DONUTS

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The induction of Natural Killer (NK) cell cytotoxicity depends upon the signaling outcome of the immune synapse formed between the NK cell and its target. During this highly regulated stepwise process, receptors, signaling molecules and the cytoskeleton rearrange into well-defined spatial structures, ultimately leading to the release of lytic granules which results in target cell death. Dynamically monitoring and influencing the course of events without disrupting the synapse has proved technically challenging. We propose the use of microcontact printing to generate arrays of artificial immune synapses (AIS) to investigate how the spatial distribution of ligands influences NK cell recognition and activation. Live time-lapse imaging of NK cells interacting with round AIS revealed that ligation of LFA- 1 alone leads to a seeking phenotype, where NK cells conserve a relatively high motility and constantly probe their close environment despite being engaged in a synapse, whereas co-ligation of CD16 and LFA-1 induced commitment of the NK cell to a single long-lasting synapse. Spatially separating ligands by presenting NK cells with donut-shaped AIS disrupted the stability of the contact, as fewer NK cells engaged in complete synapses covering the entire AIS.

However, for NK cells that managed to spread symmetrically over either dot- or donut-shaped AIS, we observed a remarkably similar spatial organization of their cytotoxic machinery, where lytic granules were found in close proximity to the microtubule-organizing center (MTOC), and where the MTOC often localized over a region void of activating ligands. This suggests that NK cells are able to integrate signaling from spatially separated ligands and respond to an area that lacks local stimuli.

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FINE CHARACTERIZATION OF HP-DM1 MAB REACTIVITY: KIR2DL1 EXCLUSIVE SPECIFICITY AND EPITOPE DEFINITION

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HP-DM1 mAb (IgG1) was produced in the laboratory of M. Lopez-Botet and selected on the basis of flow cytometry, functional and biochemical analyses, consistent with its reactivity with KIR2D molecules. To better define its specificity, we performed immunofluorescence analyses of NK cells and cell transfectants using this novel mAb in comparison with other anti-KIR2D antibodies. In NK cells from KIR2DL1pos/S1pos donors, HP-DM1 showed a staining pattern similar to 143211 mAb, suggesting the recognition of KIR2DL1. We deeply studied HP-DM1 specificity on HEK-293T cells transiently transfected with plasmids coding for different KIR2D molecules. HP-DM1 exclusively recognized KIR2DL1, and was different from other commercially available mAb, including 143211, which also stains KIR2DS5. The comparison of the extracellular amino acid sequences of different HP-DM1pos vs HP-DM1neg KIR2D molecules revealed that HP-DM1 epitope is, most likely, located in the D1 domain and allowed the identification of 6 amino acid positions possibly relevant for its binding. By site-directed mutagenesis approach and staining of HEK-293T transiently transfected with plasmids coding the mutated KIR2DL1 molecules, we defined M44, S67, R68 and T70 as residues crucial for HP-DM1 staining. Based on these results, it can be predicted that HP-DM1 recognizes all the KIR2DL1 allotypes identified to date, with the exception of KIR2DL1*022, characterized by K44, a change that also modifies its HLA-C recognition (from C2 to C1), and of KIR2DL1*020, characterized by G67. Notably, in immunofluorescence analyses, HP-DM1 (anti-KIR2DL1) used in combination with EB6 or 11PB6 (anti-KIR2DL1/S1 and anti-KIR2DL3*005), 143211 (anti-KIR2DL1/S5) and HP-MA4 (anti-KIR2DL1/S1/S3/S5) mAb allowed a more accurate identification of the different KIRpos NK cell subsets. Finally, using NK cells from KIR2DL1pos/S1pos/S5pos donors in reverse-ADCC assays, we confirmed that only HP-DM1 mAb exclusively reacts with an inhibitory receptor (i.e. KIR2DL1), while 143211, 11PB6 and HP-MA4 triggered lysis. Thus, HP-DM1 mAb is a unique reagent that specifically recognizes KIR2DL1 molecules, and it is valuable to be included in the panel of anti-KIR2D antibodies to more precisely characterize the NK cell phenotypic repertoire.

11

NATURAL IMMUNITY HLA-DR EXPRESSION IN NK CELLS IS INDUCED BY BOTH EXTRACELLULAR CYTOKINES AND SELF-PRODUCED IFNγ AND ASSOCIATED WITH HIGH METABOLIC ACTIVITY

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Normally, human peripheral blood contains minor subpopulation of HLA-DR-expressing NK cells. However, this subset readily expands in vitro under stimulation with certain cytokines and other stimuli, both due to induction of HLA-DR expression de novo and high proliferative capacity of HLA-DR-positive NK cells. As NK cells are not canonical APCs, it is little known about the molecular mechanisms causing MHC class II expression increase in these cells. The aim of this study was to elucidate some aspects of the HLA-DR induction pathway in human NK cells and check the concomitant metabolic changes.

Isolated peripheral NK cells were stimulated with soluble IL-2 and IL-21 to induce HLA-DR-positive NK cells expansion. In a series of experiments with specific inhibitors, we have shown that the percentage of HLA-DR-positive NK cells mostly depends on ligation of the IL-21 receptor and partly – of the IFNγ receptor. Thus, apart from IL-21, the increase of HLA-DR expression is stimulated by IFNγ, produced by NK cells themselves in response to cytokines. This fact explains the positive correlation between IFNγ production and HLA-DR expression in NK cells, shown earlier by us and other scientific groups. Next, we have demonstrated that, inside the cell, STAT3 and ERK1/2 transcription factors are involved in induction of HLA-DR expression, but not STAT1. In this case, signals from self-produced IFNγ and external IL-21 may synergize through MAPK/ERK pathway, with additional signaling through STAT3 from IL-21 receptor.

Besides, we have established that both freshly isolated HLA-DR+ NK cells and HLA-DR+ NK cells, acquired after 6- day stimulation with IL-2 and IL-21, demonstrate higher level of ATP and mitochondrial mass compared to HLA-DR– counterparts. Thus, HLA-DR expression in NK cells is associated with increased metabolic activity in vivo and in vitro.

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KIR ALLELES IDENTIFICATION FOR HSCT CLINICAL PRACTICE

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Killer-cell immunoglobulin-like receptors (KIRs) are NK receptors playing key role in regulation of NK cells activity. KIRs gene content is heterogeneous among human individuals and the polymorphism can affect strength of inhibitory signal or level of surface expression which may be controlled also by copy number of genes. KIR genotyping became very important in hematopoietic stem cells transplantation where the presence/absence of KIR gene has impact on outcome of transplanted patients and knowledge about KIRs alleles seem to be next standard criteria for selection of optimal donor.

The use of detailed information about KIRs genotype in routine hematology clinical practice is currently very complicated from the point of very time-consuming data analysis and human resource with deep knowledge. Due to KIR polymorphisms and extensive gene copy number variation (CNV) the KIR allele identification is a very complex task.

To obtain information about KIR distribution in Czech population and for routinely using of KIR genotyping an algorithm for the KIR alleles identification was proposed, primarily based on knowledge from The IPD database (release 2.8.0 Nov 2018) and German Bone Marrow Donor File (DKMS - Deutsche Knochenmarkspenderdatei) published studies.

First verification of designed algorithm was performed on reference cell lines AMALA, BOB, HO301 and KASO11 (from Fred Hutchinson Center, Seattle, USA) with known KIRs genotype. Our own sequencing analysis was performed using NEBNext® Ultra™ II FS DNA Library Prep Kit and NEBNext® Multiplex Oligos for Illumina - Dual Index Primers Set 1 (both New England Biolabs, USA) and Illumina Miseq sequencer (Illumina,USA).

The algorithm identified group of alleles for each gene (e.g. KIR2DL1*0030201/0030202/.../037) among which the corresponding known KIRs genotype is present (with minor deviation on the 3DP1 and 2DL5B genes). The results are consistent within the protein serie/coding-region level. But the specificity of our method is not sufficient now, due to very high similarity of some alleles (e.g. KIR2DL1 has more over 20% alleles higher similarity than 99.90% according to Levenshtein distance), and has to be increased.

To improve the results, detailed information about gene/alleles similarity, use of CNV results, KIR linkage equilibrium, ethnicity, haplotypes/genotypes and frequency knowledge are being incorporated. Verification to additional reference cell lines as well as interlaboratory comparison using primary samples is planned.

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KIR+ NK CELLS SPECIFICALLY SENSE PATHOGENIC DNA

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Antigen presenting cells use the CD205 and TLR-9 receptors to sense viral and bacterial infections. These receptors recognize single-stranded DNA containing unmethylated CpG motifs, characteristic of microbial DNA and rare in mammalian DNA. Though nearly half of human NK cells express CD205 and TLR-9, little is known about the role of exogenous DNA uptake in NK cell immunity. To determine what variables influence the NK cell uptake of microbial DNA, we measured the combined activity of CD205 and TLR-9 by culturing human NK cells with fluorescently-tagged CpG+ oligodeoxynucleotides (ODNs). By varying the nucleotide sequences of the ODNs, we found that human NK cells prefer exogenous DNA with 6-base pair spacers between CpG motifs. Using this sequence, we explored if cytokines alter the uptake of exogenous DNA. Treatment with IL-15 or GM-CSF effectively doubled the amount of DNA bound to NK cells. Despite increasing the expression of CD205 on the NK cell surface, IL-2 acted as a suppressor of DNA uptake, abrogating the effects of both IL-15 and GM-CSF. Most surprising was that NK cells expressing Killer Immunoglobulin-like Receptors (KIR) bound, on average, 20 times more exogenous DNA than KIR NK cells. The enhanced appetite of KIR+ NK cells for exogenous DNA was maintained in both inflammatory and immunosuppressive cytokine conditions. KIR+ NK cells in particular are thus seen to sense pathogenic DNA, a function that is tuned by the balance between IL-2 and IL-15. Our findings suggest that the tandem detection of missing-self and microbial DNA is essential for NK cell immunity.

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MULTIPLE LAYERS OF DISEQUILIBRIUM IN KIR AND HLA LOCI OF PATIENTS WITH BILIARY TRACT CANCERS

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BACKGROUND & AIMS:
Bile duct cancers (BDC) are rare tumors of the liver and biliary system with dismal prognosis. Natural killer (NK) cells, innate effector lymphocytes enriched in human liver, infiltrate these tumors and yet fail to control disease progression suggestive of defective NK cell tumor recognition and elimination. NK cell responsiveness is regulated by the genetically diverse family of killer cell immunoglobulin-like receptors (KIRs) that recognize distinct HLA class I ligands. The aim of this study was to characterize the genetic architecture of KIRs and their ligands in patients with BDC.

METHODS:
We here introduce a combined multidimensional characterization of the genetic architecture of KIRs and their ligands in a case-control-controlled setting involving Swedish patients with BDC followed up to eight years after diagnosis (n=148), two geographically matched (n=204+900) and six geographically unmatched healthy control cohorts (n=2917).

RESULTS:
Our methodology allowed us to identify multiple layers of disequilibrium within the KIR locus of patients with BDC. Specifically, BDC had a lower prevalence of KIR2DL3, associated with disequilibrium in centromeric A/B and B/B haplotypes. Moreover, the genotypes of KIRs and the association between KIRs and their ligands differed between patients and controls, resulting in a profound altered balance between activating and inhibitory KIRs. Finally, the number of activating KIR genes, and in particular KIR3DS1, was associated with gallbladder cancer.

CONCLUSIONS:
As compared to healthy donors, patients with BDC have a different genetic architecture in their KIR and HLA loci. This work highlights the need of multidimensional analysis of the KIR-HLA module with potential implications on immunosurveillance.

15

NK-CELL MEDIATED RECOGNITION OF HIV-1 INFECTED CD4 T CELLS IS ASSOCIATED WITH EXPRESSION OF A SPECIFIC SET OF NK-CELL RECEPTORS

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NK cells express a large set of activating and inhibiting receptors regulating NK-cell functions. In particular HLA class I-recognizing NK-cell receptors such as KIRs have been implicated to impact the course of various diseases, most prominently HIV-1 infection. Yet, little is known regarding involvement of NK-cell receptors that are independent of the host's HLA class I background in the control of HIV-infection. Therefore, we sought to identify novel NK-cell receptors that enable NK cells to recognize HIV-1-infected CD4 T cells.

Enriched peripheral blood NK cells from healthy human individuals were co-incubated for 5 h with autologous HIV-1- infected (NL4-3) CD4 T cells (n=19) and K562 (n=18), 721.221 (n=12), or Raji (with anti-hCD20-hIgG1, n=14) cells as controls. Surface expression of CD107a was measured via flow cytometry as a surrogate parameter of NK-cell activation. NK cells were then individually stained for 346 surface markers and analyzed by flow cytometry. Expression levels of the assessed surface proteins were then analyzed for association to NK-cell activation (median expression difference of 10 % points between CD107a+ and CD107a- NK cells).

NK cells exhibited higher CD107a expression levels after exposure to any type of target cell than no-target controls. Following co-incubation with HIV-1-infected CD4 T cells 31 of the 346 individually assessed surface receptors were differentially expressed between CD107a+ and CD107a- NK cells (median expression difference > 10 % points). These comprise known activation markers i.e. CD69 as well as NKG2D. Moreover, we also identified activation-associated receptors, including CD230, that have not been reported in the context of NK-cell mediated cytotoxicity so far. Additionally, three of the investigated 346 surface receptors showed association to NK-cell activation after exposure to autologous HIV-1 infected CD4 T cells but not to K562, 721.221 or Raji cells.

The findings of this study show that our approach is an appropriate procedure to identify target-cell specific NK-cell receptors. Moreover, our data suggests that NK cells may require a specific set of surface receptors to respond with degranulation towards HIV-1 infected CD4 T cells. The identification of novel NK-cell receptors involved in the recognition of HIV-1 infected cells may lead to new strategies in NK-cell based immunotherapies for HIV-1 cure approaches.

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QUANTITATIVE ANALYSIS OF NK CELL REACTIVITY USING LATEX BEADS

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Natural Killer (NK) cell responses are regulated by many different surface receptors. While we can determine the overall positive or negative effect of a given receptor on NK cell functions, investigating NK cell regulation in a quantitative way is challenging. To quantitatively investigate individual NK cell receptors for their effect on NK cell activation, we functionalized latex beads that have approximately the same size as lymphocytes. The latex beads were covalently coated with antibodies against various activating NK cell receptors or control antibodies via a chemical crosslinker. Alternatively we used recombinant ligands that were bound to the beads via biotin-streptavidin interaction. The exact amount of antibodies or recombinant ligands bound to one bead was determined with the aid of a flow cytometry based quantification kit. We used different concentrations of antibodies or recombinant ligands to generate beads with a range of different surface densities of these proteins.

This enabled us to stimulate NK cells via defined receptors in a clean, quantifiable and controllable system.

NK cells were stimulated with those beads and the resulting effector functions were analyzed. Using antibody coated beads we found that the ITAM coupled receptors appeared to be most potent with CD16, NKp30 and NKp46 inducing the strongest maximal degranulation. Moreover, CD16 and NKp44 needed the lowest stimulation to reach halfmaximal degranulation. In addition to degranulation we also detected the production of IFN-γ and MIP-1. The induction of polyfunctional NK cells, degranulating and producing one or both of the cytokines, was also most pronounced after stimulation of the ITAM coupled receptors CD16, NKp30 or NKp44. These data demonstrate that activating receptors differ in their sensitivity and efficacy to activate NK cells.

NK cells were even more efficiently stimulated when we used beads coated with recombinant ligands. Less MICA was needed to reach half-maximal degranulation compared to antibody against NKG2D. Combining activating ligands with the adhesion molecule ICAM-1 reduced the amount of ligand needed for half-maximal degranulation even more. By titrating and combining different ligands we plan to further evaluate the quantitative requirements for the activation of NK cells.

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ROLE OF THE MUCOLIPIN CALCIUM-CHANNEL FAMILY IN VESICULAR TRAFFICKING AND NATURAL KILLER CELL FUNCTION

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Natural Killer cells execute their function by secretion of a wide range of effector molecules stored within various vesicular compartments. Calcium signaling is the most critical signaling pathway that governs vesicular homeostasis, trafficking and secretion. By analyzing calcium signaling on a sub-cellular level, we recently showed that lysosomes actively contribute and modulate NK cell effector functions. Interference of calcium-flux from the lysosomal compartment attenuates NK cell degranulation and cytokine production. Interference of PI(3,5)P2 signaling, or siRNAmediated silencing of the lysosomal PI(3,5)P2-gated calcium channel TRPML1, potentiated the lysosomal granzyme B storage and increased NK cell effector functions. Here, we extend these findings and show that TRPML2, another member of the mucolipin family, is highly expressed in NK cells and increase with NK cell differentiation. Imaging experiments suggest that TRPML2 is localized upstream in recycling endosomes, compatible with a role at an early stage in the endo-lysosomal life cycle. Si-RNA silencing of TRPML2 leads to slightly enhanced NK cell degranulation and IFN-γ production. Conversely, ML1-SA2 stimulation, an agonistic and highly-specific small molecule for TRPML2, leads to intracellular calcium mobilization without affecting granzyme B content. In ongoing experiments, we use a series of ultrasensitive single fluorescent protein-based calcium indicators, such as the GCaMP6f to visualize the organization and dynamics of intracellular communication over multiple spatial and temporal scales. Ultimately, these studies will unravel the molecular pathways that regulate intracellular communication between organelles in NK cells. A better knowledge of these signaling pathways may lead to new means to optimize the functionality of NK cells for cancer immunotherapy.

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SINGLE-CELL FATE MAPPING REVEALS CLONAL DYNAMICS OF ADAPTIVE NK-CELL RESPONSES

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Upon antigen exposure a small population of T cells, which harbors T cell receptors (TCRs) specific to the relevant antigen, is selected to rapidly expand. After antigen clearance this expanded T-cell population contracts to 5–10% of its peak size. Importantly, the number of T cells remaining after this contraction phase is considerably larger than that of antigen-specific T cells available in the naive T-cell compartment. This numerical increase of antigen-specific T cells, through clonal selection, clonal expansion and preferential clonal maintenance, is one of the key factors underlying adaptive immunity and immunological memory. However, the antigen-driven evolution of the TCR repertoire does not stop at the selection of antigen-specific T-cell clones per se. It is instead characterized by the further selection of certain T-cell clones within the antigen-specific T-cell population. This phenomenon, which has been termed TCR repertoire focusing, is thought to be driven by the enhanced expansion and reduced contraction of T-cell clones, whose TCRs bind to the relevant antigen with optimal affinity.

Interestingly, an antigen-dependent enrichment of lymphocytes recognizing certain target structures has also been identified for Natural killer (NK) cells in the context of cytomegalovirus (CMV) infection. Whether these “antigenspecific” NK cells respond uniformly to CMV infection or show distinct clonal dynamics similar to those found in T cells remains unknown. In our studies we used retrogenic color- barcoding and single-cell adoptive transfer to track clonal immune responses derived from individual Ly49H+ NK cells during murine cytomegalovirus (MCMV) infection. We found that clonal expansion of single NK cells varied dramatically. The observed variability could not be attributed to the additional presence or absence of inhibitory Ly49 receptors in responding clones. Instead, NK-cell clones showed distinct levels of Ly49H receptor expression that correlated with the degree of clonal expansion and persistence during the contraction phase. Furthermore, NK-cell clones sorted for high or low expression of Ly49H clonally maintained their Ly49H expression levels. Thus, akin to adaptive processes shaping an antigen-specific T-cell receptor repertoire, the Ly49H+ NK-cell population adapts to MCMV infection. This is achieved by preferential expansion and maintenance of NK-cell clones expressing higher levels of Ly49H.

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SPECIFIC ALLELES, GENE COPY NUMBER AND C2-LIGAND STATUS DETERMINE KIR2DL1 EXPRESSION ON NK CELLS

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The functional fate of a NK cell is dependent on the balanced expression of different cell surface receptors, with inhibitory and activating KIRs being important players. For this analysis, we focused on KIR2DL1 expression, the only inhibitory KIR recognizing the HLA-C2 epitope. We applied multi-parameter flow-cytometry analyzing peripheral blood NK cells from 180 healthy individuals. In concordance with published data, we found KIR2DL1*001/002, *003/034, and *004 to be the most common alleles among all analyzed samples. Further, our results suggest that gene copy number not only regulates the pool size of KIR2DL1+ NK cells but calibrates KIR2DL1 protein expression level in an alleledependent manner. The protein expression level as well as the frequency of KIR2DL1+ NK cells in donors with one KIR2DL1 gene copy turned out to be highest in KIR2DL1*001/002, followed by *003/034 and *004, with the latter showing the lowest KIR2DL1 cell surface expression as well as the lowest frequency of KIR2DL1+ NK cells in peripheral blood. For donors with a KIR2DL1 gene copy number of two, the “additional” KIR2DL1 allele impacts both cell surface expression level and KIR2DL1+ NK cell frequency again. Although the proportion of KIR2DL1+ cells among the entire NK population is increased regardless of the allele added, the median KIR2DL1 protein expression was positively (*001/002) and negatively (*004) affected by the specific “second” allele. Furthermore we observed an allele-independent calibration of KIR2DL1 expression by the HLA-C2 ligand status: KIR2DL1 cell surface expression level is highest in donors bearing C1/C1, medium in C1/C2 and lowest in C2/C2 across all KIR2DL1 alleles. Finally, we assessed NK subpopulations defined by CD8-coexpression. CD8 expression on NK-cells may increase their cytolytic capacity. Notably, regulation of KIR2DL1 expression on the two subpopulations of NK-cells defined as CD8neg and CD8dim was similar. Also, we did not observe a significant influence of age and sex of the donors on KIR2DL1 expression level and KIR2DL1+ NK cell frequency. Together with results from functional assays, these data may help to assess the level of NK cell inhibition mediated by KIR2DL1 for various immunological responses to infections and malignancies.

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THE EFFECT OF ACUTE AND SUSTAINED BETA2-ADRENERGIC RECEPTOR STIMULATION ON THE FUNCTION OF NATURAL KILLER CELLS

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Epinephrine is a neurotransmitter, which is produced by the adrenal glands and adrenergic neurons. It plays a crucial role during acute stress in the so-called fight-or-flight response. Additionally, epinephrine can act on cells of the immune system. The number of lymphocytes in peripheral blood increases after acute exposure to epinephrine. More importantly, the outcome of cancer therapies and major surgery can be influenced by epinephrine-mediated effects on the immune system.

Natural Killer (NK) cells are innate lymphoid cells that are involved in the control of viral infection and tumors. NK cells respond to epinephrine mainly through beta2-adrenergic receptor (beta2AR) signaling, which leads to increased cAMP levels in the cytoplasm. Our data show that beta2AR stimulation affects signal transduction of activating NK cell receptors. As a result, acute exposure of NK cells to epinephrine reduces NK cell cytotoxicity and production of interferon-gamma. More importantly, epinephrine blocks the affinity increase of the adhesion protein LFA-1, which can be induced by inside-out signaling through the stimulation of activating NK cell receptors. The beta2AR mediated inhibition can be reverted by the PKA inhibitor H89. This suggests that PKA signaling after beta2AR stimulation interferes with LFA-1 mediated NK cell adhesion. Interestingly, chronic exposure to beta2AR agonists did not interfere with beta2AR expression, but completely prevented the inhibitory effects of acute epinephrine stimulation on NK cell functions. Therefore, acute and chronic exposure to epinephrine can have very different effects on the function of immune cells, which may explain some effects of acute and chronic stress on the immune system

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UNPARALLELED RAPID EVOLUTION OF KIR GENES BETWEEN RHESUS AND CYNOMOLGUS MACAQUES

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The killer cell immunoglobulin-like receptors (KIR) are expressed on NK-cells and module immune responses through interactions with MHC class I molecules. Previous characterization of the KIR genes in human and rhesus macaques (Macaca mulatta), who share a common ancestor approximately 25-33 million years ago, showed rapid evolution of the KIR gene cluster, which is reflected by few orthologs, variation in haplotype architecture, and differential gene lineage expansion. In part, this rapid evolution could be explained by a diverged MHC repertoire, and by pathogendriven selective pressure.

In the present study, we used Single Molecule Real-Time (SMRT) sequencing on a PacBio Sequel platform to characterize the KIR genes of cynomolgus macaques (Macaca fascicularis). Rhesus and cynomolgus macaques share a common ancestor that lived approximately 1-3 million years ago. The habitats of both species share a hybrid zone, in which bidirectional introgression has been observed. Also, natural barriers distributed the species into geographically distinct populations, such as the Indian and Chinese rhesus macaques, and the Mauritian cynomolgus macaques, and resulted in intraspecific variation.

Comparison of the KIR genes revealed that rhesus and cynomolgus macaques share only a few KIR orthologs, whereas most of the KIR repertoire is species-specific. Even more, different populations of the rhesus and cynomolgus macaques show abundant variation in their KIR gene repertoire, indicating intra-species evolution at the population level. This variation is mainly reflected by differential gene distribution, and population-specific KIR gene content. These observations suggest an unparalleled rapid evolution of the highly polymorphic KIR gene cluster in closely related macaque species that live in different habitats.

SESSION 2
NK Cell Development and Differentiation

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A20 CONTROLS NK CELL HOMEOSTASIS THROUGH REGULATION OF MTOR PATHWAY AND TNF

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The ubiquitin-editing enzyme A20 (encoded by the TNFAIP3 gene) is a well-known regulator of immune cell function and homeostasis. As the main negative regulator of the NFκB pathway, it provides protection against profound inflammation, thus playing a key role in several auto-immune disorders. In addition, A20 protects cells from death in an ill-defined manner. While most studies focus on its role in the TNF-receptor complex, we here identify a novel component in the A20-mediated decision between life and death.

By making use of the Cre-loxP technology, we generated the NK-A20 mouse line that specifically lacks A20 in NKp46- expressing cells. Loss of A20 in NK cells lead to spontaneous NK cell death and severe NK cell lymphopenia in all organs examined. The few remaining NK cells showed an immature, hyperactivated phenotype, hallmarked by the basal release of cytokines and cytotoxic molecules. A20-deficient NK cells showed to be hypersensitive to TNFinduced cell death and could, at least partially, be rescued by a combined deficiency with TNF (both with a chemical and a genetic approach). Unexpectedly, also rapamycin, a well-established inhibitor of mTOR, strongly protected A20- deficient NK cells from death, and further studies revealed that A20 restricts mTOR activation in NK cells.

Together, these data establish an indispensable role for A20 in NK cell activation and homeostasis. Most importantly, this study maps A20 as a crucial regulator of mTOR signaling, and underscores the need for a tightly balanced mTOR pathway in NK cell homeostasis. Being exquisitely sensitive to balances between inhibitory and activating signals, we postulate that NK cells are particularly vulnerable to any dysregulation of these balances and therefore prone to death upon loss of a crucial regulator like A20.

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ARRESTED NK CELL DIFFERENTIATION IN PATIENTS WITH DIVERSE CLINICAL SUBTYPES OF NEUTROPENIA

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Neutrophils are innate cells that have been suggested to play a critical role in terminal differentiation of NK cells. Whether this is a direct effect or a consequence of global immune changes with effects on NK cell homeostasis remains unknown. Here, we used high-resolution flow and mass cytometry to examine NK cell repertoires in 54 adult neutropenic patients, 11 neutropenic children, and 28 healthy age- and gender-matched controls. Diagnoses included T-cell large granular lymphoma and congenital, ethnic, and familial neutropenia, with autoimmune and idiopathic neutropenia being most common. A subgroup of neutropenic patients had lower frequencies and absolute numbers of NK cells, yet increased frequencies of CD56bright among NK cells. Moreover, their CD56dim compartment was characterized by a block in differentiation, with a relative lack of NKG2A-CD57+KIR+ NK cells. In line with the differentiation arrest, no expansion of adaptive NK cells could be detected in CMV-seropositive patients from this subgroup. Furthermore, CD56dim NK cells showed increased frequencies of Ki-67, CD38, HLA-DR, Tim-3 and TIGIT+ cells suggestive of proliferation, activation and exhaustion. The systemic imprint in the NK cell repertoire was associated with a blunted tumor target cell response with inefficient killing and a lower proportion of multifunctional CD56dim cells. Systems level immune mapping by mass cytometry revealed that the dramatic changes in the NK cell compartment were rather isolated with no impact on T-, B- or myeloid cells. Serum protein profiling of 239 proteins showed upregulation of pathways related to apoptosis and cell turnover, as well as immune regulation and inflammation including higher levels of IL-10, IL-18 and IL-27. Indeed, CD56dim NK cells displayed decreased Bcl- 2/BIM ratio, suggesting a shift towards pro-apoptotic pathways. RNA sequencing of the NK cell compartment further revealed that the differentiation arrest was linked to increased expression of the transcription factors TCF7 and LEF1 and genes involved in proliferation and cytokine signaling. Notably, the majority of patients with perturbed NK cell compartment exhibited high-grade neutropenia, overall suggesting that the profoundly altered NK cell homeostasis was tightly connected to the severity of the underlying etiology.

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CD27-CD70 INTERACTIONS PROMOTE MATURATION OF LIVER-RESIDENT NK CELLS BY CD8+ T CELLS

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Previous studies have shown a unique subset of NK cells characterized by CD49a+CD49b- phenotype, which is resident in the liver and can confer a memory-like response. Liver-resident NK (LrNK) cells and conventional NK (cNK) cells also differ in terms of their progenitor origin and their developmental requirements. However, despite recent progress in this field, the maturation program of LrNK cells and its underlying mechanisms remain unknown.

In this study, we found that the absence of CD8+ T cells results in phenotypic changes in LrNK cells without affecting their number. LrNK cells of CD8-/- mice expressed relatively lower KLRG1, CD11b, CD11c, but higher levels of CD27, CD127 and CD49d than those of WT mice.Expression of cytotoxic-associated molecules, such as Grzms, TRAIL, was reduced in LrNK cells of CD8-/- mice, which were less efficient in killing Yac-1 target cells than those of WT mice. However, the phenotype and functions of cNK cells were not significantly affected by CD8 deficiency. Adoptive transfer and culture experiments revealed that CD27- LrNK cells, which are reduced in CD8-/- mice, were more mature than CD27+ LrNK cells. The provision of CD8+ T cells to CD8-/- or Rag1-/- mice led to the restoration of LrNK cell phenotype and function, suggesting that CD8+ T cells promote LrNK cell maturation. Co-culture with CD8+ T cells induced immature CD27+ LrNK cells to convert into mature CD27- LrNK cells. Additionally, the blockade of CD70- CD27 interaction abrogated the ability of CD8+ T cells to promote the maturation of LrNK cells. Consequently, our study revealed that CD8+ T cells promote the maturation of LrNK cells via direct cell contact, thus providing new insight into the key mechanisms mediating LrNK cell maturation.

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DISTINCT HLA-E PEPTIDE COMPLEXES MODIFY ANTIBODY-DRIVEN EFFECTOR FUNCTIONS OF ADAPTIVE NK CELLS AND RECONFIGURE COMPONENTS OF INTRACELLULAR SIGNALING

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Adaptive NK cells are characterized by profound alterations in multiple signalling molecules, transcription factors and epigenetic modifications compared to canonical NK cells. While their existence is associated with prior exposure to HCMV, key questions regarding their regulation and function remain open. A large proportion of adaptive NK cells express the activating receptor CD94/NKG2C binding to HLA-E, a non-classical HLA-molecule presenting a limited set of peptides. To address these questions, we relied on a highly reductionistic system with recombinantly expressed single-chain peptide-beta2-microglobulin-HLA-E complexes, whereas functional analyses were conducted with sorted NK cell subpopulations. This approach allowed us to circumvent the pitfalls and complexities inherent to studies relying on peptide-pulsed HLA-E transfectants and/or analysis of adaptive NK cells as a part of bulk NK cell populations. We show that adaptive NK cells discriminate subtle differences between HLA-E-peptide complexes with exquisite specificity. Recognition of one particular HLA-E peptide ligand, VMAPRTLFL, derived from the leader sequence of HLA-G led to selective upregulation of CD25 in adaptive but not in canonical NK cells. Prolonged exposure to an environment displaying HLA-E[pHLA-G] complexes, enriched adaptive NK cells with low FceRg expression, increased proliferative activity and resulted in a heightened capability for antibody-dependent cellular cytotoxicity (ADCC) and IFN-gamma responses compared to other HLA-E peptide complexes. Ongoing studies decipher the impact of distinct HLA-E-bound peptides on the expression levels of key adaptor molecules and transcription factors in adaptive NK cells. Our study suggests that recognition of alterations in the HLA-E ligandome via the activating receptor NKG2C profoundly influences heterologous effector mechanisms, proliferation and intracellular signatures of adaptive NK cells.

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EOMES IS CRITICAL FOR MATURE MURINE AND HUMAN NK CELL HOMEOSTASIS AND FUNCTION

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Eomes is a transcription factor critical for regulating NK cell development, as mice with hematopoietic or ILC-specific deletion are NK cell deficient. However, this loss of the NK cell lineage precludes genetic loss-of-function studies, leaving a gap in our knowledge about Eomes's role in mature NK cell homeostasis and function. To address this, we generated mice with ILC-specific and tamoxifen-inducible Eomes deletion, and used CRISPR/Cas9 to delete Eomes from mature human NK (hNK) cells.

Eomes-deleted mice exhibited an acquired NK cell deficiency, with predominant loss of stage III and lesser effects on terminally differentiated stage IV NK cells. Mechanisms responsible for this include impaired in vivo NK cell maturation from Stage III to Stage IV, increased apoptosis of stage II and III cells, as well as selective alterations of stage III cell division. In regards NK cell cytotoxicity, mice with induced Eomes deletion exhibited a lack of in vivo rejection of B2M- /- cells and their in vitro cytotoxicity against RMA-S targets was also defective, while interestingly their IFN-γ and CD107a responses were intact. These data demonstrated a critical role for the Eomes program in mature murine NK cells homeostasis and rejection of MHC class I deficient targets.

We next investigated primary hNK cells from healthy donors via genetic deletion of Eomes using CRISPR/Cas9, where we obtained >90% efficiency of Eomes deletion in both CD56bright (stage IV) and CD56dim (stage V) NK cells. Consistent with our inducible mouse model, subset specific defects in cytotoxicity in Eomes-/- hNK cells were observed. CD56dim NK cells do not require Eomes for IFN-γand TNF production upon stimulation by tumor cell line or cytokines. In contrast, Eomes-deleted CD56bright NK cells have impaired IFN-γ response to cytokine stimulation. For both CD56dim and CD56bright NK cells, Eomes was dispensable for cytotoxicity against K562 tumor targets in vitro. Notably, we observed a compensatory upregulation of T-bet (median 1.7-fold in CD56bright, $P<0.002$; 1.25-fold in CD56dim, $P<0.002$) in Eomes-/- hNK cells, that may provide a redundant "fail safe" for Tbox transcription factordependent gene expression and the hNK cell functional program.

Thus, induced Eomes deletion in mature murine NK cells had profound effects on stage II/III NK cell homeostasis and function, while the impact of Eomes deletion on hNK cells was limited to CD56bright NK cells and more modest. Further investigation of inducible ILC-specific deletion of Eomes and T-bet on responses to viral infection and tumors are warranted.

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GLUCOCORTICOIDS PROMOTE NK CELL DIFFERENTIATION AND PREVENT ILC3S DEVELOPMENT FROM HUMAN CD34+ HEMATOPOIETIC STEM CELLS

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Allogenic hematopoietic stem cell transplantation is utilized in the therapy of malignant diseases of the hematopoietic system to reconstitute lymphopoiesis or hemopoiesis compromised by myeloablative therapy. Glucocorticoids (GCs) are steroid hormones which are used in their syntetic forms (corticosteroids) as anti-inflammatory agents in the clinical practice. Serious complications of allogeneic hematopoietic stem cell transplantation, such as graft-versus-host disease (GVHD), are often treated by giving GCs to patients. While it has been shown that GCs enhance hematopoietic stem cells homing and engraftment, their effects on the plasticity of Innate Lymphoid Cell (ILC) precursor differentiation has not been investigated so far.

CD34+ hematopoietic stem cells isolated from the peripheral blood apheresis of Granulocyte colony-stimulating factor (G-CSF)-mobilized healthy donors were cultured in vitro with Stem cell factor (SCF), Fms-like tyrosine kinase 3 ligand (Flt3L), Interleukin 15 (IL-15) and IL-7 to induce the differentiation towards Natural Killer (NK) cells and ILC3s, which share a common developmental pathway. We found that GCs qualitatively regulate the differentiation of the NK/ILC3 precursors, characterized by a CD34- CD56+ CD161+ CD94- immunophenotype. GCs promote the development of Eomes+ CD94+ NK cells with a high cytotoxic potential but low cytokine production, while inhibiting the differentiation of functional Rorgt+ CD127+ IL22-producing ILC3s. Mechanistically, we found that GCs downregulate the expression of the Aryl hydrocarbon receptor (AHR), a transcription factor required for NK/ILC3s precursor differentiation into ILC3s.

Identifying the effects of GCs on immune cell reconstitution after hematopoietic stem cell transplantation may help to improve their use in the treatment of transplantation complications.

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GRADUAL ACQUISITION OF NK CELLS MATURE PHENOTYPE AFTER AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION IN MULTIPLE MYELOMA

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INTRODUCTION:
Autologous hematopoietic stem cell transplantation (aHSCT) is a well-established therapy in the treatment of a wide range of cancers, such as multiple myeloma. An effective and rapid reconstitution of the immune system after transplantation is a key factor for treatment success. Specially, higher number of natural killer (NK) cells at day 15 after the aHSCT has been associated with better prognosis. However, the implication of NK cells in the success of aHSCT is still poorly understood. Furthermore, the identification of novel and better biomarkers associated with the prognosis of the disease and the success of the transplantation is needed.

METHODS:
Samples from patients with multiple myeloma were obtained from Cruces University Hospital, Donostia University Hospital and Galdakao Hospital. Samples were collected before and up to one year after aHSCT. Using multiparametric flow cytometry, the expression of surface, cytolytic and proliferation markers, in addition to transcription factor expression was determined on NK cell subsets. Functional assays were also carried out to measure degranulation and cytokine production.

RESULTS:
Right after the aHSCT the percentage of CD56bright NK cells increases, while the frequency of CD56dim and CD56neg NK cells decreases. CD56dim and CD56neg NK cells express a more immature phenotype immediately after aHSCT, identified by a high frequency of NKG2A+ and a lower percentage of CD57+ NK cells, which persists until more than a month after the aHSCT was performed. This immature phenotype can also be confirmed on CD56dim NK cells by a decreased expression of Granzyme B immediately after aHSCT. In the case of CD56bright cells, a rare CD57+ subset is found right after the aHSCT. Furthermore, all NK cell subsets have high in vivo proliferation rate immediately after aHSCT, as demonstrated by the expression of Ki67. However, all subsets are less activated (CD69+) at this time point. The percentage of adaptive NK cells is also measured after aHSCT. Finally, NK cells exhibit functional capabilities (degranulation and production of IFNγ and TNF) although right after aHSCT these capabilities tended to be lower.

CONCLUSION:
NK cells do not express a mature phenotype and complete functional capabilities right after aHSCT. This mature phenotype is acquired gradually after aHSCT.

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IL-1 AND NOT IL-15 RESCUES IMPAIRED NK CELL CYTOTOXICITY IN PATIENTS WITH STAT5B MUTATION

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INTRODUCTION:
Signal transducer and activator of transcription (STAT) 5b is critical for NK cell development, maturation, survival, and proliferation. STAT5b deficient patients have low numbers of NK cells with poor lytic function leading to viral susceptibility. In this study, we explored the lytic granule polarization and target cell killing in human STAT5b deficient NK cells.

METHODS:
We used multiparametric immune profiling to dissect the effect of Stat5b deficiency on NK cell developmental phenotype in STAT5b-deficient patients. Cytokine production was evaluated by FC. Conjugate formation of NK cells to K562 target cells was evaluated by FC and confocal microscopy. NK cell cytotoxicity was measured by standard Cr51 release assay.

RESULTS:
STAT5b-deficient patients showed a significant decrease in total NK cell numbers. Impaired NK cell maturation was characterized by low cytokine production, low expression of adhesion ligands and activation markers important for lytic synapse formation and granule convergence, higher levels of immature NK cell markers and abrogated terminal maturation. Stat5b-deficient NK cells that did form conjugates had decreased convergence of lytic granules to the microtubule-organizing center (MTOC) affecting the NK cell cytotoxic capacity. Interestingly, granule convergence and cytolytic function was restored after IL-2 stimulation but not IL-15 stimulation.

CONCLUSIONS:
Human NK cells deficient in STAT5b have abnormal development. The absence of STAT5b leads to aberrant maturation as well as impaired early activation events in NK cell lytic synapse formation. IL-2 and not IL-15 partially rescues the killing capability. Thus, these data suggest restored granule convergence and partial improvement in killing likely happens through an alternate non-canonical IL-2 pathway.

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KIR PROMOTER DEMETHYLATION IS AN ACTIVE PROCESS CONTROLLED BY TET DIOXYGENASES AND FACILITATED BY VITAMIN C

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Killer immunoglobulin-like receptors (KIR) are expressed in a tightly regulated, stochastic manner after commitment of cells to the NK cell lineage. While it is well established that DNA methylation controls allele-specific KIR gene expression in human NK cells, the mechanisms that regulate KIR promoter methylation are unknown. Using cell sorting and bisulfite sequencing with primers specific for KIR genes with variegated expression, we show that KIR promoters are heavily methylated in CD56bright NK cells and become increasingly demethylated through a continuum of differentiation that ends with a CD56dimCD94low phenotype. Ex vivo culture of sorted CD56bright NK cells with vitamin C revealed a dose-dependent increase in KIR promoter DNA demethylation and KIR surface expression that could be blocked using a small molecule inhibitor of the vitamin C transporter SVCT2. Vitamin C has recently been identified as an enzymatic catalyst for members of the ten-eleven translocation (TET) family, which function as dioxygenases and play a key role in active DNA demethylation. We show that TET2 and TET3 bind proximal KIR promoters in primary human NK cells, and this binding is significantly enhanced during culture with vitamin C. Intriguingly, RUNX3 was also markedly enriched within KIR promoters in NK cells cultured with vitamin C. Previous work has shown that a single nucleotide substitution in the RUNX3-binding site within the proximal promoter of an allele of KIR2DL5 is associated with gene silencing that could be reversed by pharmacological demethylation with 5- aza-2'-deoxycytidine. Thus, RUNX3 may play an important role in recruiting TET2 and TET3 for KIR promoter demethylation. To confirm the association between activation of TET3 by vitamin C and induction of KIR expression, we transduced NK92 cells with either a control vector containing GFP alone or with a TET3 overexpression vector. Treatment of the TET3 overexpressing NK92 cells with vitamin C led to a significant induction of KIR surface expression, while control NK92 cells treated with vitamin C remained KIR-negative. To determine whether vitamin C treatment was associated with genome wide alterations in DNA methylation and large-scale transcriptional changes, we performed whole genome DNA methylation arrays and RNA-seq on primary NK cells cultured with and without vitamin C. To our surprise, the DNA methylation and transcriptional profiles were nearly identical between the treated and untreated groups. Our work provides new mechanistic insights into KIR transcriptional regulation.

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MESENCHYMAL STROMAL CELLS AND NOTCH LIGANDS SUPPORT THE SEQUENTIAL ACQUISITION OF CD94 AND NKP80 DURING HUMAN NK CELL DEVELOPMENT

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Natural killer (NK) cells are cytotoxic innate lymphoid cells (ILCs) that mediate innate immune responses against infection and malignancy. In humans, NK cells develop from common ILC precursor cells (ILCPs) that are enriched in secondary lymphoid tissues. Downstream of ILCPs, developing NK cells sequentially acquire surface CD94 and then NKp80 expression, the latter of which correlates with EOMES, perforin, and IFN-gamma expression. It is not yet known how these steps in human NK cell development are regulated. Previous work in the field has shown that Notch signaling can modulate NK cell development and function. We hypothesized that Notch regulates human NK cell development from ILCPs. To test this hypothesis, we first cultured tonsil-derived ILCPs for 14 days with human interleukin (IL)-7 in the presence or absence of murine OP9 bone marrow-derived mesenchymal stromal cells lacking or overexpressing the human Notch ligand, delta-like-1 (DL1). ILCPs cultured with OP9 stroma produced significantly higher percentages of CD94+NKp80- NK cells compared to ILCPs cultured in IL-7 alone (10.1 ± 1.3% versus 0.29 ± 0.08%, n = 19, $p < 0.001$). Furthermore, ILCPs cultured with OP9-DL1 stroma gave rise to significantly greater percentages of CD94+NKp80+ cells compared to ILCPs cultured with OP9 stroma (3.79 ± 0.45% versus 0.79 ± 0.15%, n = 19, $p < 0.001$). These results suggested that OP9 stromal cells and Notch signaling supported the sequential acquisition of CD94 and NKp80, respectively, during in vitro NK cell differentiation from ILCPs. Next we cultured tonsil-derived CD94+NKp80- immature NK cells with IL-7 and either OP9 or OP9-DL1 stroma. Immature NK cells cultured in the presence of OP9-DL1 stroma generated significantly greater percentages of NKp80+ cells in the presence of OP9-DL1 stroma compared to in the presence of OP9 stroma (13.1 ± 1.5% versus 4.30 ± 0.74%, n = 18, $p < 0.001$). In these experiments, treatment with the Notch inhibitor DAPT significantly inhibited the acquisition of NKp80 compared to DMSO vehicle control (3.97 ± 0.85% versus 17.1 ± 2.9%, n = 9, $p < 0.001$). Similar to tonsilderived mature CD94+NKp80+ NK cells, culture-derived CD94+NKp80+ cells expressed EOMES, perforin, and IFNgamma. Collectively, these data support a mechanistic model in which mesenchymal stromal cells and Notch signaling can promote the sequential acquisition of CD94 and NKp80 during human NK cell differentiation.

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MOUSE GENETIC BACKGROUND INFLUENCES NK CELL FUNCTION THROUGHOUT THEIR FUNCTIONAL MATURATION PROCESS

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NK cells constitute a population of innate lymphoid cells important in antitumor immunosurveillance and viral clearance. NK cells acquire their functions through a four-step functional maturation process defined by the sequential expression of CD27 and CD11b, with the CD11b+CD27- phenotype representing the most mature stage. We have demonstrated that NK cell functional maturation is influenced by the mouse strain genetic background, where NOD mice exhibit less functionally mature NK cells (CD11b+CD27-), compared to their B6 counterparts. This decrease in NK cell functional maturation in NOD mice is in line with previous work showing that NOD NK cells exhibit less cytotoxic activity. To extend on these findings, we aimed to determine whether the decrease in NK cell cytotoxic activity was a direct consequence of a reduction in CD11b+CD27- functionally mature NK cells. The expression of key cytotoxic effectors (IFN-γ, TNFα and Granzyme B) was analyzed by flow cytometry throughout the four-step functional maturation process. We surprisingly observed a significant decrease in IFN-γ, TNFα and Granzyme B throughout the NK cell functional maturation in NOD relative to B6 mice. The decrease in NK cell cytotoxic effector functions were also observed in NOD.Rag-/- vs B6.Rag-/- mice, and are thus independent of T cells, B cells, and autoimmunity. NOD NK cells also do not up-regulate NKG2D in response to inflammatory signals. Finally, to determine if these phenotypic traits impact NK cell function in vivo, we assessed their ability to eliminate B16 melanoma in F1. Rag-/-.IL2Rg-/- (B6.Rag-/-.IL2Rg-/- x NOD.Rag-/-.IL2Rg-/-) mice. To account for MHC differences, we compared NK cells from B6.H2g7 and NOD mice. Tumour growth was significantly greater in mice injected with NK cells from NOD as opposed to B6.H2g7 mice. Altogether, this study demonstrates that the genetic background can influence NK cell functional maturation and bear a significant impact on NK cell function. Additional studies are required to identify which genetic polymorphisms or molecular pathways determine NK cell function.

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NATURAL KILLER CELL PRIMING BY SHORT-TIME IL-25 STIMULATION IS LONG LASTING AND MEDIATED VIA JAK3 AND REACTIVE 1 OXYGEN SPECIES

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Interleukin-15 (IL-15) plays an essential role in natural killer (NK) cell biology, including their development, maturation, homeostatic proliferation and priming. Priming of NK cell function by IL-15 is a particularly interesting process, since it represents a modulation of signaling thresholds in mature NK cells that have adaptive features. Dendritic cells (DC) are able to prime NK cells in lymph nodes by means of trans-presentation of IL-15, but the dynamics and the molecular mechanism of NK cell priming by IL-15 is not fully characterized. Intriguingly, more than 99 % of the NK/DC contacts in lymph nodes are shorter than 15 minutes, suggesting that NK cell priming is a rapid event. Here, we show that 5 minutes of IL-15 pre-treatment was sufficient to augment IFNy production, degranulation and calcium flux, induced by subsequent activating receptor crosslinking. Phosphoflow experiments demonstrated that IL-15 stimulations not only induced phosphorylation of conventional IL-15 receptor downstream molecules, such as STAT5, p38 and ERK, but also signaling molecules directly downstream of ITAM-encoded activating receptors, such as LCK and SLP-76. A proximal crosstalk between IL-15R and activating receptors in NK cells was suggested by a dampening effect of IL-15 priming by inhibitors of JAK3. Short-time priming effects were also modulated via reactive oxygen species. Furthermore, the priming effect on IFNy production and degranulation was long lasting, since augmented calcium flux responses were detected for 3 hours after removal of IL-15. Our study extends understandings of NK cell biology provide useful information relevant for the production of potent NK cells for immune therapies.

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NEONATAL BLOOD CONTAINS AN ID3+CD117- NK CELL PROGENITOR THAT PREFERENTIALLY DIFFERENTIATES INTO KIR+ NK CELLS

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The final stages of NK cell development are characterized by the expression of CD94:NKG2A followed by more mature NK cells downregulating NKG2A and generating complex KIR repertoires. Although in vitro models of NK cell differentiation generally lead to efficient generation of NKG2A+ NK cells, further maturation into KIR-expressing NK cells is only poorly supported. In the present study, we identified a novel lin-CD127+CD117- NK cell progenitor in cord blood (CB) that is closely recapitulating NK cell repertoires in vivo. Using OP9-DL1 stroma cell support, the majority of NK cells generated in vitro from lin-CD127+CD117- progenitors expressed KIR, either with or without co-expression of NKG2A. The latter NKG2A-KIR+ subset constitutes a hallmark of late NK cell stages that are commonly not generated from other progenitor sources. Notably, the frequency of the novel progenitor population significantly decreased with gestational age suggesting a preferential role during fetal development. Next, transcriptional profiles of the novel lin- CD127+CD117- subset were generated by RNAseq and compared to those of stage 2 (CD34+CD117+), stage 3 (CD34-CD117+), and stage 4 (CD94+CD56bright) progenitors from tonsils as well as CB. Among the factors that are described to regulate NK cell fate, expression of inhibitor of DNA binding 3 (Id3) was exclusively found in the novel CD127+CD117- progenitor from CB whereas the other NK cell progenitors mainly expressed ID2. Unexpectedly, the population exhibited also surface expression of the T cell markers CD5 and CD6 and showed frequent recombination of V and V TCR segments but no surface expression of the TCR complex. The latter phenotype might suggest a thymic origin of the lin-CD127+CD117- progenitor subset. Together with the strong expression of the chemokine receptor CCR7, a model emerges in which lin-CD127+CD117- progenitors constitute recent thymic emigrants that circulate in neonatal blood before entering secondary lymph nodes (SLN) for final maturation. Early seeding of this novel progenitor population to SLN might be instrumental for later expression of diversified KIR repertoires in the periphery.

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NK CELL DEVELOPMENT IN HSCT PATIENTS RECEIVING T CELL-DEPLETE GRAFTS: A MODEL TO STUDY THE EMERGENCE OF NKG2C+ NK CELLS

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The expansion of NKG2C+ NK cell populations after HCMV infection in healthy individuals or after HCMV reactivation in the hematopietic stem cell transplantation (HSCT) setting has been well-documented. Pre-existing NKG2C+ NK cells have been successfully expanded in vitro. However, the mechanisms underlying the emergence of these cells remain unknown.

HSCT with CD34+-selected allografts provide the most physiological environment to study NK cell development in context of human cytomegalovirus (HCMV) infection due to the high rates of HCMV reactivation in HCMV-seropositive individuals. We investigated NK cell development in HSCT patients receiving CD34+-selected (T-cell depleted or TCD, n=97), unmodified (T-cell repleted, n=57) and umbilical cord blood (UCB, n=52) allografts by flow cytometry at sequential time points after stem cell infusion (day 15, 30, 60, 100, 200, 270 and 365). We found that in all recipients of TCD allografts, most NK cells remain NKG2A+ (>90%) after HCMV reactivation, which occurred between day 20 and 60 post-transplantation. It was only after detection of T cells, occurring between day 50 and 200 posttransplantation, that NKG2C+ NK cells emerged. Once T cells became detectable, NKG2C+ NK cells expanded rapidly to high frequencies (34.6±18.1%). For recipients that did not experience HCMV reactivation, NK cells remained NKG2A+ before and after T cell reconstitution. In contrast, in recipients of unmodified allografts and UCB grafts, NKG2C+ NK cell expansion was not observed consistently with lower frequencies after HCMV reactivation (19.4±17.3% and 17.0±13.3%), where T cells were detectable in the patient immediately following transplantation. In recipients with TCD allografts we observed a fast transition in NK cell phenotype from most NK cells expressing CD56brightNKG2A+CD16+KIR+ to NK cells expressing CD56dimNKG2A-CD16+NKG2C+FCεR1+CD57+KIR2DL+ after HCMV reactivation and T cell reconstitution. Interestingly, 'adaptive' NKG2C+FCεR1-CD57+ NK cells were detected in only a small number of patients and then only at late time points post-HSCT, predominantly in recipients of TCD allografts.

Given these results, we hypothesize that the absence of T cells in the early phases of TCD allograft transplantation results in longer exposure to and a higher likelihood of NK cells to be primed by HCMV, leading to a more frequent and rapid expansion of NKG2C+FCεR1+ NK cells after T cell reconstitution.

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REGULATORY CIRCUITRY OF HUMAN NK CELL DIFFERENTIATION

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Understanding the epigenetic landscape underlying human natural killer (NK) cell differentiation can provide insight into novel features of NK cell biology, gene regulation and functional diversity. Here, we examined the transcriptional and epigenetic profiles of seven human blood NK cell subpopulations spanning NK cell differentiation and education alongside CD8+ T cells. These include CD56bright NK cells, CD56dim subpopulations defined by NKG2A, CD57 and KIR phenotypes as well as cytomegalovirus infection-associated adaptive cells. Combining ATAC-seq and H3K27ac ChIP-seq we characterized open-chromatin regions associated with transcription factor (TF) binding and active enhancers that dictate subset specific gene expression. These landscapes illustrate an NK cell differentiation axis with CD56bright cells that segregated CD56dimNKG2A+CD57- NK cells from other subpopulations whilst adaptive NK cells pivoted towards cytotoxic CD8+ T cells. Analysis of sequence specific TF motifs at open chromatin regions overlapping active enhancers revealed RUNX, Bach2 and TCF signatures within the early NK cell compartment alongside enrichment of NF-kB. A framework of differentially regulated enhancers, gene expression and additional ChIP-seq of selected TFs was used to construct de novo regulatory networks at key stages of NK cell differentiation. This indicated maintenance of NK cell identity by reciprocal gene regulation with Runx2 and Bach2 acting as hubs in CD56bright NK cells. In comparison, PLZF promoted canonical CD56dim NK cell gene programs together with Bcl11b. Accumulation of Bcl11b throughout the NK cell differentiation axis correlated with their epigenetic and transcriptional progression towards T cells. An important role for Bcl11b was further corroborated by analyses of a BCL11B haploinsufficient patient which displayed impaired CD56dim NK cell differentiation. Together, we provide a contextualized and comprehensive epigenetic perspective of peripheral NK cell differentiation, define regulatory circuitry and uncover mechanisms of subset specific functional competence. Our results highlight an unexpected role for Bcl11b in promoting differentiation towards adaptive NK cells.

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SINGLE CELL PROFILING REVEALS UNIQUE STAGES OF NK CELL DEVELOPMENT AND DIFFERENTIATION IN THE BONE MARROW

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Natural killer (NK) cells are potent cytotoxic lymphocytes that provide critical host defense against pathogens and malignancy. Current understanding of the molecular and cellular events that define the stage-wise progression of NK cell development is limited. Here, we use single-cell RNA sequencing (scRNA-seq) to investigate heterogeneity within the developing NK cell compartment in the bone marrow of mice. Our analyses reveal multiple subpopulations of bone marrow NK cells, representing distinct states of NK cell differentiation and development. These subpopulations express distinct surface markers, transcriptional and epigenetic factors, metabolic enzymes, trafficking and signaling molecules, and cell cycle regulators. We identify the Inhibitor of Apoptosis Protein (IAP) family member, Birc5, as a biomarker for two NK cell subpopulations with an immature and pro-proliferative phenotype. Cell-specific deletion of Birc5 in developing NK cells in vivo leads to a near-complete loss of NK cells in the bone marrow and peripheral organs. Mechanistically, we find that Birc5 is required for cell cycle progression, survival, maturation, and genomic stability in developing NK cells. We show that IL-15 and mTOR signaling postively regulate, and TGF-β signaling negatively regulates, Birc5 expression in NK cells. Collectively, our data reveal novel and distinct states of NK cell development in mice, and define an essential role for Birc5 in generation of the NK cell compartment in vivo. These studies not only provide important insights into the molecular events that underlie NK cell development, but also have the potential to inform NK cell-based therapies aimed at treating cancer and infectious disease in humans.

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THE COLLABORATIVE CROSS: ACCESSING THE GENETICS BEHIND NATURAL KILLER CELL DIVERSITY

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The laboratory mouse serves as a model organism in immunologic research allowing to study the diverse cell-types in depth from lymphoid and non-lymphoid tissues under naïve and pathogenic conditions inaccessible from human subjects. Mouse inbred laboratory strains have the additional advantage to possess little genetic diversity assuring stable phenotypes in controlled environments. However, advantageous on the one hand, this restricted genetic diversity fails to model the breadth of immune variation observed in humans – both under steady-state and disease conditions.

To study this immune variation in NK cells and the genetics behind it, we investigated the NK cell compartment in more than 30 lines from the Collaborative Cross (CC). The CC represents a large collection of recombinant inbred lines derived from 8 parental strains including classic laboratory inbred lines, disease models, as well as wild-derived lines from the three major Mus musculus sub-species, domesticus, musculus, and castaneus.

Our analyses revealed great variation of all phenotypic and functional traits under steady-state condition and upon tumor cell challenge. For example, we found that the absolute numbers of NK cells at steady-state varied by a factor of 25- to more than 50-times between the CC lines depending on the organ. Differentiation states of NK cells as measured by differential cell surface expression of CD27, CD11b and KLRG1 also revealed a great degree of variation present within the CC. Quantitative trait locus-mapping allowed to identify several genomic loci associated with the observed variation in various traits, including NK cell numbers and the expression levels of key NK cell markers, including NKp46.

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TUNING OF NK-SPECIFIC HLA-C EXPRESSION

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A complex system regulating HLA-C expression in NK cells, driven by an NK-specific promoter that produces alternatively spliced variants of the 5´-UTR has been recently identified. Distinct patterns of NK-specific HLA-C transcript splicing are observed in bone marrow, spleen, and peripheral blood, with a high proportion of untranslatable mRNAs lacking exon 1 observed in immature NK cells. In addition, the exon content of the NK-specific 5´-UTR varies across HLA-C alleles, with some exons being allele-specific. In order to investigate the possibility that allelic variation in the 5´-UTR modulates HLA-C expression levels, cDNAs containing three distinct classes of 5´-UTR were compared. Subtle changes in 5´-UTR content had a significant effect on the expression of HLA-C*03, -C*04, and -C*12 cDNA clones, suggesting that alternative splicing can fine-tune the level of protein expression. Chimeric constructs were generated to assess the contribution of the 5´-UTR versus the coding region. This results indicated that differences in protein expression levels between alleles were primarily associated with the HLA-C coding region. Although the HLAC*06 allele has a 5´-regulatory region that is identical to HLA-C*12, it is expressed at a much higher level. The high expression of HLA-C*06 was associated with differences present in the peptide-binding groove encoded by exon 2. Although the impact of allele-specific alternative splicing of NK-Pro transcripts on protein levels is modest when compared with the effect of changes in peptide-loading efficiency, it may represent an additional regulatory mechanism to fine-tune HLA-C levels within NK cells in distinct tissue environments or at different stages of maturation in order to achieve optimal levels of missing-self recognition.

SESSION 3
Innate Lymphoid Cells

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ACTIVATION OF HUMAN INNATE LYMPHOID CELLS BY ACTIVATING RECEPTORS

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Innate lymphoid cells comprise cytotoxic NK cells and different subsets of helper ILCs (ILC1, ILC2, ILC3), mainly described to produce and respond to cytokines. ILC3s have been shown to produce IL-22 and IL-17 and to express RORγt. In human, ILC3s can be further subdivided according to the expression of natural cytotoxicity receptors (NCR). Upon stimulation with cytokines (IL-1β and IL-23), NCR+ ILC3 mainly produce IL-22 and NCR- ILC3s produce IL-17. In addition, there is emerging evidence that receptors expressed on the ILC surface might also mediate ILC responses. The NCR family consists of three members, NKp30, NKp44 and NKp46. Recently, B7-H6 was identified as the tumor cell surface-expressed ligand for NKp30. Accordingly, NKp30 expressed on NK cells has been shown to mediate potent killing of B7-H6+ tumor cells. In addition to NK cells, helper ILCs also express NKp30 but the function, in particular on ILC3s upon interaction with its tumor-expressed ligand B7-H6, is still poorly understood.

In this project, we aim at investigating the effector potential of activating receptors on human ILC3s and their relevance for a putative anti-tumor immune response. We observed that ILC3s were able to respond to tumor cell lines, overexpressing or endogenously expressing B7H6, resulting in the production of high levels of IFN-γ and GMCSF. Inhibiting NKp30 with blocking mAbs revealed an involvement of NKp30 in ILC3-mediated anti-tumor response. Further studies will address anti-tumor activity of tumor-infiltrated ILC3. Our results reveal novel insights into the functionality of activating receptors on ILCs and might help exploiting activating receptors expressed by ILCs for the immunotherapy of cancer in the future.

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ALTERED FREQUENCY AND EFFECTOR FUNCTIONS OF INNATE LYMPHOID CELLS IN MULTIPLE SCLEROSIS

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Multiple sclerosis (MS) is an inflammatory autoimmune disease of the central nervous system (CNS) in which genetic and environmental factors contribute to the breakdown of peripheral immune tolerance. Innate lymphoid cells (ILC) are sentinels of different microenvironments that contribute to immune regulation. Based on their functions and/or cytokine profiles, distinct ILC subsets are considered to be the innate counterparts of the corresponding T-cell subsets. While NK cells mainly fulfill immune-regulatory functions, other ILC-subsets such as type 3 ILCs may drive MS pathology by secretion of pro-inflammatory cytokines including IL-17A, IL-22, and GM-CSF.

Investigating lesion topography in MS patients we could show that proportions of peripheral IL-17 / IL-22-producing lymphoid tissue inducer (LTi) cells, the innate counterpart of TH17 cells were enhanced with exclusively cerebral lesion development. Additionally, increased frequencies of LTi in the cerebrospinal fluid (CSF) of MS patients suggests a contribution of this ILC subset in CNS inflammation. Functional characterization of ILC in the blood as well as the CSF of MS patients and controls revealed disease-related alterations in their cytokine profile; thus, suggesting a role of this subset in MS incidence and/or progression. Additionally, further characterization of their functional properties in the CSF of MS patients and controls by genome wide sequencing using CITE-Seq revealed further disease-related alteration in the ILC compartment. Finally, immune-profiling demonstrated strong and differential effects of distinct immune-modulating therapies on ILC subsets in vivo, further implying the importance of ILCs in CNS autoimmunity.

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GROUP 1 ILCS CHARACTERIZATION AND PLASTICITY IN HEALTHY AND INFLAMED MURINE CNS BARRIERS

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BACKGROUND:
Innate lymphoid cells (ILCs) has emerged as a new family of innate cells with a fundamental role in restoration of tissue integrity, protection against infiltrating pathogens and, tumor immune-surveillance among others. However, studies have also reported on pathogenic roles for the ILCs, particularly in autoimmune diseases, such as multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). Here, we focused on group 1 ILCs, that includes the previously known natural killer (NK) cells and helper like ILC1s, which present phenotypical and cytotoxic differences that may account for previously reported contradictory results with respect of their role in MS pathogenesis.

OBJECTIVE:
To investigate the presence and dynamics of group 1 ILCs (ILC1s, intILC1s and NK cells) in CNS barriers in healthy conditions compared to neuroinflammation.

RESULTS:
We have shown that a fraction of previously considered immature NK cells (CD3-NK1.1+CD27+CD11b-) display an ILC1 phenotype. Indeed, we observed that a quarter of the total NK1.1+ cells present in the CNS are Tbet+Eomes-CD49a+CD49b- ILC1s and CD49a+CD49b+Tbet+Eomes+/- intILC1s. Interestingly, these ILC1s were found not just in brain parenchyma and meninges but also in choroid plexus (CP), where they appear highly overrepresented in steady-state (about 80% of NK1.1+ cells) and disease conditions (about 66 % of NK1.1+ cells). During EAE, ILC1s undergo phenotypical alterations as they modulate the expression of TRAIL and CXCR3, without changing the expression levels of IFN-γ and TNF-α. However, ILC1s did not proliferate in response to inflammation inside the CNS, what could indicate a differentiation of NK cells into ILC1s during inflammation. Preliminary results of in vitro studies showed that splenic NK cells cultivated with IL-15 and TGF-β acquired an increased expression of CD49a without losing of CD49b and Eomes, indicating that NK cells are not fully converting to ILC1s but to intILC1s, which shared features with both NK cells and ILC1s. What is of great interest now if to analyze the immune modulatory and excitotoxic ability of this converted intILC1s and ILC1s in order to understand their role during MS.

CONCLUSION:
The phenotypical characterization of CNS-ILC1s showed the presence of different cell subsets within CNS barriers in healthy but also inflamed conditions, in which alterations of their numbers and phenotype together with plasticity between these groups are observed. Moreover, our data points to a potential immunomodulatory role of these cells.

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GROUP 1 INNATE LYMPHOID CELL DYSREGULATION IN ACUTE MYELOID LEUKEMIA

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Acute myeloid leukemia (AML) is an aggressive hematologic malignancy with a 5-year survival rate of 27.4% despite modern clinical advances. Previous research established the importance of natural killer (NK) cells in controlling or eliminating AML; however, we previously demonstrated that AML inhibits NK cell development and function. Mechanistically, we also showed that AML cells are capable of secreting agonists for the aryl hydrocarbon receptor (AHR) transcription factor whose activation inhibits NK cell maturation and cytolytic functions, thus promoting innate immune evasion by AML. AHR is also necessary for the development and/or maintenance of other ILC subtypes, including ILC1s. In contrast, we have demonstrated that antagonism of the AHR pathway restores NK cell maturation and also enhances AML sensitivity to NK cell-mediated killing, suggesting that AHR inhibitors could provide clinical efficacy in the treatment of AML.

Given these findings, we hypothesized that AML actively inhibits the production of cytolytic NK cells while promoting the development of other ILCs. To test this hypothesis, we evaluated liver-derived group 1 ILCs in a murine model of AML, which contains an Mll partial tandem duplication and homozygous Flt3 internal tandem duplication mutations. We observed a 3-fold increase in the ratios of NCR+CD49a+CD200r+ ILC1s to NCR+CD49a-CD200r- NK cells in the livers of AML-bearing mice. These increased ratios were observed using both spontaneous (0.14:1 +/- 0.02 WT versus 0.6:1 +/- 0.17 AML, n=5, $p<0.05$) and transplant AML (0.12:1 +/- 0.02 WT versus 0.36:1 +/- 0.08 AML, n=5, $p<0.05$) models. The liver ILC1s from both WT and AML-bearing mice were capable of producing IFN-gamma upon stimulation; however, only the ILC1s from AML-bearing mice were also capable of producing IL-2. To begin to address the mechanism of skewed ILC1:NK cell ratios in AML, we assessed the effects of AML on human ILC precursor differentiation ex vivo. Co-culture of human blood-derived ILC precursors with AML cell lines resulted in the formation of CD49a+CD200R+ ILC1-like cells at increased frequencies (22% of CD45+ cells +/- 0.1% with AML vs. 7.5% +/- 1.5% without AML $p <0.05$). Furthermore, formation of this ILC1-like population was inhibited upon treatment with an AHR inhibitor in the presence of AML cells (5.3% +/- 4.3%). Collectively, these findings support a model whereby AML-mediated AHR activation skews group 1 ILC differentiation towards an ILC1 phenotype, possibly resulting in decreased immune surveillance by NK cells. This mechanism provides one reason why AML relapse remains a frequent challenge for clinical success.

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HYPOXIC REGULATION OF NKP46-EXPRESSING INNATE LYMPHOID CELL FATE IN GUT HOMEOSTASIS AND DEFENSE

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Innate lymphoid cells (ILCs) are abundantly located at mucosal sites including the gastrointestinal tract where they contribute to gut homeostasis, through promotion of tissue integrity and induction of protective immune responses. In the gut, the mucosa is in direct contact with microbial ecosystems and the crosstalk between ILC and microbiota play a crucial role in the preservation of a healthy microenvironment and the prevention of gut inflammation. Interestingly, the mucosa in the gut is characterized by low oxygen tension often described as “physiological hypoxia” and oxygen levels further decrease upon colitis. Exposure of cells to low oxygen requires activation of Hypoxia-inducible factors (HIFs), a family of oxygen-sensitive transcription factors, which control cellular adaptation to hypoxia. HIFs, with HIF-1 and HIF-2 as the most prominent members, regulate the transcription of hundreds of genes that encode proteins involved in literally every aspect of cellular or whole body-homeostasis. Our group studies the impact of a hypoxia on the function of NKp46-expressing innate lymphoid cells that comprise ILC1 (including Natural Killer cells) and a subsets ILC3 by genetic targeting of HIFs in NKp46-expressing cells. We observe that HIF-1 deficiency in NKp46- expressing ILCs changes the ILC landscape in the gut towards an increased ILC3/ILC1 already under steady-state conditions. Along with this, the intestinal cytokine profile shows decreased expression of TNFα and INFγ but increased expression of protective IL-22. Furthermore, we observe that HIF-1 deficiency in NKp46-expressing ILCs changes the microbiota composition as characterized by an increased Firmicutes/Bacteroidetes ratio. Finally, in a model of chemically induced colitis HIF-1 deficiency in NKp46-expressing ILCs protects against body weight loss, shortening of the colon length and loss of tissue integrity. In summary, this strongly suggests that oxygen levels and the HIF pathway play a crucial role in ILC differentiation in the context of gut homeostasis and colitis.

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INNATE LYMPHOID CELL DEVELOPMENT IN THE HUMAN INTESTINE

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INTRODUCTION
Following broad exposure to exogenous antigens after birth, the mucosal immune system in infant intestines is characterized by rapid modifications. We have previously shown that immune cell populations in the intestine including NK cells undergo sizeable changes in the first year of life. At present little is known regarding other innate lymphocyte cell (ILC) populations during this dynamic phase of life. ILCs have been described to play an important role in host defense as well as in immune mediated diseases. To determine the ontogeny of intestinal ILCs we characterized and compared ILC populations between infant and adult intestine samples.

METHODS
Human intestinal tissues were processed within 6 hours after procurement during surgeries to correct gastrointestinal congenital abnormalities, reconstruction of ileostomy or tumor resection. Lymphocytes were isolated from epithelium and lamina propria layers. Cell populations were analyzed by 15- parameter flow cytometry (BD LRSFortessa).

RESULTS
Innate lymphoid cells, defined as viable Lineage (Lin)-CD127+CD161+ lymphocytes, were divided into three subpopulations: ILC1s (Lin-CD127+CD161+CRTH2-c-Kit-), ILC2s (Lin-CD127+CD161+CRTH2+) and ILC3s (Lin- CD127+CD161+CRTH2-c-Kit+). ILCs were most numerous in lamina propria layers whereas less ILCs were detected in epithelium of infant and adult intestines. The total ILC population decreased during infancy. In particular frequencies of ILC2s changed, with high numbers at birth and very low numbers in adult intestines. Intestinal ILCs had a tissueresidency phenotype with high CD69 and moderate CD103 expression. ILC3s and ILC1s highly expressed CD56, while ILC2s had a low CD56 expression. Nkp44 was highly expressed on ILC3s, moderate on ILC1s and lowest on ILC2s. Our ongoing studies include analysis of chemokine receptors and functional responses such as cytokine production by ILCs.

CONCLUSION
Our data suggests that ILC populations undergo relevant changes during the first months of life, with high ILC numbers at birth that subsequently decrease during infancy. These dynamic changes in innate lymphoid cell populations might play a crucial role in host defense, development of tolerance, as well as tissue (re)modelling.

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PROFILING INNATE LYMPHOID CELLS IN COLLABORATIVE CROSS MICE TO DECIPHER GENETIC CONTROL OF DIVERSITY

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Innate lymphoid cells (ILC) are involved in many immune processes, from mucosal protection against pathogens to tissue homeostasis and repair. Most studies on murine ILCs have been performed in the classic C57BL/6 background to minimize the impact of genetic diversity on experimental outcomes. In contrast, we seek to maximize genetic diversity to uncover novel genetic traits involved in the homeostasis and function of ILCs.

To this aim, we are working with a model of genetically diverse mouse strains: the Collaborative Cross (CC) mice. These strains were generated by intercrossing 5 classical laboratory strains (A/J, C57BL/6J, 129S1/SvImJ, NOD/ShiLtJ, and NZO/HlLtJ) and 3 wild-derived strains (CAST/EiJ – M.m. castanus, PWK/PhJ – M.m. musculus, and WSB/EiJ – M.m. domesticus): as a result, the genome of each CC strain represents a unique mosaic of the founder's genomes. Therefore, by combining a high genetic diversity with the availability of many genetically identical individuals, the CC strains represent a novel and promising model to study the genetic factors driving ILC diversity.

We monitored several parameters of the three major ILC subsets: NKp46+ T-bet+ Eomes- ILC1, GATA3+ ILC2 and RORgT+ ILC3, in five different organs. Our results show an extensive phenotypic variation for each subset, in terms of absolute numbers, distribution, surface markers as well as transcription factors expression, in all the organs, across the 32 CC lines we analyzed. By correlating this substantial variation to each CC strain's genomic structure, we aim to perform Quantitative Trait Loci (QTL) mapping and identify new genetic factors involved in the regulation of ILC homeostasis and function.

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TGF-BETA DRIVES CONVERSION OF ILC2 TO AN IL-13-PRODUCING ILC3 IN THE HUMAN LIVER

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BACKGROUND:
Innate lymphoid cells (ILCs) have been shown to critically modulate homeostasis and inflammation in numerous tissues. However, little is known regarding ILCs in the human liver.

MATERIAL & METHODS:
Intrahepatic ILCs (non-fibrotic liver (n=18), cirrhotic liver (n=14)) were phenotypically and functionally characterized by flow cytometry in comparison to tonsil (n=6), colon (n=14), and peripheral blood (n=32) ILCs. Single cell gene expression analysis was performed using Chromium Single Cell 3' RNA Sequencing (10x Genomics, Inc). In functional experiments, the effects of recombinant cytokines on ILC plasticity as well as the impact of ILC-derived cytokines on human hepatic stellate cells (HSC) was studied.

RESULTS:
ILC3 represented the major intrahepatic ILC population and displayed a phenotypically distinct profile in comparison to ILC3 found in the other compartments. In contrast to tonsil, colon, and peripheral blood ILC3 we found liver ILC3 to be characterized by low IL-22/IL-17A expression but to produce substantial amounts of the ILC2-specific cytokine IL-13. In contrast to liver ILC2 (CD117(-)CRTH2(+)CD56(-)GATA(+)NCR(-)RORgt(-)) these cells were CD117(+),CRTH2(-),CD56(+/-),GATA(-)NCR(+/-)RORgt(dim) and could not be stimulated by IL-33 +/-TSLP. This ILC3-like phenotype could be confirmed by means of 3' RNA Seq analysis, revealing distinct expression of multiple ILC3-specific signature genes. As the percentage of both total and IL-13(+) ILC3 was found to be significantly increased in patients with chronic liver disease, we speculated that the altered composition of the ILC microenvironment might form the basis for the occurrence of these atypical cells. Indeed, we found that in presence of TGF-β, one of the most prominent drivers of hepatic fibrogenesis, liver ILC2 acquired an ILC3-like phenotype while preserving their capacity to produce IL-13, thus highlighting the dynamic nature of the intrahepatic ILC pool. In addition, treatment of primary human HSCs with rhIL-13 as well as co-culture with stimulated liver ILC3 resulted in increased expression of fibrosis-associated cytokines such as IL-8 and CXCL1, thereby underlining their impact on hepatic fibrogenesis.

CONCLUSION:
Taken together our data indicates that the (diseased) human liver microenvironment gives rise to a specific subset of ILC3-like cells that is capable of producing the pro-fibrotic cytokine IL-13 and, thus, may itself be involved in modulating progression of chronic liver disease.

SESSION 4
NK cells and Metabolism

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ASSESSMENT OF NK CELL MITOCHONDRIAL METABOLISM IN ME/CFS PATIENTS

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Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a complex, multisystem disease that causes longterm severe fatigue, pain and disability, and affects more than 24 million people globally. Several studies have found that this disease may be triggered by a combination of factors such as major life stressors, infection (viral and bacterial), toxin exposure, immunodeficiency, nutritional deficiencies, genetic susceptibility (FcRs and KIRs), and several others. ME/CFS patients have persistent low natural killer (NK) cell activity. NK lymphocytes are a critical first defense against pathogens and cancer. ME/CFS patients have difficulties controlling herpesvirus infections and many develop non-Hodgkin's lymphoma. Mitochondrial metabolism is crucial for immune cell function. Mitochondria dysfunction has been previously reported in ME/CFS, but it is not known whether the NK cells of these patients have altered mitochondrial metabolism that affect their activity and contribute to ME/CFS pathogenesis. More importantly, there is currently no efficient method to diagnose ME/CFS or assess efficacy of therapeutic interventions. The Bioenergetic Health Index (BHI) has been developed as promising and reliable surrogate readout of human health by measuring the bioenergetic status of immune cells. Variations in bioenergetic function in patient's immune cells can reflect both metabolic stress and the mutable role of these cells in ME/CFS immunity and pathogenesis. In our study, we observed that the two main energy-generating mitochondrial pathways, oxidative phosphorylation and glycolysis (bioenergetics parameters; BHI), are deregulated in ME/CFS NK cells and non-ME/CFS NK cells infected with herpesviruses. Moreover, we observed alterations in the morphology and membrane potential of the mitochondria of these NK cells. Our findings indicate that these mitochondrial features can affect ME/CFS NK cell function and contribute to the severity of disease. To date, this is the first metabolism assessment of NK cells in ME/CFS and as potential new diagnostic tool for the disease.

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HYPOXIA-INDUCIBLE FACTOR 1 CONTROLS NATURAL KILLER CELL METABOLISM

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The metabolism of naïve Natural killer (NK) cells relies mainly on Oxidative Phosphorylation (OXPHOS) to meet their energetic demand. During activation, NK cell metabolism switches from OXPHOS to glycolysis and the production of lactate. This metabolic reprogramming plays a crucial role for the activation of NK cells and preventing this metabolic switch impairs their effector function.

NK cells have to operate in different oxygen environments (pO2 lung = 100 mmHg, bone marrow = 40-50 mmHg, spleen = 20-25 mmHg), and tumour-infiltrating NK cells are often exposed to severe hypoxia (pO2 below 10 mmHg). Cellular adaptation to low oxygen levels is mediated by Hypoxia-inducible factors (HIFs), a family of oxygen-sensitive transcription factors. HIFs, with HIF-1 and HIF-2 as the most prominent members, regulate the transcription of hundreds of genes that encode proteins involved in every aspect of cellular homeostasis, including metabolism. In most cell types that face hypoxia, HIF-1 engages a metabolic switch from OXPHOS to anaerobic glycolysis. In addition, HIFs can play a role in the regulation of other metabolic pathways such as glutaminolysis, citrate metabolism and fatty acid synthesis.

Our group has previously demonstrated that HIF-1 is crucial for NK cell homeostasis in the spleen as well as activation and effector function of tumour-infiltrating NK cells. In order to gain more mechanistic insight, we now address the metabolic impact of HIFs in NK cells by using mouse models with NK cell-specific deletions for the HIF pathway members. Most of the metabolic studies are based on NK cells that have been expanded in vitro with IL-2 over several days. Hence, to assess the metabolic requirements of NK cells in their natural environment under steadystate, “in situ” conditions, we performed untargeted metabolomics directly on freshly isolated splenic NK cells.

Interestingly, besides a modest reduction in lactate, our results show a depletion of the amino acid pool, including tryptophan, arginine, glutamine and glutamate along with a reduction of intracellular citrate levels in HIF-1-deficient NK cells. RNA sequencing results on activated NK cells further corroborate the impact of HIF-1 on NK cell amino acid metabolism. Moreover, metabolic profiling with the Seahorse analyser shows a decrease in oxygen consumption in naïve and cytokine-stimulated NK cells under normoxic and hypoxic conditions when HIF-1 is absent.

In summary, our results suggest that HIF-1 plays a pivotal role in NK cell metabolism during steadystate NK cell homeostasis as well as during NK cell activation.

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IL-15 PRIMING ENHANCES HIF-1A PRODUCTION IN NK CELLS THROUGH STAT3

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Natural killer (NK) cells mediate innate host defense against microbial infection and cancer and thus need to adapt to inflammatory hypoxia which is characteristic for these tissue lesions. We have reported previously that exposure of human peripheral blood NK cells to interleukin-15 (IL-15) for 6 h (priming) synergizes with hypoxia in the transcriptional activation of the adaptive hypoxia inducible factor 1α (HIF-1α) und glycolysis pathway responses (Velásquez et al., J Biol Chem 2016;291:12960-77). Here, we tested the hypothesis that this enhancement relied on a synergistic augmentation of HIF-1α levels through an mTOR mediated increase in its production in response to IL-15 priming and concomitant hypoxia dependent stabilization. We found that IL-15 priming increased HIF1A gene expression. At the same time, polyubiquitinated forms of HIF-1α were detectable already under normoxia in the presence of a proteasome inhibitor (MG132), and priming synergistically supported hypoxia dependent HIF-1α protein accumulation. Rapamycin fully inhibited priming induced phosphorylation of mechanistic target of rapamycin kinase (mTOR) downstream targets. However, mTOR inhibition only partially reduced the priming dependent increase in HIF1A mRNA levels and did not prevent synergistic IL-15/hypoxia interactions at the level of HIF-1α protein or target gene expression including key glycolytic genes. Importantly, rapamycin hardly affected priming dependent glycolytic flux. Inhibition of STAT3 phosphorylation by S3I-201 similarly reduced the priming dependent increase in HIF1A mRNA but essentially abolished HIF-1α protein accumulation and glycolytic flux. We conclude that IL-15 priming supports cellular adaption to hypoxia through STAT3 dependent induction of HIF-1α production while mTOR plays a minor role in this process. Two potential therapeutic targets in cancer, STAT3 and HIF-1α, thus appear to be mechanistically linked in human NK cells during IL-15 priming.

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IL-18 UPREGULATES AMINO ACID TRANSPORTERS AND FACILITATES AMINO ACID-INDUCED mTORC1 ACTIVATION IN NATURAL KILLER CELLS

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Upon inflammation, NK cells undergo metabolic changes to support their high energy demand for effector function and proliferation. The metabolic changes are usually accompanied by an increase in the expression of nutrient transporters leading to increased nutrient uptake such as glucose, iron and essential amino acids. Previously, we have demonstrated that IL-18 is a potent cytokine that can enhance the expression of the nutrient transporter CD98/LAT1 for amino acids independently of mTORC1 pathway and thereby induce a dramatic metabolic change, associated with an increased proliferation of NK cells. Notably, treatment of IL-18-stimulated NK cells with leucine activates the metabolic sensor mTORC1, indicating that the high expression of amino acid transporters induces amino acid-driven mTORC1 activation. Following our novel finding of IL-18 in upregulating nutrient transporters on NK cells and thereby inducing metabolic changes including the mTORC1 activation by amino acids, we currently aim to upregulate those transporters on NK cells by genetic modification as an innovative means to generate NK cells with effective anti-tumor activity. Proliferation, metabolic changes and effector functions of these modified NK cells will be evaluated. Taken together, our result will determine whether NK cells genetically modified to upregulate the nutrient transporter CD98/LAT1 will achieve improved glycolytic metabolic and anti-tumor activity. In order to be effective players in antitumor immunity, NK cells need to survive in the metabolically hostile conditions of the solid tumor microenvironment, where they have to compete for nutrients with metabolically active cancerous cells. We expect that combining our approach of augmenting the glycolytic metabolic pathway of NK cells with the enhanced tumor recognition of CAR-NK cells could maximize the efficacy of cancer immunotherapy.

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INSULIN RECEPTOR SIGNALLING REGULATES NK CELL RESPONSES AGAINST VIRAL INFECTION

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The insulin signalling pathway (ISP) is required for many cellular processes, including differentiation, proliferation, and apoptosis. Insulin, the key hormone that controls glucose and lipid metabolism, activates the ISP through the insulin receptor (INSR). Interactions between the immune system and the insulin pathway have been shown to occur frequently, suggesting the importance of the ISP in regulating aspects of immune function. However, the specific roles of the INSR on natural killer (NK) and T cells at steady state and during inflammatory settings have been largely unexplored. NK cells, much like CD8+ T cells, are lymphocytes capable of potent cytolytic responses through perforin and granzyme production, and the secretion of inflammatory cytokines such as IFN-gamma. Although they are generally found under the umbrella of innate immunity, NK cells have recently been found to exhibit numerous adaptive immune features, including antigen specificity, clonal expansion, and the generation of long-lived memory cells. Here, we show that following mouse cytomegalovirus (MCMV) infection, NK cells deficient in the INSR were unable to undergo clonal expansion as efficiently as wild-type NK cells, and represented a smaller pool of effector and memory NK cell in both lymphoid and non-lymphoid organs. This defect in Insr-/- NK cells expansion could be attributed to their inability to proliferate proficiently. Furthermore, stimulation of splenocytes ex vivo with IL-12 and IL- 18 showed that NK cells lacking the INSR produced less IFN-gamma than wild-type NK cells. Following reconstitution, MCMV-infected and uninfected mixed bone marrow chimeric mice (mBMC) also showed that INSR-deficient NK cells were less able to repopulate mice compared to wild-type NK cells, suggesting the defects observed within the Insr-/- NK cells may be cell-intrinsic. These data together suggest that NK cells require the INSR for proper NK cell function during the progression of MCMV infection.

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INVESTIGATING THE METABOLIC PATHWAYS THAT ARE CRUCIAL FOR NATURAL KILLER CELLS RESPONSES

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Cytokine activated NK cells acquire an elevated metabolic phenotype that is essential for robust NK cell anti-tumour responses. We have characterized the metabolic changes that occur in NK cells following cytokine stimulation. Within 18 hours of IL2/IL12 cytokine stimulation NK cells dramatically increase rates of glycolysis and OXPHOS and these changes are associated with an up-regulation of the glycolytic machinery and increased mitochondrial mass. This increased metabolism supports cellular biosynthesis and energy production. Interestingly, NK cells are found to adopt a novel metabolic configuration that has not been described for any other lymphocyte subset. NK cells do not use the TCA cycle to drive OXPHOS, as is described in textbooks, but instead use the glucose-fueled citrate malate shuttle. Our research has revealed the signal transduction pathways leading to this metabolic phenotype. The transcription factors Srebp and cMyc are essential for cytokine-induced metabolic and functional responses in murine NK cells. Srebp is required for the metabolic switch to the citrate-malate shuttle and cMyc promotes mitochondrial biogenesis and the increased expression of the glycolytic machinery.

The importance of additional metabolic pathways for NK cell responses are now being revealed. Srebp activity is also required for the polyamine pathway in cytokine stimulated NK cells. Inhibiting flux through the polyamine pathway results in reduced levels of glycolysis and OXPHOS and the inhibition of NK cell proliferation. Concomitant with these metabolic defects these NK cells have reduced effector functions, including reduced IFNgamma production and cytotoxicity.

Together, these discoveries help us to understand why NK cells are dysfunctional in various disease states including cancer and obesity where NK cell metabolism is found to be significantly perturbed.

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METABOLIC CONTROL OF NK CELL ACTIVATION AND MEMORY FORMATION

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Natural killer (NK) cells are innate lymphocytes capable of forming long-lived memory after mounting a response against virus infection. We have previously characterized many of the key cytokines, receptors, and transcription factors that control formation of NK cell memory using the mouse cytomegalovirus (MCMV) model. In light of the growing body of evidence that changes in metabolism are critical in the activation and differentiation of innate and adaptive immune cells, we investigated the importance of metabolic regulation in NK cell activation and memory formation in vivo. Here we demonstrate that nutrient uptake, glycolysis and oxidative respiration increase significantly in NK cells following MCMV infection. Aerobic glycolysis allowed NK cells to increase the production of intermediates of the glycolytic pathway that are important substrates for multiple cellular processes. We decided to take a genetic approach to further dissect the importance of glycolysis for NK cell function. The enzyme Ldha regulates the conversion of pyruvate to L-lactate, allowing cells to perform aerobic glycolysis, thus we generated mice containing NK cell-specific deletion of Ldha. Ldha deficiency doesn't impact NK cell development or homeostasis, however loss of Ldha greatly inhibited NK cell-mediated control of MCMV and formation of memory. In addition, we observed a severe defect in the rapid proliferative ability of Ldha deficient NK cells, along with defective cellular activation and IFN-γ production. In summary our data suggests regulation of glycolysis is critical to NK cell function and memory formation. Elucidating the pathways that regulate glycolysis in NK cells will greatly improve our ability to manipulate NK cells for therapeutic purposes.

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METABOLIC REGULATION AND IFN-γ PRODUCTION DIFFERENCES BETWEEN RECEPTOR AND CYTOKINE STIMULATION

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Natural killer cells play important roles in immune responses to tumors and viral infections. Recent work from our group and others in the field of immunometabolism has underscored the importance of metabolism in the production of robust NK cell effector functions, and in regulating immune responses in general. However, mechanisms of metabolic regulation of NK cell activation and function are still poorly understood. We have previously shown that metabolic inhibition has a negative effect on IFN-γ protein production downstream of receptor (NK1.1) ligation, but no effect is observed downstream of IL-12 and IL-18 stimulation in murine NK cells. This negative effect can be reversed with IL-15 priming. Here, we confirm these findings in human NK cells. We have also performed RNA-seq analysis of murine NK cells, which revealed almost 5000 differentially expressed genes between the cytokine and receptor stimulation conditions, as well as significant differences in their active metabolic modules. Although IFN-γ protein is detected at similar levels in the two conditions, IFN-γ transcript was only upregulated in the cytokine condition, as confirmed by PCR and RNA-seq analysis. This suggests that IFN-γ protein is produced from already present transcript in the receptor ligation condition. When new RNA transcription was inhibited with actinomycin, a significant decrease in cytokine-stimulated IFN-γ protein compared to NK1.1 ligation was observed. However, in contrast to T cells, metabolic inhibition seems to be independent of GAPDH binding to AU-rich elements within the 3' UTR of IFN-γ transcript. Metabolic inhibition of murine NK cells lacking these AU-rich elements in the 3' UTR showed no significant difference in the percentage of IFN-γ positive NK cells compared to wild type. Collectively, our studies highlight the inherent differences in metabolic regulation of IFN-γ production between cytokine and receptor stimulation in NK cells.

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METABOLIC REPROGRAMMING OF HUMAN IL-12/15/18-PREACTIVATED NK CELLS

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INTRODUCTION:
NK cells acquire memory-like behavior following a brief (16-18 hours) in vitro stimulation with IL-12, IL-15 and IL-18. After a resting period, cytokine-induced memory-like (CIML) NK cells exhibit higher effector functions, which make them a promising tool in cancer immunotherapy. Our hypothesis predicts that these increased effector functions are coupled to changes in cell metabolism. Here, we have studied the metabolic reprogramming of CIML NK cells.

METHODS:
Peripheral blood mononuclear cells were collected from healthy donors and NK cells were isolated following negative magnetic selection. Cells were stimulated with IL-12, IL-15 and IL-18 (10, 100, 50 ng/mL, respectively) for 16-18 hours and then cultured with low doses of IL-15 (1 ng/mL) or IL-2 (20 IU/mL) for 7 days. Nutrient transporters expression, glucose uptake and mitochondrial mass were analyzed by flow cytometry. Glycolysis and oxidative phosphorylation (OXPHOS) were studied with XF Seahorse Analyzer.

RESULTS:
NK cells up-regulate nutrient transporters expression following cytokine stimulation. Also, CIML NK cells increase both glycolytic rate and OXPHOS, and their metabolic preferences shift towards the glycolytic pathway during the culture period of 7 days. This shift is especially noticeable when CIML NK cells are cultured in the presence of IL-2. Mitochondrial spare respiratory capacity is also increased following cytokine stimulation, although this change is not maintained after the incubation period of 7 days. Interestingly, there is no difference in the mitochondrial mass immediately after the 16-18 hours of stimulation, but it tends to increase during the following 7 days. This increment in mitochondrial mass is especially evident when CIML NK cells are cultured in the presence of IL-2.

CONCLUSION:
Human NK cells modify their metabolism following cytokine stimulation, and the presence of low doses of IL-15 or IL-2 can contribute to maintaining or increasing these metabolic changes.

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NK CELL DYSFUNCTION IN OBESITY: A SILVER LINING?

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Obesity is associated with increased risk of at least 13 different malignancies, and is now the second-largest cause of preventable cancer in the UK. NK cells kill cancerous cells and have recently been shown to be dysfunctional in obesity. This may be one mechanism by which obesity and cancer are linked. Here, we report that NK cells in the spleens and livers of obese mice express less perforin and are less able to degranulate than those from lean controls, and that this effect is more pronounced in the liver. We also observe decreased NK cell degranulation in the livers of obese humans, compared to their lean counterparts. The NK cells of obese mice were less able to kill malignant targets, both ex vivo and in vivo. NK cells from obese animals were metabolically altered compared to those from lean individuals, mimicking the metabolic profile of the liver-resident relatives of NK cells, ILC1. NK cells from obese animals also express lower levels of the transcription factor Eomes and higher levels of the inhibitory receptors CD200R1 and LAG3, again similar to ILC1. This increased expression of inhibitory receptors led us to consider the possibility that there may be some advantage to reduced NK cell cytotoxicity in obesity, and that it may accrue particularly to the liver. In support of this idea, perforin-deficient mice suffer from less obesity-associated liver damage than wild type animals. This suggests that NK cell subfunction in obesity may be beneficial in the liver, but that this comes at the cost decreased ability to control malignancies.

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PERTURBED METABOLIC FITNESS OF NK CELL SUBSETS DURING CHRONIC HIV-1 INFECTION

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Dynamic regulation of metabolism is important for maintaining homeostasis and can directly influence immune cell function and differentiation. We have recently described a reconfiguration of the NK cell pool in HIV infection that is driven in part by HCMV coinfection/reactivation, leading to expansions of NK cells with adaptive features and altered functionality. We hypothesise that HIV infection and variable levels of chronic immune activation are associated with environmental perturbations that disrupt NK cell metabolism, differentiation and function. The metabolic signature of adaptive versus canonical NK cell subsets during chronic HIV-1 infection, comparing to NK cells from HIVseronegative HCMV-seropositive individuals was addressed in the resting state and following exposure to different activating stimuli.

Adaptive NK cells from individuals with viraemic HIV infection, despite having larger mitochondrial mass, exhibited lower mitochondrial potential and reduced mitochondrial metabolism (Oxygen consumption rate (OCR)) compared to HIV seronegative CMV+ controls, suggestive of loss of mitochondrial integrity and defective NK cell OXPHOS during chronic HIV infection. Moreover, adaptive NK cells were found to have lower mTORC1 activity, highlighting the potential role of the mTOR pathway in regulating the metabolic fitness and functionality of NK cells subsets during chronic infection. In keeping with alterations in lipid profiles and fatty acid concentrations in HIV infection, NK cells from HIV infected individuals stained more strongly for lipids compared to matched HIV negative controls. These findings are reminiscent of exhausted virus-specific CD8 T cells during chronic viral infections and reports of lipidinduced metabolic defects through mTORC1 inhibition in dysfunctional NK cells in obesity. Targeting metabolic pathways/reversal of metabolic defects has great potential as an effective strategy for pharmacologically reinvigorating NK cell responses to enhance antiviral immunity.

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ROBUST METABOLIC CHANGES DRIVEN BY REPETITIVE IL-15 EXPOSURE IMPACT NK CELL FUNCTION: RETHINKING THE WAY WE DOSE

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IL-15 is being investigated in many cancer clinical trials to improve NK cell survival, expansion and function. Our lab has reported that NK cells treated continuously (3 days on-3 days on-3 days on) with high dose IL-15 (10ng/ml) are exhausted compared to NK cells treated intermittently (3 days on-3 days off-3 days on) with IL-15. The main defects observed were in NK cell function, proliferation and metabolism. Cellular metabolism is emerging as a key factor in the control of NK cell responses. We conducted an untargeted metabolomics screen to measure levels of metabolites in NK cells treated continuously (IL15-continuous) and intermittently (IL15-gap) with IL-15. The IL15-gap cells have higher levels of oxidative phosphorylation which is mediated in part by fatty acid metabolism. These cells have less fatty acids (specifically acyl carnitines), indicative of increased fatty acid demand. When the IL15-gap cells are treated with Etomoxir (5M), an inhibitor of fatty acid metabolism, they are less functional and produce less IFN. This is in contrast with previous studies where Etomoxir had no effect on cytokine-activated NK cells however those studies were carried out over a much shorter time frame. The IL15-gap NK cells have higher levels of glucose and glutamine metabolism to generate energy for biosynthetic purposes. An example of this is increased levels of phospholipids, the largest proportion of cell membrane lipids, indicating that these cells are poised for activation and proliferation. In addition, the IL15-gap cells have higher levels of aspartate compared to IL15-continuous cells. In cancer cells, aspartate availability has been shown to be a limiting factor for proliferation. The proliferative defect is reflected in NK cells from ovarian cancer patients treated weekly with N-803, formerly ALT-803, an IL-15 super agonist. Mass cytometry analysis of NK cells from the peritoneal cavity showed an initial increase in the proliferation marker Ki-67 followed by decreases over 4 doses of N-803. This corresponds to NK cell number and ex vivo cytotoxicity against K562 and OVCAR3 cells. There is more target cell lysis after the first dose of N-803 compared to the last dose. Overall this data demonstrates that repetitive IL-15 exposure leads to altered usage of metabolites and metabolic pathways. Given that these pathways regulate NK cell function and proliferation, these findings have enormous translational potential to inform IL-15 dosing strategies and to develop NK cell products that are most efficient in the tumor microenvironment.

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SREBP TRANSCRIPTION FACTORS ARE ESSENTIAL FOR NK CELL METABOLISM AND EFFECTOR FUNCTION

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It is now appreciated that NK cell metabolism and effector function are inextricably linked. Upon stimulation with cytokines, NK cells undergo robust metabolic changes including an upregulation of glycolysis and OXPHOS, which is necessary for adequate effector responses. Sterol Regulatory Element Binding Protein (SREBP) transcription factor, previously known for its conventional role in de novo lipid synthesis, has emerged as a novel key regulator of NK cell glucose metabolism. SREBP mediates the conversion of glucose into cytosolic citrate via the citrate-malate shuttle (CMS) in a distinct metabolic pathway that is required for elevated rates of glycolysis and OXPHOS. Inhibition of SREBP activation with the cholesterol metabolite, 25-hydoxycholesterol (25-HC) or inhibition of the CMS with BMS303141 impairs cytokine-induced NK cell metabolism and effector functions. Thus, this highlights the importance of SREBP in promoting a distinct metabolic configuration that is essential in regulating NK cell function.

27-hydroxycholsterol (27-HC) is another cholesterol metabolite that is implicated in a spectrum of diseases, in particular breast cancer, but is not generally considered as an inhibitor of Srebp transcription factors. Rather, 27-HC has been shown to be an endogenous selective estrogen receptor modulator (SERM) as well as a liver X receptor (LXR) ligand that can promote the growth of ER+ tumours and increase metastatic burden in mouse models of breast cancer. Interestingly, we have made the novel finding that 27HC is a potent inhibitor of Srebp activity in NK cells. Based on this discovery, we have investigated how 27HC affects NK cell metabolism and NK cell-mediated anti-tumour responses. Indeed, cytokine-stimulated NK cells treated with 27-HC had impaired cellular metabolism and reduced IFN-γ and Granzyme B production. Therefore, these data have identified 27-HC as a potent modulator of NK cell metabolism and activity, highlighting a potential new mechanism of action for this cancer-promoting metabolite.

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TGFβ DRIVES MITOCHONDRIAL DYSFUNCTION IN PERIPHERAL BLOOD NK CELLS DURING METASTATIC BREAST CANCER

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Natural Killer (NK) cells play a key role in immune defense against cancer. However, their functions become progressively impaired during cancer, which impacts their potential use in immunotherapy e.g. Trastuzumab in breast cancer. NK cell dysfunction may also leave a cancer patient susceptible to infection and metastasis. Hence, there is urgent demand to better understand NK cell dysfunction in cancer. We have previously shown that freshly isolated peripheral blood NK cells from metastatic breast cancer patients have altered cellular metabolism. Nutrient receptors fail to upregulate in response to cytokine and the same is observed for the activity of mTORC1, a master regulator of NK cell metabolism. Seahorse analysis indicates profound defects in both glycolytic and mitochondrial (MT) metabolism in IL2 stimulated patient NK cells. Confocal imaging and flow cytometry analysis on direct ex vivo NK cells from patients indicate clear structural and functional differences in NK cell mitochondria. This is characterised by increased MT mass, MT membrane potential, and MT ROS. Further more, the upregulation of ATP5B, a subunit of complex 5 in the electron transport chain, in response to cytokine is impaired. Importantly, blocking elevated levels of TGFβ improved levels of oxidative metabolism in patient NK cells. This was accompanied by increased mTORC1 activity, nutrient receptor expression, and IFNγ production. Overall we have described for the first time the metabolic dysfunction of NK cells in human cancer, and essentially, the ability to target and improve this metabolism in order to increase NK cell anti-tumour function. These findings have important implications for the design of future NK cell based immunotherapies.

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A MICROCHIP PLATFORM FOR THE EVALUATION OF SERIAL KILLING MECHANISMS AND DYNAMICS IN INDIVIDUAL NK CELLS

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Natural Killer (NK) cells consist of a phenotypically complex group of sub-populations, many of which have shown high plasticity. Despite being traditionally classified as part of the innate immune system, some NK cells have even been found to exhibit characteristics of adaptive cell types, including antigen specific recognition and long-term memory. Methods for single cell analysis, such as flow cytometry, mass cytometry and single cell sequencing, have improved the characterization of these inherently heterogeneous populations. However, most of these techniques give a static picture of the molecular content in individual cells and provide limited information about how phenotype translates into function. These experimental approaches also require large amounts of material, often impossible to obtain in a clinical setting. We have developed a microchip platform for assessing cellular function and dynamics, with focus on resolving the heterogeneity in cytotoxic response between individual NK cells. This microchip approach allows detailed characterization of individual cells within larger populations, yet only requires a small starting material of a few hundreds to thousands of cells. Using our platform, we have investigated the efficiency and dynamics of NK cell cytotoxicity, as well as the influence of different receptor stimuli on NK cell serial killing ability. We have also combined the microchip platform with the use of fluorescent reporter molecules for granzyme B and caspase 8 activity, enabling the study of cytotoxic pathways utilized by individual NK cells. Together with protein analysis using flow cytometry, our approach provides new insight in the functional heterogeneity within NK cell sub-populations.

SESSION 5
NK cells and Anti-Tumour Immunity

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A NOVEL GENETIC TOOL TARGETING TISSUE-RESIDENT CYTOLYTIC INNATE LYMPHOCYTES INVOLVED IN CANCER IMMUNOSURVEILLANCE

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Work from our laboratory has previously identified populations of cytolytic innate lymphocytes and innate-like T cells (ILTCs) that reside in the healthy mammary gland and expand in response to cell transformation in an oncogenedriven murine model of cancer. These cells are tissue-resident, can directly kill tumor cells in a perforin-dependent manner, and are dependent on the cytokine IL-15. We observed that these innate and ILTC populations share a gene expression program that is distinct from circulating NK cells and conventional T cells. Following up on these initial findings, we confirmed that one such gene, granzyme C (GzmC), indeed demonstrated a pattern of protein expression that was restricted to these tissue-resident cells. Given the lack of specific genetic tools to target these tissue-resident lymphocytes, we generated a knock-in Cre mouse line targeting the GzmC locus. Within the tumor-infiltrating immune compartment in the PyMT model of spontaneous breast cancer, expression of GzmC-Cre mirrored expression of the GzmC protein, marking tissue-resident cytolytic innate lymphocytes and ILTCs, with no expression in circulating NK cells or conventional T cells. We employed the GzmC-Cre mouse line to explore the relevance and requirements of GzmC-expressing cells in PyMT tumors. Diptheria toxin-mediated deletion of GzmC-expressing cells in tumor-bearing mice depleted ILTCs and tissue-resident but not circulating cytolytic innate lymphocytes and lead to accelerated tumor incidence and growth. Ongoing work employing mouse genetics aims to clarify what cues define the tumor tissue niche that supports GzmC-expressing cells and which signals are required for the differentiation and/or maintenance of these cells. Overall, these data describe a novel genetic tool that targets cytolytic tissue-resident innate lymphocytes and provide further evidence that these cells play a critical role in cancer immunosurveillance.

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ADOPTCELL®-NK: A NEW CLASS NK CELLS MANUFACTURED IN ACCORDANCE WITH GMP/GCTP THAT CAN ELIMINATE THE SOLID TUMORS

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BACKGROUND

Cancer immunotherapy has been established as a new therapeutic category since the recent success of immune checkpoint inhibitors and a type of adoptive immunotherapy, namely chimeric antigen receptor-modified T cells (CAR-T). Clinical trials of CAR-T targeted to solid tumor fail to show apparent antitumor activity, suggesting possible critical factors in the tumor microenvironment that impede the current CAR-T strategies. We've developed a cutting-edge, simple, and feeder-free method to generate highly activated and expanded human NK cells from peripheral blood (Saito S, et al. Hum Gene Ther 2013), and have been conducting further investigation why our new type of NK cells, named as GAIA-102, are so effective to kill malignant cells.

MATERIALS AND METHODS

Cryopreserved PBMCs purchased from HemaCare Corporation were processed by using LOVO and CliniMACS® Prodigy (automated/closed systems). CD3+ and CD34+ cells were depleted by CliniMACS® beads, and the cells were cultured with high concentration of hIL-2 for 14 days in our original closed system. Then, we confirmed the expression of surface markers, CD107a mobilization and cell-mediated cytotoxicity against various tumor cells and normal cells with or without monoclonal antibody drugs in vitro and antitumor effects against SKOV3 in vivo. We also analyzed clustering gene expression of GAIA-102 and compared the pattern with any other WBCs containing CAR-T cells.

RESULTS AND DISCUSSION

Importantly, we've found that our GAIA-102 involves CD56bright/CXCR3+/CCR5+/CCR6+ population containing both CD16high and CD16dim/- subpopulations with high amount of perforin and granzyme B, unlike any types of NK cells from peripheral blood. More importantly, GAIA-102 has proven its high killing activity against various malignancies regardless of HLA and ligands of activating receptors (e.g. MICA/B, B7-H6, CSV, etc.) expression. Surprisingly, GAIA-102 could eliminate solid tumor both in vitro and in vivo, even if those tumors were socalled "cold tumor", comprising MDSC or Treg.

We here demonstrated that GAIA-102, a novel CD3–/CD56bright/CD57– immature phenotype of NK-like cells generated using a novel culture method, shown efficient accumulation, retention, and elimination of multiple tumor spheroids. These findings indicate that GAIA-102, that do not require any genetic modification, have a great potential to become an upward-compatible modality over CAR-T strategy, and a new and promising candidate for adoptive immunotherapy against solid tumors. We now just started GMP/GCTP production of this new and powerful cells and first-in-human clinical trials in use of GAIA-102 will be initiated on 2020.

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AN IMAGE-BASED PLATFORM FOR NK-CELL RESPONSE AGAINST SOLID TUMOR MODELS

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Tissue micro-engineering is important for modelling 3D biological structures to bridge the gap between in vivo and in vitro in cellular and pharmaceutical research. Regular 2D in vitro cell cultures do not reproduce the complex 3D microenvironment of cells, extra cellular matrix and soluble factors which governs cell behavior and tissue function. The tumor microenvironment is also influencing the tumor ligand repertoire as well as immune cell response. Recently, many multicellular tumor spheroid (MCTS) culture methods has been developed based on either anchored-dependent (e.g. scaffolds) or anchored-independent (e.g. hanging drop, low-attachment surfaces) strategies but many of them does not address the challenges of adequate imaging. High quality on-chip imaging is desirable in immunology for studying dynamic immune cell response against solid tumors and their infiltration rate.

Here we present an on-chip MCTS culture platform based on ultrasonic radiation forces where spheroids are formed from a single cell suspension. 100 MCTS were parallelly cultured in a multi-well microplate with a flat glass bottom for optimal imaging capabilities. The silicon multi-well microplate consists of a silicon wafer with 100 etched micro-wells (350 µm x 350 µm, 300 µm deep) bonded to a glass plate and mounted onto the ultrasound transducer. Stable MCTS are formed during 24h of continuous ultrasound exposure and subsequent passive incubation.

The ultrasound based MCTS culture system does not influence the tumor cell NK-ligand repertoire compared to a passive MCTS culture technique and is thus a valid model system to study tumor-NK cell interaction. To investigate the NK cell response against renal cell carcinoma A498 MCTS, unstained and IL-15 activated NK cells were seeded into the microwells containing 96h old A498 MCTS and focused onto the tumor surface by ultrasound forces for 24h. After the initial 24h of co-culture during ultrasound focusing, the multi-well microplate is placed and imaged in an environment-controlled widefield microscope between 24-48h and 72-96h. Since fluorescent dyes were avoided due to the long imaging time, a machine learning based classification tool was used to segment NK cells, living tumor mass and dead tumor mass in the bright field images. The unstained cells also allowed a detailed confocal end-point analysis of NK cell infiltration after on-chip staining and MCST clearing.

In summary, we have developed a MCTS culture platform optimized for imaging-based studies of the NK cell response against solid tumors on a chip where both dynamic and detailed data can be acquired.

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CD137 (4-1BB) COSTIMULATION COUNTERACTS TGF-β1 IMPAIRMENT OF ANTIBODY-DEPENDANT NK CELL ANTI-TUMOR RESPONSES

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We have recently shown an association between tumor-infiltrating NK cells and response to anti-HER2 therapeutic antibodies (i.e. trastuzumab, pertuzumab) in breast cancer patients, supporting their contribution to treatment efficacy. In addition, NK cell dysfunction owing to immunosuppressive factors (i.e. TGF-β1) has been related to disease progression in metastatic patients. Hence, strengthening NK cell function and persistence in the tumor microenvironment is envisaged as a relevant option for enhancing the clinical efficacy of anti-HER2 antibodies.

Microarray analysis of CD16-stimulated human NK cells identified CD137 (4-1BB) as the TNFRSF member showing the highest induction upon activation. Co-culture with trastuzumab-coated SKBR3 cells triggered NK cell degranulation and CD137 expression, in the absence of PD1 or CTLA-4. ADCC-induced CD137 expression was sustained by autocrine TNF-α signaling through TNF-RI/TNFR-II, as evidenced by the effect of specific blocking mAbs. NK cells from individuals harboring adaptive NKG2C+ NK cell expansions displayed a greater CD137 upregulation, according to their proficient ADCC and TNF-α secretion. Remarkably, CD137 co-stimulation in CD16-activated NK cells by the agonist mAb urelumab reversed TGF-β1 inhibition of IL2-dependent proliferation in a dose-dependent manner, restoring CD25 (IL2Rα) expression and NKcell numbers. Indeed, gene ontology analysis of signature genes from CD16-activated NK cells after 5 day-treatmentwithTGF-β1 in the presence or absence of urelumab, identified cellular growth and proliferation, death and survival, cellinteraction and immune cell trafficking as biological processes significantly enhanced upon CD137 co-stimulation. Inaddition, urelumab-treatment prevented TGF-β1-induced differentiation of CD16-activated NK cells towards an ILC1-like profile, partially preserving their cytotoxic potential (e.g. NKG2D and GzmB) while maintaining the acquisition oftissue-resident features (e.g. HOBIT, CD103, CXCR3) as indicated by GSEA of transcriptomic data and corroborated by phenotypic analysis. Accordingly, CD137 co-stimulation in CD16-activated NK cells preserved their subsequent anti-tumor function by sustaining CCL5 secretion, direct and antibody dependent tumor cell cytotoxicity and IL-12- dependent IFN-γ production, regardless of TGF-β1. Of note, addition of trastuzumab into multicellular suspensions obtained by mechanical/enzymatic digestion of treatment-naïve fresh human breast tumor specimens recapitulated the induction of CD137 in the absence of PD1 in tumor-associated CD16+ NK cells, whereas no changes in CD137 and PD1 were detected in CD4+ and CD8+ T lymphocytes. Overall, our data reveals CD137 as a costimulatory receptor capable of overturning TGF-β1 immunosuppression by promoting activated NK cell proliferation and sequential tumor cell killing, providing the rationale for combinatorial therapeutic strategies including CD137 agonists for enhancing the efficacy of tumor antigen-specific antibodies.

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COMBINATION OF HERPES-SIMPLEX BASED ONCOLYTIC VIRUSES WITH NATURAL KILLER (NK) CELLS IN HEMATOLOGICAL MALIGNANCIES

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Oncolytic viruses (OV) are genetically modified viruses that specifically infect and lyse tumor cells. So far, OV monotherapy shows only limited benefit in clinical practice. However, it holds great potential in combinatorial treatment approaches. Natural killer cells recognize and kill infected, stressed or malignant cells without prior antigen exposure. Patients who lack NK cells are highly susceptible to severe and recurrent herpesvirus (HSV) infections. Thus, the combination of HSV based OVs (HSV-OV) and NK cells has a strong rationale.

We aim to explore the synergy between clinically applied HSV-OV and NK cells. Our main interest is Multiple Myeloma (MM) - an aggressive hematological malignancy of the B-cell line. It is known that NK cell functionality is highly impaired in MM patients. Recent NK cell-based immunotherapies against MM have shown encouraging results. We hypothesize that HSV-OV may increase NK cell functions and improve therapy outcome.

To test this, we have infected both MM cell lines and primary NK cells from healthy donors with HSV-OV at different OV doses and measured their viability and phenotype at several different time points. We show that HSV-OV directly infects and lyses tumour cells while sparing NK cells from OV mediated killing. Our data furthermore indicate that HSV-OV activates primary NK cells and increases their cytokine release and killing ability at an early time point. We further elucidated the basis of this activation by an extensive phenotypic analysis of HSV-OV treated primary NK cells. We plan to extend our study to NK cells, taken from patients with MM. These data will help to define the potential and accessibility of combinatorial HSV-OV and NK cell therapies. Both therapies are feasible and safe for patients with MM.

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COMBINING CAR ENGINEERING AND CRISPR-CAS9 GENE EDITING OF A CHECKPOINT MOLECULE IN NK CELLS FOR THE TREATMENT OF B-CELL MALIGNANCIES

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Exciting clinical results using CAR-T cells against CD19 have led to FDA approvals for B-cell malignancies. However challenges remain, including the logistic hurdles of generating autologous cellular therapy products, toxicity of CAR-T cells, and disease relapse. These limitations underscore the need to develop novel cellular products that are universal, safe and more potent. Natural killer cells are attractive contenders since they exert potent cytotoxicity against tumors and unlike T cells, do not cause GVHD or toxicity in the allogeneic setting. Our group has developed a strategy to genetically modify cord blood derived NK cells to express a CAR, ectopically produce IL-15, and express inducible caspase 9 (iC9) as suicide gene to address potential safety concerns. We have initiated a first-in-human, phase I/II clinical trial of iC9/CAR19/IL15 NK cell therapy in patients with relapsed/refractory B cell malignancies.

We now propose to further enhance the potency of CAR-NK cells by blocking intrinsic checkpoint molecules. Cytokine inducible SH2 containing protein (CIS), encoded by the CISH gene, is as an important checkpoint in NK cells and is upregulated in response to IL-15. We hypothesized that CIS may act as a potent checkpoint in our iC9/CAR19/IL15 NK cells since they continuously produce IL-15, and that targeting this pathway would enhance their antitumor activity.

We showed that CISH is upregulated in iC9/CAR19/IL15 NK cells. To examine the functional consequences of CISH deletion in our CARNK cells, we developed a protocol combining ribonucleoprotein (RNP)-mediated gene editing and retroviral transduction with the iC9/CAR19/IL15 construct. Gene editing efficiency was >90%. CISH knockout resulted in significantly enhanced function of iC9/CAR19/IL15 NK cells against Raji lymphoma evident by increased cytokine production (TNFa p=0.007, IFNg p=0.033) and degranulation (CD107a p=0.003) compared to controls. Moreover, CISH KO iC9/CAR19/IL15 NK cells killed Raji more efficiently. RNA sequencing confirmed enrichment of JAK/STAT signaling, TNFα and IFN-γ response in CISH KO iC9/CAR19/IL15 NK cells compared to Cas9 control counterparts, providing a molecular mechanism for their enhanced effector function. Moreover, in an NSG mouse model of Raji lymphoma, the antitumor activity of a single dose of CISH KO iC9/CAR19/IL15 CB NK cells was significantly better than that of Cas9 controls; (p=0.003).

These data indicate the presence of a novel interaction that regulates the functional activity of CARNK cells that could be targeted to improve adoptive NK cell therapy. We are in the process of scaling up this approach in our GMP facility for translation to the clinic.

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CYTOKINE-INDUCED MEMORY-LIKE NK CELLS EXHIBIT ENHANCED AUTOLOGOUS RESPONSES TO METASTATIC MELANOMA

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The treatment of metastatic melanoma (MM) has been revolutionized by immune checkpoint blockade (ICB). However, response rates are 50% and nearly half of initial responders will develop refractor disease. NK cells contribute to cancer immunoediting but are frequently deficient or dysfunctional in patients with cancer, including melanoma. To better define the potential of NK cells for immunotherapy in MM patients, circulating and metastasesinfiltrating NK cells from MM patients were evaluated using high dimensional mass cytometry. Data from 3 MM patients demonstrated the presence of metastases-infiltrating NK cells, most of them being CD56bright. Compared to non-involved lymph node (LN)-resident NK and blood NK cells, metastatic-LN-infiltrating NK cells displayed reduced expression of activating receptors (NKG2D, DNAM1, NKp30, NKp44 and NKp46) and increased granzyme B. These infiltrating NK cells had a greater frequency of a mature phenotype (CD57+NKG2A-). These data suggest that while NK cell can traffic to the site of melanoma metastases, endogenous patient NK cells lack an effective anti-melanoma response, potentially adapted to an immunosuppressive tumor microenvironment. We therefore hypothesized that strategies to enhance NK cell functionality and recognition may restore anti-melanoma NK cell responses. Memorylike (ML) NK cells differentiated after IL-12, IL-15, and IL-18 activation represent a promising, safe NK cellular therapy for acute myeloid leukemia (Romee R et al, Sci Transl Med, 2016). Furthermore, studies of ML NK cell biology indicate an ability to ignore self-inhibitory KIR receptors, providing a rationale to test autologous ML NK cells for cancer therapy. Human ML NK cells generated from healthy donors displayed enhanced IFN-g response ($p<0,01$) and killing ($p<0,0001$) of the melanoma DM6 cell line compared to control NK cells . Autologous ML NK cells generated from the blood of MM patients also displayed an enhanced ability to produce IFN-g ($p<0,01$) and kill ($p<0,0001$) autologous melanoma targets in vitro, compared to autologous control NK cells. Blocking experiments revealed that mechanisms whereby ML NK cells recognized melanoma targets included NKG2D and NKp46, as their blockade decreased IFN-g ($p<0,05$) and specific killing by 51Cr release. ($p<0,001$). In vivo experiments to evaluate efficacy of ML NK cells to control metastatic melanoma and local tumor growth are ongoing. Collectively, these findings suggest that autologous ML NK cells could be harnessed as a cellular therapy strategy for MM patients, especially those failing ICB and other T cell-directed immunotherapies, and warrants further investigation.

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DARATUMUMAB IN COMBINATION WITH URELUMAB TO POTENTIATE ANTI-MYELOMA ACTIVITY IN LYMPHOCYTEDEFICIENT MICE RECONSTITUTED WITH HUMAN NK CELLS

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CD38 is a transmembrane protein expressed on all malignant plasma cells in multiple myeloma. Daratumumab is an anti-CD38 fully human IgG1 mAb approved for multiple myeloma treatment. One of the proposed mechanisms of action is the induction of antibody-dependent cellular cytotoxicity (ADCC) mediated by NK cells. CD137 is a TNFR family surface glycoprotein expressed on activated lymphocytes and agonist monoclonal antibodies have been shown to enhance anti-tumor activity on NK and T cells. NK cells from peripheral blood of healthy donors and from bone marrow of multiple myeloma patients acquire surface CD137 expression in the presence of solid-phase-attached daratumumab and when encountering a daratumumab-coated CD38+ tumor cell line. In this setting, addition of the agonist anti-CD137 mAb urelumab enhances NK-cell activation increasing CD25, PD-L1, Granzyme B expression and IFNγ production. However, in vitro ADCC is not increased by the addition of urelumab both in 4h or 24h lasting experiments, although a slight CD107 increment was observed on the NK cells treated with urelumab and daratumumab in a 24h cytotoxicity assay. To study urelumab-increased daratumumab-mediated ADCC activity in vivo, we set up a mouse model based on the intravenous administration of a luciferase-transfected multiple myeloma cell line of human origin, human NK cells and daratumumab to immuno-deficient NSG mice. In this model, intravenous administration of urelumab 24h after daratumumab delayed tumor growth and prolonged mice survival. Considering these results as a whole, we conclude that even in absence of effects on CD8 T cells, addition of urelumab to daratumumab treatment may improve therapeutic anti-tumor outcomes.

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DEVELOPING A GMP-COMPLIANT, FULLY AUTOMATED PROCESS TO GENERATE CAR NK CELLS IN A CLOSED SYSTEM FOR CLINICAL USE

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Major advances have been made in harnessing natural killer (NK) cells in cancer immunotherapy in recent years. Regulated by their germ-line encoded activating and inhibitory receptors, NK cells can recognize and eliminate tumor cells rapidly without prior sensitization. Clinical evidence has shown that donor-derived NK cells have low risk in inducing graft-versus-host-disease (GvHD), thus making them ideal for allogeneic transplantation.

Sharing similar killing machinery with T cells, NK cells can also be redirected by chimeric antigen receptors (CARs) against tumor cells. To facilitate the clinical application of CAR NK cells, here we developed a highly efficient, fully automated process to generate CAR NK cells under good manufacturing practice (GMP)-compliant conditions in a closed system by using CliniMACS Prodigy device. The process covers the complete procedure of NK cell manufacturing, including separation, activation, gene modification and expansion/cultivation. High purity of NK cells (mean 93%) could be achieved with CD3-positive cell depletion followed by CD56-positive cell enrichment, resulting in a mean 4.2 log depletion of T cells.

Purified NK cells were cultivated with a feeder cell-free protocol including cytokines together with NK MACS GMP Medium, and transduced with baboon envelope glycoprotein pseudotyped lentiviral vector constructs. After 14 days of culture a mean transduction efficiency of 49.8% CAR+ cells was achieved resulting in 5.2 – 9.5*10E8 CAR+ NK cells (n=6).

In summary, we developed a novel process for automated NK purification, transduction and cultivation in a closed GMP compatible system. The high level of automation enables standardized, consistent and operator independent genetic engineering of NK cells for therapeutic applications.

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DEVELOPMENT AND VALIDATION OF KLRB1C (NK1.1) RABBIT MONOCLONAL ANTIBODIES FOR IMMUNOHISTOCHEMISTRY

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Natural killer (NK) cells are innate immune cells involved in the first-line defense against malignant and virally infected cells. NK cells can kill tumor cells without prior sensitization or recognition of specific tumor antigens. Therefore, harnessing NK cell activity is a promising cancer immunotherapy approach. Mouse models are important tools for validating candidate cancer immunotherapies. Various markers are used to identify NK cells in mice including Klrb1c, a type II membrane glycoprotein and a member of the NKR-P1 family of cell surface receptors. The best known Klrb1c antibody is NK1.1 (PK136), a mouse monoclonal antibody widely used to identify mouse NK cells by flow cytometry and to deplete NK cells from mice in vivo. Due to the polymorphic nature of Klrb1c and how it was developed, NK1.1 (PK136) can only be used in certain mouse strains like C57BL/6, but not other strains like BALB/c.

Furthermore, the NK1.1 (PK136) antibody is not capable of detecting NK1.1 by immunohistochemistry (IHC) in formalin-fixed paraffin embedded (FFPE) tissues, an indispensable tool for cancer immunotherapy research. Lack of suitable IHC-validated antibodies that can identify mouse NK cells in the tumor microenvironment has been a hindrance for NK research. Therefore, we developed Klrb1c rabbit monoclonal antibodies validated for IHC using transfected cell pellets, normal mouse tissues, and mouse tumor models. In addition, we demonstrate that our Klrb1c antibodies recognize NK cells in mouse strains that do not have the alloantigen for the NK1.1 (PK136) antibody.

Finally, in addition to IHC, we demonstrate utility of these antibodies for flow cytometry and immunofluorescence. The availability of these Klbr1c antibodies will facilitate NK cell research in mouse models for immunotherapy.

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DONOR-DERIVED HUMAN NK CELLS EXPRESS FIBROBLAST ACTIVATION PROTEIN(FAP). INHIBITINGFAP INCREASES NK ACTIVATING CYTOKINES AND IMPROVES IMMUNOTHERAPY EFFICACY IN MURINE MODELS OF PANCREATIC CANCER

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Immunotherapy has been largely ineffective in pancreatic cancer, partially due to the dense stromal fibrosis surrounding the tumor that creates a n immunosuppressive microenvironment. The main cellular component of this fibrosis, activated pancreatic stellate cells (aPSCs), are marked by elevated expression of fibroblast activation protein (FAP). Here we investigate the relationship between aPSCs, human natural killer (NK)cells and FAP. To assess the relationship between aPSCs and N K cells w e used a novel i n vitro co-culturing system that utilizes primary donorderived PSCs and human NK cell lines, NK92, NKL, YT and KHYG-1. We tested the ability of NK cells to kill aPSCs using CytotoxGlo and Annexin V assays. We monitored FAP expression using rt-qPCR, western blot and FAP activity assays. We demonstrate that the human NK cell line (NK92) is activated by and kills aPSCs. Upon direct contact with PSCs, PSCs downregulate FAP expression and NK92 cells upregulate FAP.We further demonstrated that FAP is expressed in a variety of human and murine leukocyte cell lines as well as healthy-donor derived NK cells.To assess the effects of FAP inhibition in vivo we used a non-specific FAP inhibitor, talabostat. Forty female C57BL/6 mice were injected subcutaneously with 1X105syngeneic MT3-2D cells (Kras+/G12D, p53+/-R172H derived from a PDAC KPC GEMM model). Once tumors reached 40-50 mm3, ten mice per group were given either 30 ug oftalabostat per mouse daily by oral gavage, 200 ug of anti-PD-1 per mouse twice a week by i.p., both, or neither. Control mice were treated with PBS. Treatment was terminated after 4 weeks and the mice were monitored, with tumor measurements occurring weekly. This experiment was repeated with 15 mice per group and tumors were collected for flow immunophenotyping and mRNA expression analysis. Blood was collected weekly to monitor cytokine levels. Talabostat enhanced anti-PD- 1 therapy efficacy in murine models of PDAC. Cytokine analysis demonstrated that talabostat treatment enhanced NK activating cytokines. Flow cytometry immunphenotyping showedcombination treatment reduced T cell infiltration but increased N K cell infiltration. This is the first report of FAP expression in multiple human and murine leukocyte lineages. It further suggests that talabostat, a non-specific FAP inhibitor, can increase circulating NK activating cytokines and enhances anti-PD-1 anti-tumor efficacy through increased recruitment of NK cells to the tumor.

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EFFECT OF IGG SUBCLASSES AND FCGR3A POLYMORPHISM ON HUMAN NK CELL EFFECTOR FUNCTIONS

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Natural killer (NK) cell-mediated antibody-dependent cellular cytotoxicity (ADCC) plays an important role in cancer immunotherapy, inflammation, and autoimmunity. NK cells express the Fc-gamma receptor (FcγR) FcγRIIIA. Studies showed an association between the single nucleotide polymorphism (SNP) V158F in FCGR3A and the efficacy of monoclonal antibody (mAb) therapy, mainly for IgG1 subclass. In addition, IgG subclasses may impact the strength of IgG-FcγRIIIA interactions. However, the molecular mechanisms regulating cellular responses upon IgG-FcγRIIIA interactions in NK cells need to be studied in order to better understand individual responses to mAb treatment. The aim of the current study was to analyze in vitro the role of IgG subclasses and FCGR3A polymorphism on the cytotoxic function of effector immune cells. For that, we performed a non-radioactive cytotoxic DELFIA assay using NK cells from healthy human donors (effectors cells), Daudi cell line (target cells) and variable concentration of anti-CD20 mAb subclasses in a 2h assay. Additionally, we measure the affinity of different anti-CD20 subclasses antibodies to CD20 and FcγRIIIa by flow cytometry. FCGR3A V158F allelic variation of the donors was performed by nested-PCR and restriction enzyme digestion (Nla III). IgG subclasses showed similar Kd but slightly different Bmax IgG1 > IgG3 ≥ IgG4 > IgG2 binding to CD20 on Daudi cells. On the other hand, IgG1 and IgG3 were the strongest triggers of ADCC, whereas IgG2 hardly lead to cytotoxicity independently of donors' SNP V158F for FCGR3A. IgG4 induced ADCC only in 158VF heterozygous donors; donors homozygous for 158VV have not been tested yet. Our preliminary data highlight the impact of IgG subclasses and SNP V158F FCGR3A on human NK cell functions

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EFFECTS OF HYPOXIA ON NK CELLS: MODULATION OF IMMUNOREGULATORY FUNCTIONS AND MIGRATION RESPONSES

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The ability of immune cells to infiltrate tumor tissues and maintain effector functions represents a critical issue to develop effective immunotherapy approaches in solid tumors. Hypoxia, which often characterizes the tumor microenvironment, can influence the function of many immune cell types thus favoring the tumor escape from the host response. However there is presently little information on its effects on NK cells. In this study we show that hypoxia can profoundly affect human NK cells and their ability to respond to different chemotactic stimuli. NK cells derived from peripheral blood were cultured for 16h or 96h in the presence of IL-2 under normoxic or hypoxic (pO2 1%) conditions and then submitted to different analyses including the gene expression profiling, the quantitation of cytokine and chemokine release, the cytofluorimetric analysis of the chemokine receptor expression, and the evaluation of the chemotactic response to specific chemokines. After exposure to hypoxic conditions NK cells showed a peculiar transcriptional profile characterized by a distinctive hypoxic signature. As assessed by Gene Ontology annotation several of the analyzed hypoxia-targeted genes were implicated in various processes related to metabolism, cell cycle, apoptosis, and immune functions including cytokine and chemokine production, and chemokine receptor expression. Hypoxia could indeed variably reduce NK cell ability to release IFNγ, TNFβ, GM-CSF, CCL3 and CCL5. Remarkably, hypoxia could also influence NK chemokine receptor pattern, by sustaining the surface expression of CCR7 and CXCR4. This effect occurred selectively (CCR7) or preferentially (CXCR4) on CD56bright NK cells, which indeed showed higher chemotaxis to CCL19, CCL21, or CXCL12. These findings may contribute to explain the rather frequent presence of CD56bright cells within the NK cell infiltrate in tumors.

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EVALUATION OF NK CELL PHENOTYPE AND ACTIVITY AS A FUNCTION OF OXYGEN CONTENT WITHIN THE ENVIRONMENT

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NK cell immunotherapies are being studied in the clinic in a number of settings, including solid tumors. For these therapies to succeed, the NK cells need to enter the tumor microenvironment (TME) and retain functionality. While some studies have assessed the impact of checkpoint signals and immunosuppressive cells on NK cells, the role of oxygen content, usually low within the TME, has not been thoroughly evaluated. In order to study this question, oxygen content and pressure was manipulated using an advanced incubator system (AVATAR control system from xcellbio) to model the tumor microenvironment (1% O2 and 0.3 or 2 PSI), the blood environment (12% O2 and 2 PSI), and the bone marrow environment (5% O2 and 0.6 PSI). Our findings were controlled to a standard 5% CO2 incubator (20% oxygen), not physiologic for any of these settings. The effect of 24 hour, 3 day, and 7 day incubations in these conditions were then evaluated. While oxygen concentration did not seem to significantly impact NK cell survival, NK cell proliferation was markedly reduced at the 1% O2 (TME) and intermediately reduced at the 5% O2 (bone marrow) when compared to the 12% O2 (blood) and standard incubator conditions. NK degranulation against K562 targets, measured by CD107a surface expression, was impacted negatively after shorter (24 hrs and 3 days) low oxygen incubations, but not at day 7. Interestingly, degranulation mediated by ADCC was not impacted by oxygen concentration. In contrast, IFNγ was decreased early (24 hrs and 3 days) with low oxygen but was increased at the 7 day time point in low oxygen when compared to higher oxygen conditions. Reverse ADCC assays using P815 cells comparing low oxygen (1%) to high oxygen (20%) showed increased IFNγ and TNFα. Degranulation was decreased with NCR triggering but not CD16 triggering, but all reverse ADCC modalities resulted in decreased target killing at the low oxygen condition. High-dimensional mass cytometry (CyTOF) analysis indicated that low oxygen, particularly at the 3 and 7 day time points, resulted in disappearance of CD56bright NK cells, increased CD69 expressing cells, lack of proliferation (Ki67), decreased expression of CD16 and NCRs (NKp44, NKp30, NKp46 and NKG2D), and low Perforin and Granzyme levels. Taken together, these results strongly support a role of oxygen content in maintenance of NK cell cytolytic activity within the TME. Ongoing studies will determine the genetic and metabolic drivers of these findings.

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IDENTIFYING MODULATORS OF NATURAL KILLER CELL ACTIVATION THROUGH A MULTIPLEXED GENETIC SCREEN

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Natural killer (NK) cells can recognize and kill virally transformed and malignant cells in the absence of antigen specificity. With their innate selectivity against transformed cells over normal healthy cells, NK cells have gained considerable attention as promising therapeutic tools for cancer immunotherapy. However, up to date only few clinically approved therapies exist that harness their potential, mainly because the activating and inhibitory receptor pathways and their interplay are still not fully understood. To systematically identify and study regulators of NK cell activation and proliferation, we have adapted CRISPR/Cas9 for single-gene and multiplexed loss-of-function studies in primary NK cells. Our screening system is based on primary NK cells isolated from transgenic mice harboring a constitutively expressed Cas9 allele in the Rosa26locus and an interferon gamma (IFN-g) reporter cassette to monitor NK activity using flow cytometry. To enable CRISPR-based loss-of-function studies in this system,we have established lentiviral vectors and transduction protocols for efficient sgRNA delivery into primary NK cells. In a first screen, we sought to identify regulators in signaling pathways mediating NK cell activation. We screened in a pooled format using deep-sequencing of sgRNAs as main readout. When we monitor the library representation over nine days of NK cell culture, we find that sgRNAs targeting generally essential genes or known regulators of NK cell proliferation and survival strongly deplete over time, providing additional proof that our CRISPR/Cas9 system is highly effective and suitable for negative-selection screening. In fractionated sorting experiments, we identified several factors that show strong sgRNA biases between hypo-responsive(IFN-g low)and hyper-responsive(IFN-g high)cells, indicating that they may function as modulators of NK cell activation. To probe their potential in modulating NK cell anti-tumor effects, novel targets arevalidated in co-culture systems andinin vivomodels.

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IMAGING NK ACTIVITY IN 3D CULTURES: THE ROLE OF PVR IN MODULATING NK CELL RECOGNITION VIA DNAM-1 AND TIGIT

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Natural Killer cells are able to recognize, follow and kill malignant cells that continuously arise in our body, limiting their potential growth and expansion. However, NK cell-based immunotherapies to treat solid tumors have been largely unsuccessful, possible due to immune-suppressive activity of the tumor environment. To increase our understanding of NK cell's role in immune surveillance of solid tumors, we have developed a microchip platform where low energy-ultrasound waves induce in vitro 3-dimensional (3D) cultures and characterize NK-tumor interactions by high-resolution imaging. Here, we combine the use of microchip-based ultrasound-induced 3D culture with traditional culture approaches to investigate whether renal carcinoma cell grown in monolayers or as spheroids are different in term of NK cell recognition and killing. We further extended the analysis to NK cells, analyzing their phenotype and subset distribution in 3D cultures. The results demonstrate profound differences between the 2D and 3D cultures, justifying the use of spheroids for studying the properties of the tumor microenvironment. Specifically, we observed a significant reduction of intracellular and extracellular PVR in 3D cultures of A498 renal cell carcinoma alone, suggesting that NK cell recognition via DNAM-1 is reduced in 3D compared to 2D cultures. We further determined NK cell viability and expression of NK receptors in both 2D and 3D co-cultures of NK cells and renal carcinoma cells, distinguishing between intra-tumoral and extra-tumoral NK cells in 3D cultures at different time-points. A downmodulation of DNAM-1 was observed in both 2D and 3D co-cultures of NK-tumor cells compared to NK alone.

Interestingly, an increased expression of TIGIT was observed only in 3D co-cultures of NK-tumor cells and not in 2D co-cultures or cultures of NK cells alone. Based on those findings, the TIGIT+ DNAM-1- NK population was characterized in 3D co-cultures of NK cells-tumor cells, revealing an increased percentage of mature NK cells with impaired cytotoxic activity and cytokine production. Thus, our data suggest that PVR-mediated activation is reduced in 3D cultures of renal cell carcinoma due to modulated protein expression of both target and NK cells. Ongoing studies are directed towards understanding how the interplay between DNAM-1, TIGIT, and PVR could affect the NK activity and infiltration in the tumor microenvironment. Our results suggest roles of TIGIT and PVR as immune checkpoints not only in the treatment of hematological cancer, but also in solid tumors.

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IMPACT OF NKG2D AND NKG2A GENE POLYMORPHISM ON NK CELL FUNCTION AND OUTCOME OF IMMUNOTHERAPY IN ACUTE MYELOID LEUKEMIA

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Natural killer (NK) cells are key effector cells in myeloid leukemia, and previous studies by us and others have shown that expression levels of the natural cytotoxicity receptors, NKp30 and NKp46 impact on prognosis and outcome of acute myeloid leukemia (AML). Studies have suggested that NKG2D gene polymorphisms account for part of the interindividual variation in NK cell function and furthermore in the prevention of cancer, but there is limited data regarding the role of NKG2D gene variants in AML. In exploratory studies of multidimensional flow cytometry data using the software package RchyOptimyx, we observed a role for NKG2D for treatment outcome of histamine dihydrochloride (HDC)/IL-2 immunotherapy in AML. We thus genotyped AML patients receiving HDC/IL-2 for the NKG2D single nucleotide polymorphism, rs1049174. These studies showed that individuals who carried at least one HNK1 allele (G/x) had improved overall survival as compared to individuals with two LNK1 (C/C) alleles.

The HNK1 alleles have been proposed to enhance NKG2D-dependent functions, but the in vitro evidence for this is scarce. Furthermore, this polymorphism is in strong linkage disequilibrium with other polymorphisms in the NK locus, including SNPs near the gene encoding NKG2A. Thus, in a second part, we use K562 variants that were genetically engineered to be killed in a highly NKG2D-dependent manner to determine the impact of NKG2D gene variants on NK cell cytotoxicity. We further type patients and healthy controls for NKG2A SNPs and determine the impact on NK cell education (granzyme B content and degranulation to K562 cells) and on clinical outcome of HDC/IL-2 immunotherapy in AML.

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INCREASED NK IMMUNOGENICITY OF BRAF INHIBITOR RESISTANT MELANOMA CELLS

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Targeted therapies and immunotherapies are reference treatment for melanoma patients. BRAF and MEK inhibitors induce rapid clinical responses in most melanoma patients bearing a BRAFV600E tumor. However, acquired resistance occurs in the majority of patients leading to relapse. Immunotherapies that active immune cytotoxic effectors induce long lasting responses in 30% of patients. In that context, combination of inhibitors with immunotherapy is a promising approach and we considered boosting Natural Killer (NK) cell tumor immunosurveillance, as melanoma cells express stress-induced molecules and activate NK cell-lysis. We have generated vemurafenib resistant (R) cells from BRAFV600E SK28 and M14 sensitive (S) melanoma cell lines. Vemurafenib resistance involved activation of p-AKT in SK28R and of p-MEK/p-ERK in M14R cells, two resistance mechanisms found in patients. Resistance was accompanied by modulation of NK ligands. Compared to S cells, SK28R displayed increase expression of NKG2D ligands (MICA, ULBP2) whereas M14R exhibited decreased ULBP2 and HLA-E (NKG2A ligand) and increased HLA-DR. SK28R and M14R cells induced higher NK degranulation and IFNγ secretion and were more efficiently lyzed by NK cells. SK28R showed increased TRAIL-DR5 expression and TRAIL-induced apoptosis whereas TRAIL-induced apoptosis of M14R was decreased. Analysis of transcriptomic datas allowed us to see the involvement of different pathways in invasivness (MET transition) and in the response to NK cells (TNFa response, INFg response). Combined BRAF/MEK inhibitors abrogated the growth of SK28S, M14S, and M14R cells, while growth of SK28R was maintained. Combined inhibitors treated S cells activated poorly NK cells but R cell lines activated polyfunctional NK cells and were lyzed with high efficiency. These findings outline the interest of combined targeted therapy and NK based immunotherapy for melanoma patients.

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INNATE-LIKE HER2-SPECIFIC-NKP30+CD8+ T CELLS EXHIBITING DUAL ANTI-TUMOR POTENTIAL AGAINST TUMOR TARGETS

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CD8+ T cell recognition of tumor cells is typically based on the detection of specific MHC–peptide complexes, while natural killer (NK) cell recognition relies on the detection of NK ligands by an array of NK receptors. There is increasing evidence that tumors are heterogeneous and frequently escape from the recognition by CD8+ T or NK cells, which represents a major challenge for the use adoptive transfer therapies for successful immunotherapy. We recently identified a population of CD8+ T cells circulating in peripheral blood of healthy donors, marked by the expression of NKp30 displaying a diverse TCR repertoire. This innate-like NKp30+CD8+ T cell population could be de novo induced by IL-15 and required the acquisition of the FceRIg adaptor after promoter demethylation. B7H6, a tumor-associated ligand for NKp30 is frequently upregulated in several tumors, making B7H6/NKp30 an important axis in tumor immunology. IL-15-induced NKp30+CD8+ T cells co-expressed other activating NK receptors and exhibited potent NK-like cytotoxic activity against tumor cells expressing B7H6, both in vitro and in vivo in a pre-clinical xenograft melanoma mouse model. To further harness the anti-tumor activity of NKp30+CD8+ T cells, we aimed at arming these cells with specificity directed against the tumor antigen Epidermal Growth Factor Receptor 2 (HER-2), overexpressed in several malignancies, such as breast cancer, ovary, gastric and colon carcinoma. We succeeded to virally transduce primary human NKp30+CD8+ T cells to specifically target HER-2. These HER-2specific- NKp30+CD8+ T cells showed enhanced responses towards tumor targets with expression of HER-2 and/or B7H6. This dual capacity to recognize and kill tumor cells, combining anti-tumor activity of both CD8+ T and NK cells, allows this unique effector population to target tumor heterogeneity, thus improving strategies against tumor escape.

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INVESTIGATING MECHANISMS OF ACTIVATING RECEPTOR-INDUCED ANTI-TUMOR DYSFUNCTION IN NATURAL KILLER CELLS

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NK cells are gaining clinical momentum as potent immunotherapeutic mediators of anti-tumor responses, but the suppressive effects of the tumor microenvironment on NK cell activity have not been fully explored. Ligands for NK cell activating receptors are abundant in many solid tumors and, in excess, are known to provide NK cells with inhibitory signals, preventing adequate tumor infiltration and clearance. Two such receptors involved in anti-tumor immunity are NKG2D and NKp46, though their mechanistic role in mediating negative signals and functional exhaustion in NK cells remains to be fully investigated. The goal of my research is to study the inhibitory effects of cellular activation on NK cell anti-cancer immunity, how this dysfunction can be rescued, and the cellular mechanisms that govern these processes. Our preliminary data have revealed a dysfunctional NK cell phenotype after 72 hours of continuous exposure to plate-bound agonists of NKG2D and NKp46, suggesting that there are physiologic limits to activation stimuli. This phenotype is characterized by decreased production of the pro-inflammatory cytokines IFN-γ and TNF-β following incubation with tumor targets cells, decreased in vitro killing of tumor targets evaluated using the IncuCyte live-imaging platform, upregulation of immune checkpoint markers, and downregulation of activating receptors. Coincident with these traits are metabolic defects consisting of decreased oxygen consumption and extracellular acidification rates, pointing towards deficiencies in mitochondrial and glycolytic health, respectively. Importantly, this work has also demonstrated that a 48-hour recovery period with low dose IL-15 is unable to restore the function of exhausted NK cells; however, the addition of low dose IL-12 to this recovery can rescue some aspects of cytokine production. Future work will investigate the underlying biology of reversible and irreversible activating receptormediated NK cell exhaustion. Ultimately, this research will inform the design of rational NK cell-based cancer immunotherapies to effectively apply NK cells to solid tumor malignancies.

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LEUKEMIC BLASTS DOWNREGULATE HLA-E EXPRESSION TO LOW-BUT TOLEROGENIC-LEVELS SUGGESTING A KEY ROLE FOR NK CELLS IN CONTROL OF PEDIATRIC ALL

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Clinical and experimental evidence suggests that acute lymphoblastic leukemia (ALL) is highly resistant to NK cellmediated killing, even in the allogeneic setting. The mechanisms mediating this resistance are poorly understood but it is likely that inhibition via HLA class I-specific NK cell receptors plays a major role in this process. To this end, we followed expression of classical HLA class I as well as the non-classical HLA-E molecule on leukemic blasts through serial time-points during the disease history of pediatric ALL patients. Unexpectedly, whereas for classical HLA class I alleles no consistent up- or downregulation was observed, HLA-E expression was decreased in leukemic blasts of all patients. Specifically, HLA-E downregulation was seen in all patients at the time of diagnosis and more importantly in all cases of relapse including relapse following chemotherapy and relapse following allogeneic stem cell transplantation. The onset of HLA-E downregulation in peripheral blood consistently met the significant increase in molecular minimal residual disease (MRD) levels in bone marrow samples. In order to better understand the paradoxical finding of HLA-E downregulation, which makes the leukemic blasts principally more susceptible to NK cell mediated killing, functional experiments were performed. Importantly, blocking of NKG2A-HLA-E interaction between healthy NK cells and primary ALL blasts using an HLA-E specific antibody made leukemic blasts susceptible to recognition by NKG2A+ NK cells, leading to a significant increase in degranulation. Thus, ALL blasts downregulate HLA-E to a specific low level that still enables full inhibition by NK cells. Vice versa, HLA-E expression on leukemic blasts could be upregulated to physiological levels by stimulation with interferon gamma. Altogether, the present study suggests that downregulation od HLA-E is a necessary condition for establishment of pediatric B-ALL and points to a decisive role of NK cells in this process. Moreover, HLA-E constitutes a novel and highly reliable marker for monitoring of minimal residual disease in peripheral blood of ALL patients.

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MESENCHYMAL STROMAL CELLS SHAPE THE MDS MICROENVIRONMENT BY INDUCING SUPPRESSIVE MONOCYTES THAT DAMPEN NK CELL FUNCTION

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Altered bone marrow hematopoiesis and immune suppression is a hallmark of myelodysplastic syndrome (MDS). While the bone marrow microenvironment influences malignant hematopoiesis, the mechanism leading to MDSassociated immune suppression is unknown. We tested whether mesenchymal stromal cells (MSCs) contribute to this process. Here, we developed a model to study cultured MSCs from MDS patients compared to similar aged matched normal controls for regulation of immune function. MSCs from MDS patients (MDS-MSC) and healthy donor MSC (HDMSC) exhibited a similar in vitro phenotype and neither had a direct effect on NK cell function. However, when MDS and HD-MSCs were cultured with monocytes, only the MDS-MSCs acquired phenotypic and metabolic properties of myeloid-derived suppressor cells (MDSCs), with resulting suppression of Natural Killer (NK) cell function, along with T cell proliferation. A unique MSC transcriptome was observed in MDS-MSCs compared to HD-MSCs, including increased expression of the reactive oxygen species (ROS) regulator, ENC1. High ENC1 expression in MDS-MSC induced suppressive monocytes with increased INHBA, a gene that encodes for a member of the Transforming growth factor beta (TGFβ) superfamily of proteins. These monocytes also had reduced expression of the TGFβ transcriptional repressor MAB21L2, further adding to their immune suppressive function. Silencing ENC1 or inhibiting ROS production in MDS-MSCs abrogated the suppressive function of MDS-MSC conditioned monocytes. In addition, silencing MAB21L2 in healthy MSC conditioned monocytes mimicked the MDS-MSC suppressive transformation of monocytes. Our data demonstrate that MDS-MSCs are responsible for inducing an immune suppressive microenvironment in MDS through an indirect mechanism involving monocytes.

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MODELING NK CELLS ANTI-CANCER ACTIVITIES IN FULL HUMAN IMMUNE SYSTEM MOUSE MODELS

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The therapeutic potential of NK-cells for the elimination of cancer cells represents the basis for several ongoing patient clinical trials. This approach can now be mimicked with full human immune system reconstitution in mouse models (hu-mouse), which provide smart and predictive preclinical endpoints to translate these new NK therapies into efficient and safe clinical drug candidates.

Using transient hydrodynamic expression of human IL15 to boost further the number of functional NK cells in the fully reconstituted CD34+ engrafted hu-mouse models is a key step. This physiological and temporal expression of hIL-15 allows the expansion of human NK cells in peripheral blood but also in other lymphoid tissues, providing numerous physiological advantages compared to the constitutive NK boost produced in hIL15 transgenic hu-mouse models. One week after the human IL15 boost, the human NK cells amount is tripled in the peripheral blood of hu-mouse and is stable for at least five weeks providing sufficient time to engraft tumor cells (subcutaneous or orthotopic injection) and assess a drug candidate anti-tumor efficacy. Significant human NK cells infiltration in the tumor is also enhanced in hIL15 boosted hu-mice. In addition, the increased efficacy of ADCC-dependent antibody drug candidates (administered prophylactically or therapeutically) in hIL15 boosted hu-mouse models as been reported with e.g. melanoma or multiple myeloma cell lines. Furthermore, human NK cells isolated from hIL15 boosted hu-mice present ex vivo activities. In summary, the hIL15 boosted full human immune system hu-mouse provide a reliable and validated in vivo antitumor models to better predict the efficacy and MOA of pre-clinical drug candidates targeting NK cells functions.

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MURINE ACUTE MYELOID LEUKEMIA ALTERS NK CELL MATURATION AND FUNCTIONS BY AFFECTING IL-15 SIGNALING

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Despite the significant progress in the treatment of Acute Myeloid Leukemia (AML), this disease remains lifethreatening due to escape to chemotherapy and to immune surveillance. Among immune escape mechanisms, subversion of anti-tumoral features of Natural Killer (NK) cells has been described. Hence, NK cells in AML display altered phenotype, maturation, gene expression and functions, thus affecting clinical outcome. However, so far the mechanisms underlying these defects remain elusive. Here, we have used syngeneic mouse model for AML to decipher these mechanisms. After leukemia progression, we showed maturation and functional defects in NK cells comparable to those described in patients. To investigate the mechanisms of these defects, we studied IL-15/mTOR signaling pathway, an important regulator of NK cell maturation and cytotoxicity. Although NK cells of leukemic mice displayed an activated phenotype, we showed that they responded significantly less to IL-15 stimulation ex vivo. Moreover, we noted that leukemic mice NK cells displayed a reduced mTOR mediated metabolism, and in line, reduced proliferation capacities in vivo. Next, we performed mRNA sequencing analysis of leukemic mice NK cells. We noted an alteration of NK cell signaling pathways. Hence, we observed a down-regulation of activating pathways, such as MAPK and STAT signaling, and an up-regulation of negative regulators, such as SOCS proteins in leukemic mice NK cells. Altogether, our data suggest an exhaustion of NK cells in AML that might be responsible for NK cell defective target cell recognition. Further investigation is required to determine whether physical interaction or immunosuppressive factors both from the leukemic microenvironment are responsible for NK cell defects.

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NATURAL KILLER CELLS OFFER DIFFERENTIAL PROTECTION FROM LEUKEMIA IN CHINESE SOUTHERN HAN

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Interactions of human natural killer (NK) cell inhibitory receptors with polymorphic HLA-A, -B and -C molecules educate NK cells for immune surveillance against tumor cells. The KIR A haplotype encodes a distinctive set of HLAspecific NK cell inhibiting receptors having strong influence on immunity. We observed higher frequency of KIR A homozygosity among 745 healthy Chinese Southern Han than 836 adult patients representing three types of leukemia: ALL (OR=0.68, 95% CI=0.52-0.89, p=0.004), AML (OR=0.76, 95% CI=0.59-0.98, p=0.034), and CML (OR =0.72 95% CI=0.51-1.0, ns). We observed the same trend for NHL (OR=0.47 95% CI=0.26-0.88 p=0.017). For ALL, the protective effect of the KIR AA genotype was greater in the presence of KIR ligands C1 (Pc=0.01) and Bw4 (Pc=0.001), which are tightly linked in East Asians. By contrast, the C2 ligand strengthened protection from CML (Pc=0.004). NK cells isolated from KIR AA individuals were significantly more cytotoxic towards leukemic cells than those from other KIR genotypes (p<0.0001). These data suggest KIR allotypes encoded by East Asian KIR A haplotypes are strongly inhibitory, arming NK cells to respond to leukemogenic cells having altered HLA expression. Thus, the study of populations with distinct KIR and HLA distributions enlightens understanding of immune mechanisms that significantly impact leukemia pathogenesis.

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NK CELL LYMPHOPOIESIS DURING THE ONSET AND PROGRESSION OF LEUKEMIA

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NK cells are key components of the innate immune system that can directly recognize and eliminate infected cells and cancer cells without previous activation or expansion. Ample data support a role for NK cells in tumor surveillance. Direct evidence derives from experiments showing that mice deficient in NK cells are more prone to tumor development (Guillerey C. and Smyth M.J., Curr Top Microbiol Immunol, 2016). Similarly, in humans an increased risk of cancer, including myeloid malignancies, has been linked to NK cell-deficiencies (Imai K. et al., Lancet 2000). NK cells from patients with myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) have poor cytotoxic functions and reduced expression of killing receptors (Fauriat C. et al., Blood 2007; Carlsten M. et al., Leukemia 2010). The severity of NK cells impairment is positively linked to more advanced cancer stages. The mechanism behind this suppression of NK cell function is not well understood, and it has not been investigated whether and how the disease environment alters NK cell development. The NHD13 mice carrying human NUP98-HOXD13 transgene represent a disease model progressing from MDS at 4-7 months to leukemia at 10-12 months (Lin Y.W. et al., Blood 2005; Chung Y.J. et al., Proc Natl Acad Sci USA 2008). Previous work demonstrated that B and T lymphopoiesis in NHD13 mice is impaired, but NK cell development has not been studied. We are using this model to investigate: 1) how different NK cell compartments are changing during the onset and progression of the disease, 2) what mechanism/ pathways are involved in NK cell-deficiencies and 3) how leukemic cells escape NK cell surveillance. We found that at the MDS stage (3-5 months), NK cell development is severely perturbed as illustrated by the dramatic reduction in the NK cell progenitor pool and nearly complete loss of mature NK cells, while at the same time the proportion of leukemic blasts expressing c-KIT is increased. Interestingly, already in young mice (1.5-2 months), before the signs of MDS could be detected, the NK cell compartment is affected: the numbers of NK cell progenitors and mature NK cells are decreased and, although NK cell activity is not significantly altered, the final NK cell maturation stages are perturbed. We are currently performing competitive transplantation, lineage potential analysis and gene profiling of progenitors and mature NK cells purified from NHD13 mice and the littermate controls to identify mechanisms involved in NK cell developmental defects.

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NK CELL-MEDIATED GRANZYME B AND CASPASE-8 ACTIVITY DURING SERIAL KILLING OF TUMOR CELLS

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Natural Killer (NK) cells are capable of responding to virus-infected and tumor cells by releasing the contents of cytotoxic granules or by engaging death receptors that initiate caspase cascades. While the mechanisms of the two pathways are well studied, the complex interplay between both has remained poorly defined. To visualize the granzyme B (GrzB) and death receptor-mediated target cell death in a time-dependent manner, we used fluorescent localization reporters that enabled us to measure the activities of GrzB and of Caspase-8 in target cells upon contact with NK cells simultaneously by life cell imaging.

We observed a rapid target cell death which was induced by GrzB and which originated from early established NK: target contacts. In contrast, cell death mediated by Caspase-8 was a result of later target cell engagements and took much longer from NK: target cell contact to target cell death. By using a microchip platform, we could investigate the serial killing activity of individual NK cells. This analysis showed that NK cells switch from mediating a fast GrzBmediated cell death in their first killing events to a slow death receptor-mediated cell death during their final tumor cell encounter. Investigating NK cells during target cell exposure, we found a clear reduction of GrzB and perforin and an increase of surface-CD95L over time, showing how the switch in cytotoxicity pathways is controlled.

To investigate the contribution of both killing pathways we deleted perforin in human NK cells. These NK cells were unable to perform GrzB-mediated serial killing and only killed once via death receptors. In contrast, using CD95 knockout target cells did not impair the GrzB-mediated serial killing while death receptor-mediated cell death was clearly reduced.

Our study demonstrates that the use of GrzB vs. death receptor-mediated target cell killing is differentially regulated during the serial killing activity of NK cells. This suggested a kinetic regulation of the two cytotoxic pathways during serial killing.

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NK CELLS SUSTAIN BREAST CANCER DORMANCY IN THE LIVER

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Failure of the conventional therapies to eradicate disseminated tumor cells (DTCs) and prevent tumor recurrence is one of the main barriers to effective cancer treatment. The increasing recognition that these dormant DTCs depend on the surrounding microenvironment suggests that targeting the latter might be a powerful alternative strategy for therapeutic intervention in metastatic cancer. Deriving and implementing such microenvironmental-targeted therapies in the clinic urges a comprehensive understanding of the cellular and molecular make-up of DTC microenvironments in a tissue-specific manner, which is currently lacking. Here we reveal the determinants of metastatic dormancy and progression of breast cancer to the liver, an often site of metastasis and most times associated with patients' cause of death. We developed a DTC live tracker that distinguishes quiescent from dividing cells and showed the liver is a preferential shelter for quiescent cells, conceding metastatic expansion only in few regional sub-niches. We then isolated hepatic niches corresponding to distinct breast cancer progression stages (dormant disease and manifest metastasis), and reconstructed their cellular composition by next generation sequencing, imaging and immunoprofiling. We identified natural killer (NK) cells as prime effectors of sustained breast DTC quiescence. Depletion of NK cells from early dissemination phase resulted in increased metastatic burden at end stages of cancer progression, whereas boosting this suppressive population prevented hepatic metastatic outgrowths. Combining stateof-the-art gene expression and bioinformatics, proteomics and engineered liver-like microenvironments, we identified secreted factors in the microenvironment-cancer cell crosstalk that are essential for suppressing NK cell function and sparking cancer cell proliferation. These findings uncover NK cells as a key unit of the liver dormant niche and show the efficacy of normalizing NK cell function within specific distant tissues to prevent overt metastases.

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NK CELL-SPECIFIC CDK8 DELETION ENHANCES ANTITUMOR RESPONSES

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Cyclin-dependent kinase 8 (CDK8) is a serine/ threonine kinase and member of the transcription-regulating CDK family. CDK8 activates or represses transcription by associating with the mediator complex or by regulating transcription factors. Oncogenic activity of CDK8 has been demonstrated in several cancer types such as colorectal cancer, breast cancer or acute myeloid leukemia (AML). Thus, targeting CDK8 represents a potential therapeutic strategy.

Because knockdown of CDK8 in a NK cell line enhances cytotoxicity and NK cells provide the first line of immune defense against transformed cells, we asked whether inhibiting CDK8 would improve Natural Killer (NK) cell antitumor responses. We investigated the role of CDK8 in NK cell function using mice with conditional ablation of CDK8 in NKp46+ cells (Cdk8fl/flNcr1Cre mice). Regardless of CDK8 expression, NK cells develop and mature normally in bone marrow and spleen. However, CDK8 deletion increased expression of the lytic molecule perforin, which correlated with enhanced NK cell cytotoxicity in vitro. This translates into improved NK cell-mediated tumor surveillance in vivo in three independent models: B16F10 melanoma, v-abl+ lymphoma, and a slowly developing oncogene-driven leukemia. Our results thereby define a suppressive effect of CDK8 on NK cell activity. We now aim to dissect the mechanism underlying the inhibitory effect of CDK8 in NK cells performing high-throughput analyses including ChIP-Seq and to address whether the effect is kinase dependent or independent.

In summary, our findings suggest that targeting CDK8 in cancer could hit two birds with one stone – enhancing NK cell activity while simultaneously blocking tumor cell proliferation.

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PRELIMINARY RESULTS OF A LARGE POPULATION-BASED STUDY INDICATE NO ASSOCIATION OF KIR-/HLA-GENOTYPE AND THE RISK OF DEVELOPING ACUTE MYELOID LEUKEMIA

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Immunogenetic disease association studies may give rise to new hypotheses on the immunosurveillance of cancer. Polymorphic killer cell immunoglobulin-like receptor (KIR) genes and Acute Myeloid Leukemia (AML) have not been investigated so far in large population studies. For this purpose we typed the KIR and HLA genotype of German patients who were diagnosed with AML and compared their results to data of 100.000 German individuals who registered with DKMS. Patient samples were retrieved from the Collaborative Biobank and the Biorepository of the Study Alliance Leukemia. The samples were genotyped for KIR and HLA with high-resolution amplicon-based Next Generation Sequencing using the standard workflow. Data on controls were taken from recently typed individuals who had registered with DKMS and whose DNA had been typed in the same workflow. Owing to the large number of controls in this setting, even small deviations from the average distribution could be revealed. Knowledge on KIRs and their cognate HLA-ligands allowed for testing of several hypotheses of Natural Killer (NK) cell mediated leukemia surveillance.

For a preliminary analysis, genotype information of 677 patients with AML was compared to data from 100.000 controls. We did not find significant differences between the two cohorts for absence/presence calling for KIR2DL1, 2DL2, 2DL3, 2DP1, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1 and 3DS1. When grouped by telomeric or centromeric gene content, the major haplotypes A/A, A/B, and B/B were almost equally distributed among patients and controls. With respect to the KIR-ligands C1 and C2, patients with AML more often exposed both ligands (51% versus 47%, ztest, p=0.02) compared to controls but significance was lost after adjustment for multiple testing. No significant differences between patients and controls were found with respect to the presence or absence of HLA-Bw4-80I/80T, the cognate ligand for KIR3DL1. Using information on KIRs and their cognate ligands, we further tested receptorligand models. KIR2DS1-positive individuals with C1/C1 or C1/C2 ligands were equally distributed among patients and controls. Also, individuals who were grouped according to their KIR3DL1-alleles and cognate ligands into stronginhibiting, weak-inhibiting, and non-inhibiting combinations were equally represented among patients and controls.

The current results suggest that the NK- and HLA-genotype is not associated with the risk for developing AML. Final results of the full analysis-set, including allele level information, and more advanced models will be presented at the conference.

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REDUCED CD160 EXPRESSION CONTRIBUTES TO IMPAIRED NK CELL FUNCTION AND POOR CLINICAL OUTCOMES IN PATIENTS WITH HCC

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Previously, we have reported that decreasing in the number of NK cells and their functional deficiency play an important role in the progression of hepatocellular carcinoma (HCC). However, the underlying mechanisms remain obscured. In this study, we analyzed the expression of CD160 on both intrahepatic CD8+ T cells and NK cells through the evaluation of peritumoral and intratumoral tissues of 279 HCC patients in addition to 20 healthy livers. In our study, significantly reduced expression of CD160 was observed on intratumoral NK cells. Patients with lower CD160 cell densities within tumor were accompanied by a worsen disease condition and higher recurrence rate. Flow cytometrysorted primary intrahepatic CD160+ and CD160- NK cells of healthy livers were analyzed by high-resolution microarray and gene set enrichment analysis (GSEA), the results indicated that human CD160+ NK cells featured functional activation, high IFN-γ production, and NK-mediated immunity. In addition, global transcriptomic analysis of sorted peritumoral and intratumoral CD160+ NK cells revealed that intratumoral CD160+ NK cells are more exhausted and produce less IFN-γ compared to peritumoral CD160+ NK cells. High level of TGF-β1 interfered with the production of IFN-γ by CD160+ NK cells, blocking of which specifically restored IFN-γ production of CD160+ NK cells to normal level. In conclusion, these findings indicate that reduced number of CD160+ NK cells, together with the functional impairment of CD160+ NK cells by TGF-β1, contribute to the tumor immune escape. Reversing the expression of CD160 or blocking TGF-β1 seems to be a promising therapeutic way in fighting liver cancer.

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SESAMOLIN CREATE AN OPTIMAL ENVIRONMENT FOR CANCER CELL SENSITIZATION BY NATURAL KILLER CELLS

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BACKGROUND AND AIM
In our previous study, we demonstrated that sesamolin can increase the level of cancer cell susceptibility to natural killer (NK) cell mediated cytolysis when it treats cancer cells. The present study attempted to demonstrate the direct influence of sesamolin on NK cells.

METHODS
Materials and reagents: Sesamolin was purchased from Nagara Science (Japan), which was purified from sesame oil. Cell lines: NK-92MI and Raji were purchased from the ATCC (USA).
Preparation of naïve NK cells: The NK cells were then isolated using immunomagnetic beads selection with 'Untouched Human NK cells' (Invitrogen, Dynal, Inc., Norway) according to the manufacturer's instructions. NK cell cytotoxicity assay: The lytic capacity of NK cells was examined in LDH assay (Takara, Japan).

RESULTS
When NK-92MI cells were treated with sesamolin, the cytolysis activities of NK cells increased depending on the concentration of sesamolin. The highest cytolytic activity of NK cells was observed when Raji and NK-92MI cells were treated with sesamolin at 20 μg/mL and 40 μg/mL, respectively. Sesamolin also increased the expression of the degranulation marker, CD107a, on the surface of NK cells and the production of immune-activation cytokine, IFN-γ, from NK cells. The effects of sesamolin on NK cells were reproduced in the naïve NK cells. We found that sesamolin effects are triggered by the result of phosphorylation of the p38, ERK1/2 and JNK pathways in NK cells.

CONCLUSIONS
This study proved that NK cell activity can be increased by the stimulation of sesamolin on NK cells as well as cancer cells.

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SMAC MIMETIC (SM)INDUCED SENSITIZATION OF RHABDOMYOSARCOMA (RMS) CELLS TOWARDS NATURAL KILLER (NK) CELL MEDIATED KILLING

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RMS is one of the major form of pediatric soft tissue cancers. Although the five-year survival rate has increased over the last decades, the aggressive subtype of metastatic alveolar RMS still has a poor outcome and an overall five-year survival rate below 20%. One underlying mechanism of cell death resistance and cancer progression in RMS cells is a dysregulation of inhibitor of apoptosis proteins (IAP). Small molecule inhibitors mimicking the endogenous IAP antagonist Second mitochondria-derived activator of caspases (Smac), i.e. SM, can restore the apoptosis inducing pathways of cancer cells. Here, we hypothesized that SM could be used to sensitize RMS cells to NK cell mediated killing.

To investigate NK cells as therapeutic strategy for treatment of RMS, co-culture experiments of RMS cells with NK cells were performed. NK cells were able to induce lysis of RMS cells. Notably, pretreatment of RMS cell with SM further increased the NK cell-mediated lysis of RMS cells. To elucidate the underlying mechanism of SM induced sensitization in RMS cells, activation status of signaling pathways was investigated. Treatment of RMS cells with SM hints towards a bimodal feedback mechanism regulating both, the canonical and non-canonical NF-κB signaling pathway.

Further, NK cells are influenced by a treatment with SM similar to the tumor cells. An increased transcription of NIK, IFNγ and TNFα hints to an activation of NF-κB signaling pathways in treated NK cells and needs further evaluation. During a cocultivation of NK and RMS cells, NK cells induce the expression of IRF1, TRAIL and IFNγ.

Previously, we could show that NK cells kill RMS cells by a TRAIL mediated mechanism and that SM sensitizes RMS cells to a TRAIL induced cell death in a synergistic manner. Further, the surface presentation of TRAIL-R2 was increased by SM on RMS cells. The increased surface presentation of TRAIL-R2 on RMS cells provide a mechanistic explanation for the observed SM induced sensitization of RMS cells towards NK cell mediated killing.

In addition, SM seem to influence the NK cell subpopulation phenotype and thereby modulate the NK cell activity. Understanding the underlying molecular mechanisms of the tumor sensitization, and how to manipulate NK cell signaling pathways, will facilitate the development of a SM – NK cell immunotherapeutic approach.

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STAT3 MEDIATED ACTIVATION OF THE AHR SIGNALING PATHWAY MODULATES NK CELL DEVELOPMENT AND FUNCTION

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The aryl hydrocarbon receptor (AHR) is an evolutionarily-conserved signaling pathway that integrates environmental cues into the regulation of cellular processes. AHR plays a critical role in hematopoietic stem cell (HSC) development, and a role in NK cell development has also been described wherein loss of AHR expression corresponds to a decline in CD56 expression during maturation. However, the role of AHR in the functional regulation of mature human NK cells remains largely unexplored outside of being a receptor for kynurenine, which mediates a suppressive signal from the tumor microenvironment. In this study, we show that mature peripheral blood NK cells expanded with IL-21-expressing feeder cells have increased AHR and CD56 gene expression, resulting in a CD56bright phenotype. Pharmacological inhibition of AHR using the purine analog, StemRegenin, decreased CD56 expression in NK cells during expansion, resulting in a CD56dim phenotype. AHR inhibition also reduced the expression classic AHR target genes AHRR and CYP1B1, and resulted in an increase in the cytotoxic function of NK cells. In contrast, treatment with the AHR agonist kynurenine attenuated anti-tumor function of NK cells. Interestingly, RNA seq showed that blocking AHR affected STAT3 and GPCR signaling pathways that are involved in migration and metabolism of NK cells. Finally, chromatin analysis (ChIP seq) confirmed AHR binding to XRE sites proximal to the promoters of genes involved in NK cell development and function, including CD56. Based on these findings, we propose a model of AHR/STAT3 pathway interactions in the regulation of mature NK cell function and CD56 expression, and postulate that generation of AHR-deficient NK cells may help overcome immune suppression imposed by the tumor microenvironment to make NK-based cell therapies more effective.

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STRONG 3DL1 INTERACTION PREDICTS FAVORABLE OUTCOME IN AML IMMUNOTHERAPY

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NK cell function is regulated through interactions between inhibitory NK cell receptors and HLA class I molecules. Studies in bone marrow transplantation of AML patients have reported that weaker interacting genetic combinations of KIR3DL1 and HLA-Bw4 predicted improved relapse-free and overall survival. In this study we looked into the impact of KIR3DL1 and HLA-Bw4 in non-transplanted AML patients receiving HDC/IL-2 for prevention of relapse in the postconsolidation phase. We genotyped all patients for KIR3DL1 allele presence (low, high or null) and corresponding HLA ligands (HLA-A, Bw4 (80I or 80T)). In contrast to previous transplantation studies, a strong receptor-ligand interaction predicted significantly improved relapse-free survival and a trend towards improved overall survival. A majority of the patients had highly expressed KIR3DL1 alleles and we observed a strong correlation between improved survival and presence of HLA-Bw4 (80I). In in vitro degranulation assays towards HLA-negative K562 cells, 3DL1+ NK cells from HLA-Bw4 (80I) donors displayed significantly higher degranulation responses as compared to NK cells from 80T donors, suggesting improved 3DL1 mediated education in 80I individuals. These differences remained in experiments when NK cells were pre-stimulated by IL-2. We also screened a cohort of AML blasts from newly diagnosed patients and observed low or moderate levels of HLA-Bw4 expression suggesting that IL-2 activated NK cells could override the inhibitory signal. In conclusion, we suggest that 3DL1+ NK cells from donors with a strong interacting receptorligand 3DL1 profile is advantageous upon IL-2 based immunotherapy in relapse prevention of AML. This is likely mediated through improved NK cell education and a moderate expression of HLA-Bw4 on malignant leukemic cells.

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STRUCTURAL BASIS FOR THE RECOGNITION OF NECTIN ADHESION PROTEINS BY THE NK CELL RECEPTORS, TIGIT, CD96 AND DNAM-1

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TIGIT, CD96 and DNAM-1 constitute a family of immune receptors that regulate the activity of NK cells towards transformed targets. The capacity of these nectin receptors to mediate target cell adhesion, immune synapse formation and regulate effector function is dependent on their recognition of nectin and nectin-like (necl) adhesion molecules, which are over-expressed in a wide variety of cancers. Within this axis, DNAM-1 is a stimulatory receptor that activates NK cell-mediated cytotoxicity and is crirical for tumour immune surveillence, while TIGIT is an inhibitory receptor that counteracts DNAM-1 activity. Whether CD96 functions as an activating or inhibitory receptor is unclear. Here, we have determined the crystal structures of all of the human nectin receptors in complex with their cognate ligands, including TIGIT:nectin-2, CD96:necl-5 and DNAM-1:necl-5. In addition, we have performed a comprehensive binding and mutational analysis of these receptors to fully characterise their ligand binding affinity and specificity. Our findings indicate that TIGIT, CD96 and DNAM-1 recognise their ligands with similar (low-micromolar range) affinity using a conserved docking topology that is reminiscent of that observed for nectin-nectin homo/heterodimer assembly. Structural and mutational analysis highlighted the important role that the 'lock and key' motifs within the first extracellular immunoglobulin domain (D1) of each receptor play in ligand binding. Moreover, we demonstrated that the C-C' loop of TIGIT dictates its ligand binding hierarchy, identified a novel motif in CD96, termed the 'ancilliary key', that is critical for necl-5 recognition, and interrogated the role of the second Ig domain of DNAM-1 in necl-5 binding. Altogether, these data significantly broaden our understanding of nectin-nectin receptor interactions and has implications for understanding the molecular basis for tumour recognition and escape.

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THE EXTRACELLULAR MATRIX PROTEIN NIDOGEN-1 IS A NOVEL SOLUBLE LIGAND FOR NK44 ACTIVATING RECEPTOR

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Interactions between activating receptors expressed by human Natural Killer (NK) cells and their corresponding ligands are involved in the recognition of tumor and virus-infected cells. In this context, a major role is ascribed to Natural Cytotoxicity Receptors (NCRs: NKp46, NKp30, and NKp44). In parallel with the identification of several NCR ligands able to activate NK cell-mediated cytotoxic activity, different soluble ligands have been shown to dampen NK cell functions, representing possible regulatory mechanisms or immune evasion strategies.

By combining two-dimensional electrophoresis and high-resolution mass spectrometry, we identified the extracellular matrix protein Nidogen-1 (NID1) as a novel ligand for NKp44 receptor. NKp44-NID1 interaction was validated by both immunoprecipitation and ELISA experiments, revealing that NKp44 recognizes NID1 in its native conformation. When released in soluble form, NID1 regulates NKp44-induced cell activation, both in the BW-NKp44/DAP12 cellular model and in polyclonal NK cell populations. More importantly, in NK cells it also decreases IFN-γ production induced by Platelet-Derived Growth Factor (PDGF)-DD following NKp44 engagement.

We also demonstrate that NID1 can be expressed on the surface of NID1-releasing cells, including different tumor cell lines, and that NKp44Fc is able to recognize NID1 exposed at the cell surface as well as NID1 released in the extracellular space. NID1 expressed at the cell surface or bound to a solid support fails to trigger NK cell cytotoxicity or cytokine release; however, it induces in NK cells relevant changes in the proteomic profiles, pointing at an effect on different biological processes. In addition, NID1 has been also detected in the extracellular vesicles (both microvesicles and exosomes) derived from HEK293T cell line and, also in this form, can be recognized by NKp44Fc .

Collectively, our results indicate that soluble NID1 may interfere with target cell recognition via NKp44, exerting a regulatory effect on NKp44-induced NK cell activation, and suggest a potential role of NID1 as a decoy NKp44 ligand. Further studies will be required to fully characterize NKp44-NID1 interactions in different biological and pathologicalm conditions.

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THE GREAT ESCAPE: ROLE OF THE ACTIN CYTOSKELETON IN CANCER IMMUNE ESCAPE

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Cancer immune evasion is a major obstacle to effective anticancer therapeutic strategies. Even though significant progress has been made in investigating the numerous strategies how tumors evade recognition and destruction by the immune system, approaches on antagonizing these escape strategies are often overcome in a short time. A new strategy to escape attacks from innate immune cells which we observed first in different breast cancer lines is the accumulation of actin at the region of the immunological synapse (IS). While reorganization of actin filaments (F-actin) is a crucial prerequisite for the formation of the IS and directed release of cytotoxic granules, these observations were so far limited to the immune cell site. Our findings indicate that a rapid actin accumulation in cancer cells can promote their survival during natural killer (NK) cell attack. Tumor cells that are competent to respond to NK cell contact with fast actin cytoskeleton remodeling survive cytotoxicity assays and can detach from the immune cell without undergoing apoptosis. The rapid accumulation of polymerized Factin at the immune synapse is accompanied by clustering of inhibitory ligands such as MHC class I or PD-L1 at the synaptic region. We report similar findings in different types of cancer as well as murine breast cancer and melanoma models. Importantly we also find an actin accumulation at the synaptic region in tumor cells challenged by cytotoxic T lymphocytes, strongly indicating a conservation of this escape strategy. In vivo data suggests that enhanced actin dynamics mediate increased resistance in immune competent mice, identifying the actin cytoskeleton as a potentially vulnerable property of cancer cells, with the possibility to target specific actin filament populations or actin-binding proteins that are selectively upregulated or show altered functions in cancers.

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TUMOR-DERIVED IL15 INDUCES A TISSUE-RESIDENT CYTOTOXIC INNATE LYMPHOCYTE RESPONSE

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Harnessing the effector function of cytotoxic lymphocytes for the treatment of cancer is an area of intense research. Thus, understanding how cytotoxic lymphocytes are regulated within the tumor microenvironment is crucial to development of new therapies. IL15 is a cytokine that plays various roles in the development and function of cytotoxic lymphocytes, including the maturation and effector function of natural killer (NK) cells as well as generation of memory CD8 T cells. Utilizing the MMTV-PyMT mouse model of breast cancer, our lab has evidence that this unique, transpresented cytokine also regulates a population of unconventional cytotoxic innate lymphocytes. Using cell type specific Cre recombinase-expressing mice crossed to mice carrying the floxed allele of IL15 and bone marrow chimeras, we have demonstrated that hematopoietic and stromal-derived sources of IL15 are dispensable for this unconventional innate cytotoxic response. However, epithelial tumor-cell derived IL15 is essential as without this source of IL15 these unconventional lymphocytes are severely diminished, and their cytotoxic capability is decreased. Human renal cell carcinoma (RCC) is an epithelial-cell derived cancer presenting as 3 major subtypes with unique histology and molecular characteristics. Clear cell RCC is the most prevalent with a 5-year survival rate of 50%, while chromophobe RCC is rare, accounting for 5% of cases with a 5-year survival of 85%. We hypothesized that there may be an immune-dependent mechanism behind this difference in survival outcome and therefore profiled fresh human tumor samples with peripheral blood and adjacent normal kidney matched controls via flow cytometry. Surprisingly, there was no induction of a CD8 T cell response nor expression of PD1 in chromophobe tumors, suggesting lack of an antigen-specific response in this subtype of RCC. However, compared to blood and normal kidney tissue, chromophobe tumors had a significant expansion of CD56bright innate lymphocytes with high expression of tissue residency molecules and granzymes, suggesting their relevance and cytotoxic capability within the tumor environment. Interestingly, chromophobe RCC has the second highest median expression of IL15 compared across cancer types. In vitro culture of CD56bright innate lymphocytes with IL15 alone was sufficient to induce expression of the integrins CD49a and CD103, granzymes, and the ability to kill target tumor cells. In addition, high expression of IL15 and IL15Ra is correlated with better overall survival in chromophobe RCC. Altogether, these data suggest that IL15 derived from tumor cells regulates a cytotoxic innate lymphocyte response.

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TUMOR-DERIVED SOLUBLE CD155 INTERFERES WITH DNAM-1 FOR NATURAL KILLER CELL-MEDIATED TUMOR IMMUNITY

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CD155 is a transmembrane molecule ubiquitously expressed on both hematopoietic and nonhematopoietic cells and its expression is upregulated upon tumor transformation. CD155 is a ligand for an activating receptor DNAM-1 and inhibitory receptors TIGIT and CD96, and is involved in tumor immune responses by T cells and NK cells.

Unlike mouse, human cells express soluble form of CD155 (sCD155) encoded by splicing isoforms of CD155 lacking the transmembrane region. We have recently reported that the serum levels of sCD155 were elevated in patients with various cancers compared with healthy people. However, the role of sCD155 in tumor immunity remains unclear. To address this issue, we generated a B16/BL6 tumor transfectant stably producing the extracellular portion of mouse CD155 tagged with the FLAG protein at the C-terminus (sCD155/BL6). When sCD155/BL6 or parental BL6 were intravenously injected into WT, TIGIT KO or CD96 KO mice, the number of lung metastasis was larger in mice injected with sCD155/BL6 than in mice injected with parental BL6. In contrast, they were comparable when sCD155/BL6 or parental BL6 were injected into DNAM-1 KO mice. Depletion of NK cells by injection with an anti-NK1.1 antibody canceled the difference in the number of lung metastasis, suggesting that sCD155 inhibited DNAM-1-mediated tumor immunity by NK cells. In vitro killing assay also showed that sCD155 interfered with DNAM-1-mediated NK cell cytotoxic activity. In addition, we found that DNAM-1 had higher affinity to sCD155 than TIGIT and CD96, suggesting that sCD155 preferentially bound to DNAM-1 rather than TIGIT and CD96.

Together, these results demonstrate that sCD155 inhibited DNAM-1-mediated cytotoxic activity by NK cells, resulting in promoting the lung metastasis of sCD155/BL6 melanoma.

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TYK2 GOVERNS TUMOUR IMMUNE-SURVEILLANCE AND ALTERS NK CELL ACTIVITY IN A KINASE-INDEPENDENT MANNER

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The non-receptor tyrosine kinase 2 (TYK2) is a member of the Janus kinase (JAK) family. In the canonical JAK-STAT signalling pathway, cytokine-activated TYK2 propagates the signal via activation of signal transducer and activator of transcription (STAT) 1-6 into chromatin. TYK2 has been reported to exhibit non-canonical functions, i.e. independent of its kinase activity, in both mice and humans. Mice expressing a kinase-inactive version of TYK2 (Tyk2K923E) phenocopy complete TYK2 deficiency (Tyk2-/-) with respect to reduced cytokine-induced STAT activation and impaired IFNg and IL-15Ra production upon IL-12 and type I IFN stimulation, respectively. However, NK cells from Tyk2K923E mice show a partial restoration of maturation in steady-state spleen and of cytolytic capacity in tumour surveillance when compared to NK cells from Tyk2-/- mice. RNA-seq revealed a major effect of loss of TYK2 or the presence of TYK2K923E on the transcriptional profile of homeostatic splenic NK cells with almost 2000 differentially expressed genes. In addition, we found differences between the transcriptomes of Tyk2K923E and Tyk2-/- NK cells, which might explain the observed intrinsic gain-of-function of Tyk2K923E NK cells. In addition, we provide evidence for kinase-independent functions of TYK2 in immune cell cross-talk. We found increased NK cell migration induced by conditioned medium from Tyk2K923E compared to Tyk2-/- CD8+ T cells. Moreover, the microenvironment of transplanted RMA-S tumours showed increased infiltration of Ly6C+CD3e+TCRb+ central memory T cells (TCM) in Tyk2K923E compared to WT and Tyk2-/- mice. TCM are known to be potent producers of IFNg. We are currently investigating whether this cell population contributes to the restored NK cell-mediated surveillance of transplanted tumours in Tyk2K923E mice.

Taken together, we report a comprehensive analysis of the chromatin landscape and transcriptional activity of Tyk2-/- and Tyk2K923E NK cells and investigate kinase-independent functions of TYK2 in CD8+ T cells and their cross-talk with NK cells.

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VISUALIZATION OF NK CELL-MEDIATED ELIMINATION OF METASTATIC TUMOR CELLS IN THE PULMONARY CAPILLARIES

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Lung metastasis is the principal cause of death in many cancer patients. Accumulated evidence indicates that natural killer (NK) cells play key roles in the defense of lung metastasis; however, little is known when, where, and how the metastatic tumor cells are eliminated by NK cells in the lung. To address these questions, we have developed highly sensitive bioluminescent and fluorescent live-imaging of lung metastasis of melanoma cells. Bioluminescence imaging revealed that NK cell-dependent elimination of melanoma cells was observed within 12 hours after injection. During several hours of intravital imaging of lung under a two-photon excitation microscope, most NK cells stayed on the endothelial cells of pulmonary capillary, but some NK cells were found to attach and/or leave from the endothelial cells. By using calcium biosensor GCaMP6s and caspase biosensor SCAT3, NK-mediated attack and apoptosis induction of metastatic tumor cells were visualized. By using transgenic mice, in which ERK biosensor is expressed only in NK cells, it was also found that NK cells exhibited ERK activation before induction of calcium flux in tumor cells. In line with this observation, NK cell-dependent metastatic tumor elimination was suppressed by MEK inhibitor treatment. In vitro experiments revealed that ERK activation upon conjugation with target cells was only detected in DNAM-1 expressing NK cells, and that optogenetic activation of ERK MAP kinase resulted in the increase of adhesiveness in NK cells. MEK inhibitor treatment drastically decreased the target cell adhesion by NK cells. Thus, ERK-dependent NK cell-mediated killing within pulmonary capillaries plays a critical role to prevent metastatic tumor cells from colonizing in the lung.

SESSION 6
NK cell Memory

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ADAPTIVE NK CELLS ARE UNIQUELY SUSCEPTIBLE TO EXHAUSTION IN RESPONSE TO CHRONIC STIMULATION

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Adaptive natural killer (NK) cells expressing the activating receptor NKG2C are long-lived and more resistant to immune suppression than canonical NK cells. Expansion of this NK cell subset in peripheral blood is only observed in cytomegalovirus (CMV) seropositive individuals. We found that chronic stimulation of adaptive NK cells through NKG2C with plate-bound agonist antibodies in the presence of IL-15 led to robust proliferation of these cells but at the expense of effector function. Chronically stimulated adaptive NK cells from CMV seropositive, but not seronegative, individuals exhibited markedly diminished IFN-γ production associated with induction of the checkpoint inhibitory receptors LAG-3 and PD-1. We also found that chronic stimulation with agonist antibodies against NKp30 or NKG2D in the presence of IL-15 induced LAG-3 and PD-1 expression on adaptive NK cells but not canonical NK cells, indicating that adaptive NK cells have an inherent susceptibility towards exhaustion under periods of chronic stimulation through activating receptors. To determine whether a more physiologic stimulation through NKG2C could also drive the upregulation of checkpoint inhibitory receptors and dysfunction, we co-cultured NK cells from CMV seropositive individuals with CMV-infected human umbilical vein endothelial cells (HUVECs). NKG2C+ NK cells activated by CMV-infected HUVECs exhibited high frequencies of LAG-3 and PD-1 expression and defective IFN-γ production. Several studies in mice have provided evidence for widespread epigenetic and transcriptional alterations in exhausted CD8+ T cells. We sought to determine whether similar epigenetic changes were also evident in chronically stimulated adaptive NK cells. To this end, we performed whole-genome DNA methylation arrays. We identified a strong signature of promoter-associated DNA hypomethylation in chronically stimulated adaptive cells. Many of these hypomethylated promoter regions matched to genes shown to be induced in exhausted CD8+ T cells. We identified an additional set of promoter regions that were hypermethylated after chronic NKG2C stimulation that match genes known to be repressed in exhausted CD8+ T cells. Thus, chronic NKG2C stimulation induces epigenetic reprograming towards an imprint indicative of cytotoxic lymphocyte exhaustion. Finally, we examined the impact of CMV reactivation on NK cells from bone marrow transplant patients. We compared NK cells from peripheral blood taken within 8 weeks of CMV reactivation (N=10) to patients without reactivation (N=21) and identified a distinct population of NKG2C+ NK cells with a LAG-3+PD-1+ phenotype in transplant recipients that experienced viral reactivation. Our work has important implications for cancer immunotherapy and the response of NK cells to viral infections.

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ADRENERGIC SIGNALING REGULATES THE NK CELL RESPONSE TO VIRAL INFECTION

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Natural Killer (NK) cells are innate lymphocytes with the ability to kill virally infected, stressed, or transformed cells through the recognition of ligands normally absent in healthy cells, or detection of missing ligands normally present. Although traditionally categorized as a component of the innate immune system, recent evidence suggests that NK cells exhibit adaptive features during their response against pathogens, such as their ability to undergo clonal-like expansion and form a pool of long-lived memory-like cells. Whereas activating receptor engagement and proinflammatory cytokines are required to drive NK cell clonal expansion, additional stimulatory signals controlling their proliferation remain to be discovered. Here, we describe one such signal that is provided by the adrenergic nervous system, and demonstrate that cell-intrinsic adrenergic signaling is required for optimal adaptive NK cell responses.

Early during mouse cytomegalovirus (MCMV) infection, splenic NK cells trafficked into the white pulp and located in close proximity to adrenergic nerve fibers. Concurrently, NK cells upregulated Adrb2 (which encodes the β2- adrenergic receptor), a process that was dependent on IL-12 and STAT4 signaling. NK cell-specific deletion of Adrb2 resulted in suboptimal NK cell homeostasis, maturation, and effector function in a mixed Bone Marrow Chimaera setting. Furthermore, Adrb2-/-NK cells showed impaired expansion and memory during MCMV challenge, in part due to a diminished proliferative capacity. Consequently, lymphopenic mice receiving NK-Adrb2-/-NK cells were significantly more susceptible to MCMV that those receiving WT NK cells.

In summary, we show that NK cell-intrinsic adrenergic signaling is required for protection against MCMV. Taken together, we propose a novel role for the adrenergic nervous system in regulating circulating lymphocyte responses to viral infection.

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CD57–NKG2C+ HUMAN NK CELLS EX VIVO AND IN VITRO

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In humans, cytomegalovirus infection is associated with the development of a subset of adaptive NK CD57+NKG2C+ cells. Despite the unique functional characteristics of the cells and the ability of rapid reactivation in the organism during infection, it is difficult to expand this subset in culture. The purpose of this work was to compare NKG2C+ NK cells at different stages of differentiation in order to find alternative possibilities for the further use of NK cells in immunotherapy. CD57–NKG2C–, CD57–NKG2C+, CD57+NKG2C–, CD57+NKG2C+ NK cell subsets were studied. NK cells were isolated from peripheral blood mononuclear cells of healthy donors by negative magnetic separation. Afterwards, the subsets mentioned above were isolated by cell sorting and cultivated in the presence of IL-2 with the addition of K562 feeder cells expressing membrane-bound IL-21 on their surface (K562-mbIL21). Ex vivo, NK cells from the less differentiated CD57–NKG2C+ subset, as well as from the “adaptive” CD57+NKG2C+, had an increased expression of the HLA-DR molecule, known as a marker of NK cells activation, of the inhibitory KIR2DL2/DL3 receptor and costimulatory CD2 molecule. In addition, CD57–NKG2C+ cells had a reduced expression of CD16, NKG2A receptors, the same tendency was observed in CD57+NKG2C+ cells. During in vitro stimulation with K562-mbIL21 and IL-2 the CD57–NKG2C+ NK cell subset demonstrated the greater proliferative activity compared to other subsets and maintained a high levels of HLA-DR and CD2 expression. After three-week cultivation, CD57–NKG2C+ subset showed high cytotoxic activity.

Thus, CD57–NKG2C+ subset demonstrated some phenotypic and functional characteristics similar to the “adaptive” CD57+NKG2C+ subset, however, it had higher proliferative activity.

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CYTOKINE-INDUCED MEMORY-LIKE NK CELLS PERSIST FOR >2 MONTHS FOLLOWING ADOPTIVE TRANSFER INTO LEUKEMIA PATIENTS WITH AN MHC-COMPATIBLE HEMATOPOIETIC CELL TRANSPLANT

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NK cells exhibit innate memory or memory-like responses following stimulation with haptens, viruses, or cytokines. We and others have established that human memory-like (ML) NK cells differentiate following a short-term activation with IL-12, IL-15, and IL-18. These ML NK cells have increased anti-tumor activity against AML and other cancers in vitro, in xenograft models, and in a first-in human phase 1 clinical trial of ML NK cells in AML (Romee R et al, Sci Transl Med, 2016). In the phase 1 trial, mass cytometry revealed a unique multi-dimensional phenotype of in vivo differentiated ML NK cells, confirmed using donor-specific HLA markers. Although adoptively transferred MHChaploidentical ML NK cells expanded and differentiated over 2-3 weeks, these cells were eliminated by recipient allogeneic immune responses, a challenge observed with all allogeneic lymphocyte therapies. Immune rejection precludes following ML NK phenotype, persistence, and function long-term in these patients. We hypothesized that ML NK cells could persist longer than 2-3 weeks in an MHC-compatible setting, and thus be able to assess ML NK cells durability. To test this idea, a phase 2 clinical trial was designed for refractory AML patients, who receive a reduced-intensity HLA-haploidentical hematopoietic cell transplant (HCT), followed by same-donor ML NK cell adoptive transfer at day 7, followed by 2 weeks of IL-15/N-803 (NCT02782546). Using viSNE, ML NK cells were confirmed as distinct from conventional NK cells, CD56hi/NKp30hi/CD62Lhi/KIR+/NKG2A+/CD57+/-, yet also inconsistent with immature NK cells arising from the HCT graft (CD56bright/KIR-/CD57-). In multi-dimensional analyses ML NK cells persisted in patients for at least 2 months (n=5) following adoptive transfer, and constituted 20- 50% of total NK cells at day 60 (n=3, 206±97 cells/µl; mean±SE). These ML NK cells appeared highly functional (56±8% IFN-γ, 20±3% TNF, 41±7 CD107a) when stimulated with tumor targets immediately ex vivo on study day 28 (n=7). scRNA-seq from ML NK cells differentiated over 21-60 days within a patient supported the idea that ML NK cells are distinct from conventional NK cells even two months post-transfer. In conclusion, a single infusion of ML NK cells resulted in a durable population of highly functional NK cells, as evidenced by multi-dimensional analyses using mass cytometry and scRNA-sequencing. These studies provide evidence that ML NK cell therapy in the MHCcompatible setting overcomes persistence barriers and provide a platform for innovation in NK cell therapeutics.

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DECONVOLUTING GLOBAL PROINFLAMMATORY CYTOKINE SIGNALING NETWORKS IN NATURAL KILLER CELLS

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Signal transducer and activator of transcription (STAT) proteins are evolutionarily conserved mediators that play critical roles in developmental and host defense pathways in the immune system. Within the natural killer (NK) cell compartment, early proinflammatory cytokine signaling mediated through STAT proteins plays a key role in promoting optimal antiviral responses. Previous and current work in the lab have described the non-redundant requirements of STAT1, STAT4, and STAT5 signaling in NK cell responses to mouse cytomegalovirus infection. Activation of these STATs are thought to be induced by distinct cytokines, i.e., STAT1 by type I IFNa, STAT4 by IL-12, and STAT5 by IL-2 and IL-15. However, the pleiotropic effects of both cytokine and STAT signaling preclude the ability to precisely attribute molecular changes to a respective cytokine and/or STAT protein. Furthermore, NK cells are exposed to all of these cytokines concurrently during infection, but we have yet to fully understand how these global proinflammatory signaling networks interact and contribute to the net effect of optimal survival, proliferation, and function. In this study, we aimed to characterize these interactions in NK cells by using a global multi-faceted approach, incorporating several high-throughput sequencing methods (RNA-seq, ATAC-seq, and ChIP-seq) that broadly interrogate both the transcriptional and epigenetic landscapes. We uncover an IL-2/15 mediated axis that preferentially overlaps with an IL-12/18 network and overall enhances IL-12/18-induced changes. These changes are reflected on both the levels of mRNA as well as chromatin accessibility, and are distinct from the mode of regulation observed in IFNa-STAT1 mediated changes. By incorporating the global transcription factor binding profiles of these STAT proteins in response to their respective proinflammatory cytokine, we have generated a network of direct transcriptional targets that include both distinct and shared modules across all three signaling pathways. Overall, our study sheds light on the crosstalk between proinflammatory cytokine signaling pathways in NK cells and will serve as a valuable resource for improving NK cell immunotherapy.

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DIFFERENTIATION AND MAINTENANCE OF NKG2C+ NK CELL EXPANSIONS

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In a proportion of people infected with human cytomegalovirus (HCMV), NK cells expressing the activating receptor NKG2C undergo expansion that partially resembles anti-viral adaptive responses. We have previously found that adaptive NKG2C+ NK cells differentially recognize distinct HCMV strains encoding variable UL40 peptides that, in combination with pro-inflammatory signals, control the population expansion and differentiation of adaptive NKG2C+ NK cells, thus identifying the long sought variable ligand inducing these expansions. Building on these findings we now aim to decipher the imprints HCMV infection has on the NK cell phenotype and differentiation, in order to understand how these characteristic expansions of adaptive NK cells are induced and maintained. Specifically, we apply high dimensional phenotyping by combinatorial flow cytometry using a combination of markers demarcating maturation or described to be affected by HCMV infection. This will enable us to understand how HCMV affects the heterogeneity of the NKG2C+ population and how this population is divided into more specific subsets with potentially different roles in functional responses and maintenance of the NKG2C+ pool. We further interrogate global gene expression by single-cell RNA sequencing to identify HCMV-induced gene expression signatures confined to individual subsets. Unsupervised clustering, differential expression analysis and trajectory inference will be applied to shed light on how exposure to HCMV skews adaptive and conventional NK cell differentiation. Finally, we plan to use the insights we gain from these datasets to isolate NKG2C+ subpopulations and assess their functional and proliferative capacity, as well as the phenotype of the cellular output these populations generate

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FRIEND RETROVIRUS INFECTION INDUCES THE DEVELOPMENT OF MEMORY-LIKE NATURAL KILLER CELLS

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Traditionally, NK cells belong to the innate immune system and are important cytotoxic lymphocytes that can recognize and eliminate virus-infected cells through their germline-encoded receptors. In infections with retroviruses, like Human Immunodeficiency Virus (HIV) or Friend Virus (FV) infection of mice, NK cells clearly mediate anti-viral activities, but they are usually not sufficient to prevent severe pathology. However, NK cells were recently reported to possess memory-like functions that were predominantly provided by hepatic NK cells. Memory properties were mainly documented in contact hypersensitivity models or during cytomegalovirus infections. However, the precise role and the physiologic importance of memory-like NK cells during retroviral infections are still under investigation. Here, we show that Friend retrovirus (FV) infection of mice induced a population of phenotypically memory-like NK cells at 28 days post infection. Upon secondary antigen encounter, these NK cells showed an increased production of the proinflammatory cytokines IFNγ and TNFα as well as the death ligand FasL in comparison to naïve NK cells. Furthermore, we found an augmented elimination of antigen-matched but not antigen-mismatched target cells by these memory-like NK cells. In adoptive cell transfer experiments, equal anti-viral activities of splenic and hepatic memorylike NK cells during the late phase of acute FV infection were found. Our results strongly imply the existence and antiviral activity of spleen and liver memory-like NK cells in FV infection, which efficiently respond upon secondary exposure to retroviral antigens. This might be important for cancer immunotherapy, the treatment of viral diseases and the improvement of vaccination strategies.

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HIGH RISK SEXUAL BEHAVIORS OF MEN WHO HAVE SEX WITH MEN IMPACT PHENOTYPE AND FUNCTIONAL FEATURES OF NK CELLS

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BACKGROUND:
Individuals with high risk behaviors has been proposed as cohorts for the study of the HIV resistance. This group includes commercial sex workers, men who have sex with men (MSM), and intravenous drug users. MSM represents an adequate cohort for the study of resistance since they have a high risk of contracting HIV-1 infection, due to several factors such as the high prevalence of HIV infection in this subpopulation, the increase in transmission efficiency through unprotected receptive anal sex, social discrimination and abuse of psychoactive substances, lack of knowledge of HIV status and limited coverage and access to prevention programs.
Within the mechanisms described to explain resistance, a greater effector activity of the NK cells has been proposed, reflected in a greater cytotoxic capacity and production of soluble factors such as cytokines and chemokines; In addition, there is evidence of a subpopulation of NK cells with "immunological memory", which, in some studies, has been observed more frequently in HESN. The study of the phenotype and function of NK cells in MSM would help to elucidate mechanisms associated with resistance. To this end, it is proposed to evaluate the phenotype and function of NK cells in high risk MSM.

METHODS:
The frequency of NK cells subpopulation including "Memory like" NK cells was evaluated by flow cytometry in peripheral blood. Effector capacity was evaluated with cytotoxic assays in co culture of PBMCs and K562 cells and with activation assays with the same stimuli were the expression of Granzyme, IFN-γ, MIP-1β and CD107a were evaluated.

RESULTS:
High risk individuals shown a higher frequency of NK cells expressing maturation markers as well as higher frequency of "Memory like" NK cells that were correlated with the magnitude of sexual exposure. Functional analysis revealed a higher cytotoxic capacity and IFN-γ production after stimulation with K562 cells. The polyfunctionality analysis revealed a similar frequency of polyfunctional CD107a+/IFN-γ+/MIP-1β+ cells between both groups; however, an NK cell population specialized in IFN- γ production in high-risk MSM individuals was observed.

CONCLUSION:
These data showed a higher effector capacity of NK cells in High risk MSM, which has been previously described in HESN cohorts, suggesting that this is a conserved characteristic among resistant individuals. A higher frequency of "Memory like" NK cells also have been found; These findings suggest that this characteristic can be involved in the natural resistance to HIV that is observed in this population.

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KINETIC OF NATURAL KILLER CELLS SHOWING AN UNCONVENTIONAL IMMUNOLOGICAL MEMORY IN HAPLOIDENTICAL HEMATOPOIETIC STEM CELL TRANSPLANTED PATIENTS UNDERGONE CYTOMEGALOVIRUS REACTIVATION

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Haploidentical hematopoietic stem cell transplantation (h-HSCT) represents a promising therapeutic approach to cure patients affected by hematologic malignancies. h-HSCT induces a state of immunologic tolerance between donor and recipient cells and it allows to rapidly find a donor for almost all patients in need. Despite the positive results in terms of overall/disease free survival and reduced tumor relapse, the HLA mismatch between donor and recipient has not been yet fully exploited and the clinical outcome of h-HSCT patients is hampered by life-threatening side effects. Among them, the human Cytomegalovirus (HCMV) infection/reactivation represents one of the major causes of morbidity and mortality after h-HSCT.

Immune cell reconstitution (IR) is certainly key in determining a positive h-HSCT clinical outcome. In this context, we demonstrated that Natural Killer (NK) cells represent the first innate lymphocytes recovering after h-HSCT, thus highlighting their role in ensuring a prompt alloreactivity early after transplant as well as protection against opportunistic viral infections. Other and we previously reported that HCMV infection/reactivation greatly impact NK cell maturation and effector-functions, by providing a rapid expansion of mature, long-lived and hyper-functional NK cells showing memory-like (ml) properties.

To investigate the impact of HCMV infection/reactivation on NK cell IR after h-HSCT, we set up a complex multiparametric flow cytometry panel and we characterized immune-reconstituting NK cells at different time points up to one year after the transplant. Data where then analyzed by an unsupervised PhenoGraph algorithm, that evaluates, at single cell level, differences/similarities of marker expression, building up clusters of phenotypically identical cells.

This system biology approach identified a subset of NK cells showing a peculiar CD158b1b2j+NKG2ANKG2C+ NKp30- phenotype that is expanded only in h-HSCT patients experiencing HCMV reactivation. Interestingly, this latter NK cell population showing memory-like functional features is maintained even after the resolution of the infection and its frequency positively correlates with HCMV viral load and with the numbers of reactivation events. These findings show in a human setting in vivo the expansion and the kinetic of those NK cells that “remember” the HCMV challenge in patients experiencing viral reactivation. Our data are important to better understand the ability of NK cells to control this life-threatening infection after h-HSCT as well as to deep our knowledge in regard to human NK cell maturation in response to viral challenges.

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NK CELLS ACQUIRE EPIGENETIC MEMORY-LIKE FEATURES FOLLOWING LPS-INDUCED SYSTEMIC INFLAMMATION

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Natural killer cells are unique players in innate immunity, and as such, an attractive target for immunotherapy. Following viral infection, NK cells display immune memory properties, defined by heightened responses to restimulation, an expansion of specific NK cell sup-populations and a protective role against re-infection. However, a similar memory to bacterial infection or systemic inflammation, and the molecular mechanisms behind NK cell memory remain elusive. In fact the long-term status of NK cells following systemic inflammation was unknown. Here we show that following LPS-induced endotoxemia in mice, NK cells acquire cell-intrinsic memory-like properties showing an increased production of IFNγ upon secondary stimulation. The NK cell memory response is apparent even under the post-endotoxemic suppressive environment and is detectable for at least 9 weeks. Importantly, we reveal a novel epigenetic mechanism essential for NK cell memory, where an H3K4me1-marked latent enhancer, uncovered at the ifng locus. Chemical inhibition of histone methyltransferase activity erased the enhancer and abolished NK cell memory. Thus, NK cell memory develops after endotoxemia, in a histone methylation-dependent manner, ensuring a heightened response to secondary stimulation and conferring protection against bacterial infection.

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TEMPORALLY DISTINCT ROLE FOR STAT5 ACTIVITY DOWNSTREAM OF IL-2 AND IL-15 SIGNALING IN ANTIVIRAL NK CELLS

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Natural killer (NK) cells are cytotoxic lymphoid cells of the innate immune system, which possess the capacity to elicit adaptive features such as clonal expansion, contraction and the formation of long-lived memory cells in response to mouse cytomegalovirus (MCMV) infection. Signal transducer and activator of transcription (STAT) transcription factors are crucial regulators of diverse NK cell functions. Our lab has previously demonstrated that several STAT family members, namely STAT1, STAT2 and STAT4, are crucial for NK cell-mediated responses to MCMV. However, the role of STAT5 downstream of interleukin 2 (IL-2) and IL-15 in antiviral NK cell responses has not been carefully dissected. STAT5 acts downstream of common gamma chain cytokines and is essential for NK cell development, maturation and homeostasis. In this study, we investigated how STAT5, downstream of IL-2 versus IL-15 signaling, acts as a transcriptional regulator during MCMV infection. We demonstrate that STAT5 expression in NK cells is required for viral control and even partial STAT5 deficiency results in a defective capacity of NK cells to generate optimal effector and long-lived memory cells in response to MCMV infection. During early MCMV infection, STAT5 induces the transcription of genes that are involved in NK cell cytotoxicity and terminal maturation. In the course of infection, we observe a functional and temporal dichotomy in IL-2, IL-15 and STAT5 signaling: IL-2, via its high affinity receptor CD25, drives initial proliferation, whereas IL-15 and STAT5 are required for the survival of effector NK cells during the contraction phase. Thus, we propose a novel model for temporally distinct STAT5 signaling in the generation of an optimal antiviral NK cell response.

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THE CD56BRIGHT CD62L+ NKG2A+ IMMATURE CELL SUBSET IS DOMINANTLY EXPANDED IN HUMAN CYTOKINE-INDUCED MEMORY-LIKE NK CELLS

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Recent studies have revealed the immunological memory of NK cells. CMV infection induces immunological memory in a subset of NK cells in vivo, but short-term in vitro cytokine stimulation (IL-12 and IL-18) also induces NK cell memory. However, cytokine-induced memory-like (CIML) NK cells much differ from CMV-induced 'adoptive' NK cells in terms of phenotype and function. Moreover, heterogeneous cell subsets within the CIML NK cells has not been elucidated. To address this, we generate and analyzed CIML NK cell subsets.

The dominantly expanded cell subset within CIML NK cells has immature CD56bright CD62L+ markers, which are expressed in a CD56bright CD16- NK cell subset. To address the lineage relationship of NK cell subsets, three resting NK cell subsets were flow-sorted then stimulated to generate CIML NK cells: 1) CD56bright CD16- (all are CD62L+) immature cells, 2) CD56dim CD16+ CD62L+ less mature cells, and 3) CD56dim CD16+ CD62L- mature cells. Whereas about 70% of non-stimulated CD56bright CD16- CD62L+ cells lost CD62L expression after one week of culture, the CIML NK cells maintain CD62L+ phenotype. In contrast, regardless of cytokine stimulation, about 30% of CD56dim CD62L+ cells lost CD62L expression. CD62L was not acquired on the CD56dim CD16+ CD62L- cells. Thus, the dominant population within CIML NK cells is derived from CD56bright CD16- CD62L+ NK cells, but not other cell subsets, possibly due to the higher proliferation capacity and responsibility to cytokines.

An inhibitory receptor NKG2A, which is expressed on almost all CD56bright CD16- subset, were co-expressed with CD62L inhibits CIML NK cell cytotoxicity against HLA-E expressing target cells. However, CIML NK cells also acquired KIR expression, which is associated with enhanced cytotoxicity. Interestingly, another inhibitory receptor LAG-3 was induced mainly on the NKG2C+ CIML NK cell populations. LAG-3 expression in CIML NK cells is increased through their maturation status; NKG2A+ KIR- (immature: 6.4%), NKG2A+ KIR+ (mature: 17.6%), and NKG2C+ KIR+ (terminally matured: 54.7%). Whereas exogenous IL-18 alone, type I interferon, or high-dose IL-2 or IL-15 did not induce LAG-3 expression on NK cells, IL-12 was a main inducer of LAG-3, and IL-18 further enhanced it.

In summary, CIML-inducing short term in vitro cytokine activation induces selective expansion of CD56bright NKG2A+ CD62L+ NK cells with the acquisition of KIR expression. LAG-3 expression is induced mainly on NKG2C+ KIR+ and NKG2A+ KIR+ cell populations. Our findings imply targeting NKG2A and LAG-3 should be considered for CIML NK cell-based immunotherapy.

SESSION 7
NK cell Immunotherapy and Immunomodulation

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A NOVEL ANTICD38-CAR CONSTRUCT INCREASES THE IN VITRO RESPONSIVENESS OF NK CELLS AGAINST MULTIPLE MYELOMA CELLS

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Multiple myeloma (MM) is a plasma cell malignancy, that currently remains incurable. The identification of CD38, a transmembrane glycoprotein overexpressed on MM cells, led to the development of target-specific therapeutics such as the FDA-approved monoclonal antibody (mAb) Daratumumab. Although a valuable treatment option for refractory/relapsed MM patients, Daratumumab has a limiting response rate of about 30%, which highlights the clinical need for novel therapeutics. A modern alternative to antibody-based technologies is the genetic modification of effector cells into expressing highly-selective chimeric antigen receptors (CARs). Although T-cells are the most commonly studied effector cells, both preclinically and clinically, NK cells may be of potential relevance due to their shorter life span and low risk of causing graft-versus-host disease. The increased attention towards CAR-NK therapies has led to multiple preclinical studies, which have already shown encouraging results. Here, we used a lentiviral approach to transduce NK cell lines with a CAR that consists of an antiCD38 extracellular domain and the intracellular domains of CD28 and CD3ζ. We show a reproducible transgene expression ranging between 11-68% depending on the NK cell line. In functional assays, CAR-positive NK cells display a 3-fold higher degranulation against the MM cell line RPMI8226, compared with CAR-negative NK cells. Furthermore, we generated genetically modified CD38- negative RPMI8226 cells using the CRISPR/Cas9 technology to test the selectivity of the CAR-NK cells. Ongoing efforts also include the introduction of the CAR into primary NK cells from MM patients. Altogether, our findings show that antiCD38-CAR NK cells are strong candidates for the immunotherapy of MM.

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A PRECLINICAL ASSESSMENT OF TUMOR INFILTRATING NATURAL KILLER CELLS FOR PEDIATRIC SOLID TUMOR MALIGNANCIES

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The past several decades have seen great strides in improving the outcomes of pediatric cancer. However, the nonhematologic, solid tumor malignancies of childhood and adolescence continue to pose great challenges for the pediatric oncologist. Despite the initial promising improvements in long-term outcomes with the use of neoadjuvant, multi-agent chemotherapy and aggressive local control, outcomes have not improved for patients with metastatic, refractory or relapsed disease. Tumor infiltrating lymphocytes (TIL) - T lymphocytes harvested from resected tumors and expanded ex-vivo for infusion back into the patient - have proved a promising treatment for certain adult solid tumor malignancies such as melanoma, non-small cell lung cancer, renal cell carcinoma, and breast cancer. However, these are not the only immune effector cells capable of infiltrating the tumor microenvironment. Natural killer (NK) cells are an essential effector cell of the innate immune system and have been shown to play a critical role in cancer surveillance and anti-tumor immune response. Around 25% of the time, the process utilized to isolate TIL from resected pediatric solid tumors - tumor fragmentation and culture in high dose (1000-6000 IU) IL-2 - leads to a mixed lineage immune effector cell culture containing TIL as well as tumor infiltrating NK cells (TINK). In order to perform a pre-clinical assessment of TINK for use as a TIL-like adoptive therapy for pediatric solid tumor malignancies, we have expanded these cells in serum free media with IL-2 and IL-21-expressing, K562 feeder cells in order to select for an NK cells. To date we have done method optimization with 4 samples, with a total of 6 samples evaluated. Evaluable NK cells were produced by 3 of the 6 samples. Phenotypically, expanded TINK are CD56-comparable, but CD16DIM compared to healthy donor NK cell control. While by calcein-release cytotoxicity assay against K562 target cells they are only slightly less cytotoxic than control, their fold expansion is significantly reduced. The expression of activating receptors responsible for direct cytotoxicity in expanded NK cells is comparable between TINK and control, which may be the reason for retained cytolytic function. If data from subsequent TINKs continue to indicate them to be a CD16DIM population with reduced proliferative and slightly reduced cytolytic capability, we would conclude that adoptive therapy with healthy donor allogeneic expanded NK cells should be seen as a better option than adoptive transfer of expanded TINK.

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AFUCOSYLATED ANTIBODY AUGMENTS CD16-MEDIATED SERIAL KILLING AND CYTOKINE SECRETION BY NK CELLS

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The ability of Natural Killer (NK) cells to perform antibody-dependent cellular cytotoxicity (ADCC) via the FcγRIII receptor (CD16) is widely used as a therapeutic mechanism for monoclonal antibodies (mAbs). We have recently demonstrated that CD16 shedding increased NK cell motility and induced sequential engagement of multiple opsonised target cells, by using pharmacological inhibitors and NK cells transfected to express a non-cleavable form of CD16 (Srpan et al., J Cell Biol, 2018). Here, we investigated CD16-mediated NK cell responses to variants of Rituximab, a therapeutic monoclonal antibody which mediates depletion of CD20-expressing B cells widely used in the treatment of non-Hodgkin lymphomas, chronic lymphocytic leukaemia (CLL) and rheumatoid arthritis. We compared an afucosylated form which has higher affinity for CD16, as well as alternative isotypes of Rituximab, including IgG2 and IgG4 with low and moderate affinity for CD16, respectively. When 721.221 or Daudi target cells were coated with afucosylated Rituximab, primary NK cell degranulation and cytotoxicity were increased by 18.3±3.9% and 26.1±7.03%, respectively, while IFN-γ secretion increased 2-3 fold, compared to wildtype Rituximab. In contrast, isotypes IgG2 and IgG4 had no impact on CD16-mediated NK cell responses. Unexpectedly, afucosylated Rituximab also triggered increased cleavage of CD16 by a disintegrin and metalloprotease-17 (ADAM17). Live-cell microscopy in home-fabricated micro-wells revealed that this led to more rapid and efficient sequential killing of opsonised targets. Thus, disassembly of the immune synapse caused by CD16 shedding is a factor determining the efficiency of NK cell responses to therapeutic monoclonal antibodies which can be manipulated with antibody design.

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AKT INHIBITION SELECTIVELY EXPANDS YOUNGER NK CELLS, INCLUDING AN NKG2A+NKG2C+ DOUBLE-POSITIVE POPULATION HIGHLY RESPONSIVE TO TUMOR CELLS

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Ex vivo expanded NK cells have immense potential as allogeneic cell therapies, but their short persistence postinfusion suggests that terminal differentiation occurs during expansion. To decouple PI3K-driven differentiation from proliferation, peripheral blood NK cells were expanded with IL-2 and IL-15 with an AKT inhibitor (AKTi) for one week. While similar numbers of NK cells were achieved, more mature NK cells were unable to divide with AKTi whereas expansion of CD56bright cells was unaffected. CD56bright cells have increased phosphorylation of STAT5, AKT, ERK, and S6 after IL-2 and IL-15 stimulation and increased total levels of the proteins compared to CD56dim. While nearly all CD56bright cells are CD57-CD62L+ (95.0±3.1%), CD57-CD62L+ cells are a much smaller proportion among the CD56dim (16.2±6.0%) and similarly have increased signaling compared to other CD56dim populations. These signaling advantages are largely maintained when stimulated in the presence of AKTi. Together, this indicates that the proliferation of less mature NK cells is not dependent on AKT, and this may be due in part to their increased signaling capacity. Accordingly, NK cells expanded with AKTi had lower expression of CD57 and CD16 and were less apoptotic, while expression of CD62L, NKG2D, and NKp46 were increased. In addition, growing NK cells with AKTi resulted in a higher frequency of NKG2A+NKG2C+ double-positive (DP) cells. In healthy donors, DP cells comprise a larger proportion of the CD56bright cells than the CD56dim cells (17.6±10.3% vs. 2.9±4.3%) and are present regardless of HCMV seropositivity. Compared to other CD56bright and CD56dim cells, DP cells have increased levels of NKG2D and NKp46, and were intermediate for levels of CD16, CD57, and CD62L. Correspondingly, DP cells were the most responsive population to K562, KG1, and 721.221 tumor targets, and intermediate in ADCC response to BE(2)N as measured by degranulation and IFNγ production in twelve healthy donors. DP cells were inhibited by HLA-E expressed on K562 cells, but were inhibited less when HLA-E presented the G*01 versus A*02 leader peptide. These DP NK cells appear to be an intermediate, transitional population between CD56bright and CD56dim NK cells, positioning them to be a potential common progenitor of CD56dim NKG2A and CD56dim NKG2C single-positive NK cells. Selective expansion of these younger DP NK cells with potent effector function against multiple tumor types through AKT inhibition may be a promising approach for NK cell therapies.

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AN ANTIBODY DESIGNED TO IMPROVE ADOPTIVE NK-CELL THERAPY INHIBITS PANCREATIC CANCER PROGRESSION IN A MURINE MODEL

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PURPOSE:
Natural killer (NK) cells are primary cells capable of responding to cancer cells, and are therefore useful as cell therapy agents. In this respect, NK cell therapy can be developed as an alternative treatment for pancreatic cancer that is resistant to chemotherapy. However, the major limiting factor of NK therapy is that tumor cells prevent a sustained anti-tumor immune response by inhibiting the homing of NK cells to tumor sites. Therefore, the key to achieving superior cancer immunotherapy depends on enhancing the trafficking of expanded NK cells to tumor tissues.

EXPERIMENTAL DESIGN:
To overcome the limitation, we aimed to develop an antibody-based NK cells homing protein, named NK cell recruiting protein-body (NRP-body). We evaluated the infiltration of expanded NK cells by NRP-body in pancreatic cancer or metastasis murine models.

RESULTS:
In the Matrigel invasion assay, NRP-body enhanced NK cell migration to tumor cells via the CXCL16 gradient, which were separated from NRP-body by furin on the surface of pancreatic cancer cells. CXCL16 induced NK cell infiltration through ERK activation of RhoA signaling. In addition, CXCL16 stimulated the antibody-mediated killing activity and degranulation of NK cells. In the PDAC models, administration of NRP-body enhanced the infiltration of transferred NK cells in the tumor tissues and reduced the tumor burden compared with controls. The overall survival was increased in NRP-body-treated mice even in metastasis models compared with NK cell only injected mice.

CONCLUSION:
Enhancement of NK cell infiltration into the tumor tissue is the key factor to overcome for successful cancer immunotherapy. Therefore, the combination of NRP-body with NK cell therapy might be an efficacious strategy for the treatment of pancreatic cancer.

TRANSLATIONAL RELEVANCE:
The main limiting factor of NK therapy is that the infiltration into the tumor site is not effective. We constructed an NRP-body by conjugating NK cell chemokine to the MSLN-scFv antibody that binds to an MSLN known to be expressed in pancreatic cancer. We have confirmed that infiltration of expanded NK cells is increased by NRP in the pancreatic and metastasis models performed to determine the validity of NRP-body. Therefore, our results suggest that increased NK cell infiltration has an important effect on overcoming pancreatic cancer

AUTOMATED ISOLATION AND ENRICHMENT OF GMP GRADE NK CELLS FOR MANUFACTURING OF CAR NK CELLS

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BACKGROUND & AIMS:
In contrast to T cells, natural killer (NK) cells show potent antitumor effects against cancer cells without GvHD (Graft versus Host Disease). Therefore, innovative development of cellular immunotherapies switches to GMP-compliant manufactured NK cells. In this work, isolation and expansion of NK cells in high number for patient treatment were displayed for CliniMACS Prodigy® (Prodigy). In-process control samples (IPC) and quality control were taken for several time points to examine cell surface markers, NK cell ability of degranulation/cytotoxicity/cytokine secretion and alpharetroviral transduction. Generation of chimeric antigen receptor expressing NK cells were redirected against CD123pos AML cancer cells.

METHODS:
For Isolation and expanding NK cells the large-scale manufacturing process was performed using the automated Prodigy. Monitoring of the NK cell growth and quality parameters were checked by determination of glucose, pH, viability, cytotoxicity and flow cytometric analysis of NKp30, NKp44, NKp46, NKG2D, CD137, CD178 and CD253. After cryopreservation NK cells were recultured for three days in order to prove revitalization, functionality and marker expression. We also defined the best time for optimal successful transduction of expanded NK cells with alpharetroviral CAR vectors (anti-CD123 CAR) by harvesting NK cells at different time points of expansion process in regard to transduction rates. The redirected effector-mediated killing activity against the AML cell line KG1a was analyzed by flow cytometry-based cytotoxicity assays and time-lapse tracking experiments.

RESULTS:
Manufacturing and clinical-scale expansion started with 8-15 x 109 leukocytes (1.1 2.3 x 109 NK cells) by using leukaphereses (n=3). Immunomagnetic separation process of NK cells led to high purities (median 99.1%) and viabilities (median 86.9%) and subsequent growth accomplished 4.2 to 8.5fold expansion rates. NK cells showed robustness and high killing activities before and even after cryopreservation which was also reflected by surface markers. Optimal CAR transduction was demonstrated for NK cells collected on day 8 or day 14 since killing and degranulation properties were excellent. Time-lapse imaging to monitor redirected effector-to-target contacts between anti-CD123 CAR NK and AML cell line KG1a showed clearly long-term effector-target interaction.

CONCLUSIONS:
We integrated the clinical-scale expansion procedure in the automated and closed Prodigy, included IPC samples, QCs and optimal time frames for NK cell transduction with CAR vectors and therefore established a standardized GMP-compliant overall process.

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BAEV AS A NEW TOOL TO DEVELOP NK BASED IMMUNOTHERAPY

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NK cells are very efficient to recognize and kill tumor cells, and could represent an interesting tool for immunotherapy. Although infusions of activated NK cells have been are safe and well tolerated, they need to be improved to increase their therapeutic potential. The modification of NK cells with chimeric antigen receptors (CAR) could improve their functions, but no efficient technique allows the transduction of peripheral blood-derived NK. The aim of this project was to develop an efficient transduction technique and produce NK cells expressing CARs. Methods. Freshly isolated NK and NK obtained from the NK cell Activation and Expansion System (NKAES) were transduced with lentiviral vectors pseudotyped with different envelope glycoproteins. NK cells were then re-expanded using the NKAES system for 14-21 days. GFP or CAR expression was assessed by flow cytometry. Viral receptor expressions were evaluated by RT-PCR and RNA-Seq. The effect of CAR expression was tested in cytotoxicity against a NK resistant pre-B-ALL leukemia cell line. Results. VSV-G-LVs resulted in poor NK transduction rate in expanded NK (15.7%), similar to MV-LVs (13.7%), while RD114 performed better (37.8%). The use of BaEV-LVs outperformed them all with a transduction rate mean of 83,4% in NKAES. The transduction level was good even at low MOI. Similarly, BaEV transduction was more efficient in resting NK (23.0%) compared to the other envelopes (10.4%, 2.1%, and 7.8% for VSV-G, MV-LV and RD114 respectively). Transgene expression was sustained for at least 21 days. BAEV receptors (ASCT1/ASCT2) expression patterns were consistent with transduction efficacy. The expression of a third generation anti-CD22 CAR or the expression of two CARs simultaneously allowed the killing of pre-B-ALL cells. Conclusions. This BAEV-LVs will allow the development of NK-based immunotherapies such as CAR-NK cells which are a promising cancer therapy. In addition to its impact on the development of NK-based therapy, The development of such a tool could also have a major impact on basic research of NK-cell biology study as it would allow the genetic modification of primary NK to study their function.

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CANINE NON-B, NON-T NK LYMPHOCYTES HAVE A POTENTIAL ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY FUNCTION AGAINST ANTIBODY-COATED TUMOR CELLS

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The antibody-dependent cellular cytotoxicity (ADCC) is a cell-mediated immune defense mechanism in which effector immune cells actively lyse antibody-coated target cells. The ADCC of tumor cells is employed in the treatment of various cancers overexpressing unique antigens, and only natural killer (NK) cells are known to be major effectors of antibody-mediated ADCC activity. Canine NK cells are still defined as non-B, non-T large granular lymphocytes because of the lack of information regarding the NK cell-restricted specific marker in dogs, and it has never been demonstrated that canine NK cells have ADCC ability against tumor cells. In the present study, we investigated whether canine non-B, non-T NK cells have ADCC ability against target antibody-coated tumor cells, using cetuximab and trastuzumab, the only human antibodies reported to bind to canine cancer cells. Activated canine non-B, non-T NK cells (CD3–CD21–CD5–TCRαβ–TCRγδ–) for 13~17 days ex vivo showed ADCC ability against trastuzumab- or cetuximab-coated target tumor cells expressing various levels of human epidermal growth factor receptor 2 (HER-2) and epidermal growth factor receptor (EGFR). Trastuzumab and cetuximab induced significant ADCC responses of canine NK cells even in CMT-U334 and CF41.Mg cells expressing low levels of HER-2 and/or EGFR, as well as in SKBR3 and DU145 cells overexpressing HER-2 and/or EGFR. The trastuzumab-mediated ADCC activity of NK cells was significantly enhanced by treatment with rcIL-21. The results of this study suggest that canine non-B, non-T NK lymphocytes have a potential ADCC function and that combinational strategies of monoclonal antibodies with either cytokines, which activate NK cells in vivo, or adoptive transfer of NK cells may be a feasible method for amplifying the efficacy of immunotherapy against malignant cancers even with very low expression of target molecules in dogs.

CHANGES IN PHYSIOLOGICAL AND ENVIRONMENTAL CONDITIONS MIMICKED USING AVATAR™ TECHNOLOGY DOES NOT ALTER ONKORD® NATURAL KILLER CELL DEVELOPMENT AND FUNCTION

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Glycostem Therapeutics (Oss, the Netherlands) investigates a Natural Killer (NK)-cell based immunotherapy (oNKord®) for the treatment of haematological malignancies and solid tumours. oNKord® is produced in a multi-step process, starting from fresh umbilical cord blood-derived CD34+ hematopoietic stem cells, isolated and expanded ex vivo and then differentiated into functional NK cells in a feeder cell-free culture system. oNKord® is committed as an off-the-shelf, allogeneic cancer immunotherapy and Phase I/II clinical trial in acute myeloid leukaemia (AML) and in multiple myeloma (MM) will begin in Q1 2020.

oNKord® cultures are maintained in vitro in standard incubation conditions, i.e. 37 °C, 20% O2 at atmospheric pressure (0 pound-force per square inch, PSI). In vivo, when infused into patients, cells will face a completely different microenvironment, which will mutate as they will reach other districts, as the solid tumour niche. Each microenvironment will bear a unique oxygen and interstitial fluid pressure condition (Muz et al, Hypoxia, 2015; Goel et al, Physiol Rev, 2011), which will profoundly affect cell homeostasis and metabolism.

To investigate oNKord® manufacturing and physiological stability in different microenvironments, we employed the Xcell Bio AVATAR™ technology, which allows to fine-tune oxygen and pressure conditions in vitro, mimicking the physiological conditions for NK cells residence and development in various organs. CD34+ progenitor cells were expanded and differentiated into oNKord® mimicking four different microenvironment conditions: bone marrow (5% O2, 2 PSI), liver/stiff tumour microenvironment (1.5% O2, 0.8 PSI), lymph node (5% O2, 0.4 PSI) and circulation (15% O2, 2 PSI), compared to the standard incubation setting. oNKord® phenotype and cytotoxic potential were analysed in all settings. Interestingly, we found that CD34+ progenitors' expansion is the most affected process, as cell replication is inhibited in higher-pressure districts (bone marrow, liver/tumour and circulation). oNKord® differentiation and cytotoxicity are more homogeneous and slightly higher in bone marrow and lymph-node, although efficient maturation is achieved in all settings. Overall, the bone marrow and lymph node conditions support best oNKord® development. This is in line with compartmentalisation of NK cells development in humans, where cells migrate to peripheral blood and other districts after reaching maturity in the lymphatic system (Eissens et al, PLoS ONE, 2012).

Our data show oNKord® stability in vivo and set the basis for further improvement of cell fitness in specific districts. Mimicking, how oNKord® cells survive and function in vivo is critical to understand and control the potential of the treatment after infusion.

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CHARGE-ALTERING RELEASABLE TRANSPORTERS ENABLE GENETIC AND PHENOTYPIC MANIPULATION OF RESTING PRIMARY HUMAN NATURAL KILLER CELLS

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Natural killer (NK) cells are highly recalcitrant to standard transfection techniques. To date, successful attempts at transfecting NK cells have relied on non-physiological activation methods that dramatically and permanently alter NK cell functional and cell surface phenotype. Charge-altering releasable transporters (CARTs) are cationic diblock oligomers that complex with polyanionic cargo and rearrange to neutral small molecules over time, facilitating cargo release while avoiding toxicity associated with persistent cations. CARTs have previously been shown to transfect primary T cells with mRNA both in vitro and in vivo. Here, we report that CARTs efficiently transfect primary human NK cells without the need for prior NK cell activation or expansion. Isolated NK cells cultured without any supplemental cytokines are robustly transfected with both CART-complexed mRNA and siRNA. Additionally, Cas9 ribonucleoprotein is sufficiently anionic to form complexes with CARTs, and these complexes can also be transfected into resting NK cells for CRISPR/Cas9-based genome editing. CARTs transfect primary NK cells two orders of magnitude more efficiently than published protocols, emphasizing the scalability of this approach. CART-transfected NK cells maintain comparable viability to untreated cells, and analysis of NK cell surface phenotype via cytometry by time-of-flight reveals that, unlike current electroporation protocols, CART transfection causes minimal reconfiguration of NK cell surface phenotype. Further, synthesis of a fluorescently-labeled CART enables convenient selection of transfected cells without requiring delivery of additional markers. Collectively, our work provides a toolkit for genetic and phenotypic manipulation of resting primary NK cells that will enable previously unfeasible work on this understudied lymphocyte subset.

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CHIMERIC ANTIGEN RECEPTOR MODIFIED MEMORY-LIKE (CAR-ML) NK CELLS EXHIBIT POTENT RESPONSES TO NK-RESISTANT CANCERS

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Cytokine-induced memory-like (ML) NK cells differentiate after IL-12, IL-15, and IL-18 activation, and exhibit potent anti-tumor response. ML NK cells are being tested in multiple clinical trials for patients with AML, inducing complete remissions in >50% of patients in a phase 1 study. However, many NK cell-resistant blood and solid cancers are poorly recognized by ML NK cells. We hypothesized that ML NK cells engineered with chimeric antigen receptors (CAR) would demonstrate improved anti-tumor responses against classically NK-resistant targets. To test this idea, ML NK cells were engineered to express an anti-CD19-CAR, and responses against NK-resistant B-cell malignancies were evaluated in vitro and in vivo. CAR-modified primary human ML NK cells (CAR-ML) were transduced with an αCD19-CD8a-41BB-CD3z-GFP (CD19-CAR-ML) lentivirus, resulting in a 15-25% transduction efficiency measured by GFP expression in vitro. CD19-CAR-ML (GFP+) and control ML NK (GFP-) cells were evaluated for functional responses to CD19-positive or CD19-negative tumor targets. CD19-CAR-ML NK cells demonstrated significantly increased IFN-γ production (44±4% vs. 15±3%, $p<0.001$); mean ± SEM) and degranulation (31±4% vs. 5±1%, $p<0.001$) against NK-resistant CD19+ Raji targets, compared to control ML NK cells. CD19-CAR-ML NK cells also exhibit a significantly increased effector response compared to CD19-CAR NK cells that were treated with IL-15 only ($p<0.05$). CD19-CAR-ML NK cells did not demonstrate enhanced responses against CD19-negative targets. Additionally, αCD33-CD8a-41BB-CD3z-GFP+ (CD33-CAR-ML) did not display enhanced responses against Raji, compared to controls (GFP-), thus demonstrating CD19-CAR-ML enhanced responses are antigen (CD19) specific. Notably, CD19-CAR-ML NK cells also exhibited significantly enhanced killing, degranulation, and IFN-γ production against primary CD19+ follicular lymphoma targets from patient lymph nodes ($p<0.01$). Autologous CD19-CAR-ML NK cells generated from lymphoma patient NK cells demonstrated significantly increased IFN-γ production ($p<0.05$) and degranulation ($p<0.01$) against their own CD19+ lymphoma targets, compared to control ML NK cells (GFP+). ML NK cells transduced with CD19-CAR were transferred into NSG mice engrafted with Raji lymphoma cells. CD19-CAR-ML NK cells reduced tumor burden measured by flow cytometry compared to CD33-CAR-transduced or control CAR-NK cell-treated mice. Notably, CD19-CAR-ML (GFP+) were increased to >70% of human NK cells from 20% in the CD33- CAR-ML recipient mice in the Raji-NSG model after 3 weeks in vivo, suggesting improved survival or expansion of antigen-specific CAR-ML NK, compared to non-transduced ML or non-specific CAR-ML NK cells in vivo. Thus, CAR expression in ML NK cells expanded anti-tumor recognition to NK cell-resistant tumors, warranting continued CAR-ML development and testing against a variety of cancers.

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CLONAL ARCHITECTURE OF EXPANDED IMMUNOTHERAPEUTIC NK CELL PRODUCTS EXAMINED USING A RHESUS MACAQUE BARCODE MODEL

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Clinical trials of adoptively transferred NK cells have employed several methods of NK cell preparation, including stimulation with cytokines or proliferation/expansion with feeder cells such as EBV-transformed B cells or K562 expressing co-stimulatory ligands and membrane-bound cytokines. Our laboratory has utilized the rhesus macaque model to clonally track the hematopoietic progeny of transplanted CD34+ stem and progenitor cells which have been transduced with "barcoded" lentiviral vector libraries. Tracking of NK cells in these animals has revealed long-lived mature NK clones which persist multiple years and wax and wane under the possible influence of pathogens. In this study, the transplanted macaque model is employed to study the changes in the clonal architecture of the NK cell population when expanded in vitro to large numbers, using culture protocols analogous to those used in clinical preparation of NK cells for infusion into humans. Specifically, stimulation of "barcoded" macaque NK cells with irradiated SMI-LCL or 4-1BBL+IL-21+K562 feeder cells with IL-2 are being investigated. Cultures of NK cells purified from the blood of barcoded macaques and cultured with SMI-LCL demonstrate proliferation of many NK clones, representing a significant proportion the NK cell clonal repertoire. Proliferation of long-lived "memory" NK clones was also observed. These experiments provide insight into the nature of the immunotherapeutic NK cell products used in several previous and current clinical trials.

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CO-EXPRESSION OF IL-15 SUPERAGONIST FACILITATES SELF-ENRICHMENT OF GD2-SPECIFIC CAR-NK CELLS AND MEDIATES POTENT CELL KILLING IN THE ABSENCE OF IL-2

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Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system that are activated rapidly upon encounter of virally infected, stressed or neoplastic cells, and play an important role in cancer immunosurveillance. Allogeneic NK cells do not carry a high risk of inducing graft-versus-host disease, allowing to base clinical protocols on donor-derived primary NK cells or the clinically applicable NK cell line NK-92. To enhance their antitumoral activity both, NK-92 and primary NK cells have been engineered to express chimeric antigen receptors (CARs) specific for different tumor-associated cell surface antigens, and early phase clinical trials are ongoing to further evaluate these approaches in cancer patients. Extending on prior work from our group, here we generated an optimized lentiviral CAR vector encoding a humanized second-generation receptor that encompasses a scFv(hu14.18) antibody fragment specific for the disialoganglioside GD2 which is expressed at high levels by neuroblastoma cells and other tumor cells of neuroectodermal origin, followed by a CD8α hinge region and a composite CD28-CD3ζ signaling domain. To enable long-term survival of transduced cells, an IL-15 superagonist sequence (RD-IL15) composed of the sushi domain of IL-15Rα (amino acid residues 31-107), a flexible linker and affinity-optimized IL-15N72D was fused to the CAR via a P2A self-cleaving peptide. Transduction of NK-92 cells with this improved vector provided the gene-modified cells with a selective growth advantage triggered by the expression of RD-IL15, resulting in self-enrichment during culture in the absence of IL-2. Importantly, the enriched NK-92/hu14.18.28.z_RD-IL15 cells displayed stable CAR expression in long-term cultures, and exhibited high and specific cytotoxicity against GD2-positive tumor cells in in vitro cell killing experiments. Ongoing work now aims at transferring this approach to ex vivo expanded peripheral blood-derived primary NK cells and evaluation of their antitumoral activity in murine in vivo tumor models

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COMBINATION THERAPY OF GEMCITABINE AND CD34+ PROGENITOR-DERIVED NK CELLS RESULTS IN SUPERIOR OVARIAN CANCER KILLING

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Ovarian cancer (OC) is the most lethal gynecological malignancy. To improve survival, novel therapeutic strategies are urgently needed. As ovarian tumors often downregulate MHC class I expression, they are prone to Natural Killer (NK) cell-mediated immunity. Previously, we demonstrated that umbilical cord blood-derived CD34+ hematopoietic progenitor cell (HPC)-NK cells are highly capable of killing OC monolayers and spheroids. Furthermore, intraperitoneal infusion of HPC-NK cells in human OC tumor-bearing immunodeficient mice significantly limits tumor progression and improves survival. However, HPC-NK cell treated mice do not remain tumor-free, indicating that further maximizing HPC-NK cell therapy is needed. Combining HPC-NK cells with gemcitabine could be an attractive treatment strategy for ovarian cancer patients, since gemcitabine is used in second-line treatment. Furthermore, gemcitabine increases expression of NKG2D ligands in various cancer types, which provide activating signals to NK cells. The goal of this study was to investigate the cytotoxic, phenotypical and functional effects of gemcitabine treatment on HPC-NK cells and OC cells. First, we established that low-dose (2.5 nM) gemcitabine kills 50-60% of OC cells after 2 days, while HPC-NK cells were relatively spared. Notably, this did not negatively impact HPC-NK cell degranulation and production of IFNγ, perforin and granzyme B. Importantly, we demonstrated that HPC-NK cells combined with lowdose gemcitabine kill 80% of OC cells after 2 days, compared to 60% by gemcitabine or HPC-NK cells alone. This additive effect on OC killing might be attributed to increased expression of NKG2D ligands ULBP2/5/6 on OC cell lines. In conclusion, HPC-NK cell therapy in combination with gemcitabine treatment could be a promising approach to treat ovarian cancer.

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COMBINING CD5-TARGETED CHIMERIC ANTIGEN RECEPTOR ENGINEERING AND GENETIC EDITING OF TGF-BR2 FOR THE TREATMENT OF T-CELL HEMATOLOGIC MALIGNANCIES

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Primary refractory/relapsed T-cell lymphoma and leukemia (TCL) patients have poor prognosis and few targeted therapies. This contrasts with B-cell acute lymphoblastic leukemia/large B-cell lymphoma patients, for whom the clinical success of CD19-specific CAR-T-cell therapy has produced two FDA-licensed products. Developing equivalent ACTs targeting neoplasms of T-cell origin is hindered by shared expression of targetable antigens on both malignant T-cells and normal T-lymphocytes, which leads to fratricidal killing of the CAR-expressing effector cells during CAR-Tcell manufacturing and potentially severe T-cell immunodeficiency upon adoptive transfer. All T-cell malignancies express CD5, making it an attractive target, however it is also expressed on normal T-cells; thus, engineering T-cells to express a CAR against CD5 would be complicated by fratricide. NK cells provide an advantage over T-cells as they lack CD5 expression and will therefore not be targeted by CAR. CD5-mediated fratricide. We have developed a strategy to genetically modify cord blood (CB)-derived NK cells to express a CAR, ectopically produce IL-15 to support NK cell proliferation and persistence in vivo, and express a suicide gene - inducible caspase-9 (iC9), to address potential safety concerns. As proof of principle, we have initiated a first-in-human, phase I/II clinical trial of iC9/CAR19/IL15-NK cell therapy following lymphodepleting chemotherapy in patients with relapsed/refractory B-cell lymphoid malignancies with promising results. We propose to extend this approach to target T-cell malignancies by genetically modifying NK cells to express a CAR against CD5. We confirmed anti-T-ALL activity of iC9/CAR.5/IL-15 transduced CB-NK cells as evidenced by increased cytokine production (TNFa p=<0.0001, IFNg p=0.0019), degranulation (CD107a p=0.0186) and enhanced cytotoxicity against both T-cell ALL cell line (CCRF-CEM) and primary leukemic cells as shown by Cr51-release assay (p=0.001 at 1:1 ratio, p=0.008 at 5:1, p=0.01 at 10:1, p=0.02 at 20:1). Moreover, the xenogeneic NSG mouse model of CCRF-CEM T-ALL, iC9/CAR.5/IL-15 transduced NK cells exerted significantly better anti-tumor activity and improved survival of mice compared to NK cells or IL-15 transduced NK cells (p=0.01). Based on our findings that TCL express high levels of immunosuppressive cytokine TGF-β, we developed a protocol combining iC9/CAR.5/IL-15 transduction and Cas9 ribonucleoprotein-mediated gene editing of TGF-βR2 to protect CAR-NK cells from the immunosuppressive tumor microenvironment. TGF-βR2 knockout resulted in significantly enhanced tumor killing by iC9/CAR.5/IL-15 transduced CB-NK cells compared to Cas9 control cells both in vitro and in vivo. These data indicate TGF-βR2 KO in iC9/CAR.5/IL-15-transduced CB-NK cells could further enhance anti-tumor activity, and equip them to evade the immunosuppressive tumor milieu.

COMPARATIVE ANALYSIS OF THE CYTOTOXIC POTENTIAL OF CYTOKINE-INDUCED KILLER AND NATURAL KILLER CELLS FOR NEUROBLASTOMA THERAPY

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BACKGROUND:
Neuroblastoma (NB) is the most common solid extracranial tumor in children. Despite therapeutic progress, prognosis is poor and innovative therapies are of medical need. Therefore, we investigated the cytotoxic potential of interleukin (IL)-activated natural killer (NK) cells compared to activated cytokine-induced killer (CIK) cells against different human NB cell lines in vitro.

METHODS:
NK cells were isolated from peripheral blood mononuclear cells (PBMCs) using CD56 enrichment or CD3/CD19 depletion kits. They were expanded ex vivo with different cytokine combinations such as IL-2, IL-15, IL-18 and/or IL-21 under feeder-cell free conditions. CIK cells were generated from PBMCs by ex vivo stimulation with interferon-γ, IL-2, OKT-3 and IL-15. A comparative analysis of expansion rate, purity, phenotype and cytotoxic activity against different NB cell lines was performed.

RESULTS:
CD56 enriched NK cells showed a median expansion rate of 4.3-fold after 10-12 days with a final frequency up to 99.0% NK cells. In contrast, the cell product after CD3/CD19 depletion consisted of a median frequency of 43.5% NK cells that expanded significantly faster with 7.5-fold and also reached up to 98.6% purity. CIK cells expanded with a median rate of 30.8-fold. NK cells showed a significantly higher median cytotoxicity against NB cells (46.6%: CD56 enriched, 53.7%: CD3/CD19 depleted) compared to CIK cells (7.2%) (E:T ratio of 5:1, 3 hours coincubation). Prolonging the cultivation after CD3/CD19 depletion to 15 days enhanced the expansion to 12.3-fold with a slightly reduced cytotoxicity (31.1% compared to 40.9%). The addition of an IL21-boost prior harvesting increased the expansion rate and cytotoxicity to median 12.6-fold and 51.5%. Further optimization of ex vivo culture using another cell culture medium led to a median 24.4-fold (compared to 9.6-fold) expansion in 15 days and comparable cytotoxicity of 52.5%.

CONCLUSIONS:
NK and CIK cell products may offer an innovative immune therapeutic option for patients with high-risk NB after allogenic stem cell transplantation. Our study revealed that NK cells have a significantly higher cytotoxic potential to combat NB. The use of IL-15 expanded and IL-21 activated NK cells developed from a CD3/19 depleted apheresis product seems highly promising for maintenance therapy and is currently evaluated in vivo in xenograft models.

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COMPARATIVE LIVE CELL IMAGING OF LYTIC GRANULE MOTILITY IN CAR- AND ADCC-MEDIATED NK CYTOTOXICITY TOWARDS BREAST CANCER CELLS

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Upon encountering a susceptible target, NK cells mediate directed cytotoxicity by exocytosis of lytic effector molecules such as perforin and granzymes. The steps leading to NK cell granule exocytosis are highly regulated. Granule exocytosis is preceded by convergence of granules to the microtubule organizing center (MTOC) and subsequent polarization of the MTOC and granules to the immunological synapse (IS). In case of antibody-dependent cell-mediated cytotoxicity (ADCC), it has been shown that signaling through the Fc receptor is critical to polarize MTOC and granules to the IS with otherwise resistant targets. Here we used spinning disk confocal microscopy for live cell imaging to analyze granule-mediated NK cell cytotoxicity in ErbB2-targeted CAR expressing NK-92 cells (NK- 92/5.28.z) and research-grade high affinity FcR expressing NK-92 cells plus Herceptin™ towards ErbB2-positive breast cancer cells (MDA-MB-453), which are resistant to parental NK-92. Interestingly, unmodified NK-92 cells in combination with MDA-MB-453 cells showed granule convergence to the MTOC, but failed to polarize MTOC and granules to the IS. In contrast, retargeting by either CAR or mAb/FcR towards the ErbB2 antigen on MDA-MB-453 enabled granule polarization to the IS resulting in highly effective cytotoxicity. Granule polarization was rapid in both the CAR and high affinity FcR expressing NK-92 cells after cell-cell contact was initiated (3 and 9 minutes respectively). These observations suggest that retargeting of NK-92 cells by either transgenic CAR or high affinity FcR expression in combination with tumor-specific antibodies confers tumor cell lysis by enabling the otherwise impaired MTOC and granule polarization to the IS which resembles the physiological exocytosis cascade observed in naturally occurring ADCC.

DEVELOPING A SILVER BULLET: CAR NK CELLS TARGETING TFH TO PROMOTE REMISSION IN PATIENTS WITH LUPUS AND OTHER AUTOIMMUNE DISEASES

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Follicular helper T cells (Tfh) are critical for vaccine and infection elicitation of long-lived humoral immunity, but exaggerated Tfh responses can promote autoimmunity and other pathologies. Dysregulated Tfh responses are central to development of lupus-like disease in mice and a cardinal feature of patients with systemic lupus erythematosus, a devastating and incurable autoimmune disease that is a leading cause of death for ethnic minority women of childbearing age. Unfortunately, no clinical interventions are currently available for selective depletion of Tfh cells to alleviate these disease conditions. We engineered a programmed death ligand 1 (PDL1)-based chimeric antigen receptor (CAR) that facilitates specific targeting of cells highly expressing human programmed cell death protein 1 (PD1), a cardinal feature of Tfh cells. CAR-expressing NK-92 cells robustly degranulate in response to plate-bound or cell-associated PD1. Of significant importance, incubation of PDL1 CAR NK cells with CD4 T cells from human tonsil results in discriminatory elimination of PD1-high Tfh and follicular regulatory T cells (Tfr) while sparing PD1-low non- Tfh memory T cells and regulatory T cells (Treg). Naïve CD4 T cells (PD1-neg) and B cells (PD1-low) were also spared. Depletion of Tfr and Tfh from the pool of tonsil CD4 T cells dramatically reduced the capacity of these cells to promote B-cell proliferation, plasmablast differentiation, and immunoglobulin production. Application of CAR NK cells to a pristane-induced model of lupus-like disease in humanized mice resulted in robust and selective elimination of PD1-high CD4 T cells. Using a drug-inducible CAR expression platform, we recently generated CAR NK cells from lupus-patient derived induced pluripotent stem cells. Given that CAR NK cells are emerging as a safe and effective alternative to CAR T cells in cancer immunotherapy, our results suggest a new, clinically translatable application of CAR NK cells for selective depletion of pathogenic Tfh cells in lupus and other disease states.

DIRECTED DIFFERENTIATION OF MOBILIZED HSCS INTO FUNCTIONAL NK CELLS WITH ENHANCED ANTITUMOR ACTIVITY

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Natural killer (NK) cells are specialized effector cells of the innate immune system that play an important role in the defense against viral infections and elimination of neoplastic cells. Unlike T lymphocytes, NK cells do not require prior sensitization and recognition of peptide antigens presented in complex with MHC molecules. Instead, natural cytotoxicity of NK cells is triggered rapidly upon appropriate stimulation, regulated by a complex balance of signals from germline-encoded activating and inhibitory cell surface receptors. Due to this intrinsic ability to efficiently detect and kill malignant cells, NK cells hold promise for adoptive cancer immunotherapy. For clinical application, usually donor-derived NK cells are isolated from peripheral blood or umbilical cord blood and expanded ex vivo with cytokines before infusion into patients. Experimentally, NK cells have also been derived from hematopoietic stem cells (HSCs) by ex vivo differentiation following different protocols. Successful ex vivo differentiation of HSCs to NK cells has been described using CD34+ cells from bone marrow, umbilical cord blood (UCB-CD34+), and more recently from induced pluripotent stem cells (iPSC). HSCs mobilized into the peripheral blood (PB-CD34+) represent another valuable starting material for ex vivo generation of NK cells. PB-CD34+ cells are easy to access, and can usually be collected at higher quantities than UCB-CD34+ cells. However, in comparison to UCB-CD34+, use of PB-CD34+ cells for ex vivo NK cell generation can be challenging. Earlier studies have reported rather poor differentiation of PB-CD34+ cells into NK cells, with decreased viability, low activity and high donor-variability. To address these shortcomings, we established a cytokine-based culture protocol that reproducibly allows the generation of mature and functional CD56+ NK cells from mobilized PB-CD34+ cells. To further aid ex vivo expansion of NK cells, we generated feeder cells coexpressing pro-NK cell factors including CD137 (4-1BB) ligand and membrane-anchored IL-15 and IL-21. Co-culture of PBMCs as well as ex vivo differentiating CD34+ cell pools with these feeder cells promoted development and marked expansion of NK cells to high purity without compromising their cytolytic activity. Importantly, feeder-cell stimulation compensated for donor-dependent variability by enabling efficient NK cell production and expansion also from donor CD34+ cells that otherwise displayed poor differentiation potential. Our findings suggest mobilized PBCD34+ cells expanded and differentiated according to this two-step protocol as a promising source for the generation of allogeneic NK cells for adoptive immunotherapy.

DISCOVERY OF A NOVEL NK CELL LINE WITH DISTINCT IMMUNOSTIMULATORY AND PROLIFERATIVE POTENTIAL AS AN ALTERNATIVE PLATFORM FOR CANCER IMMUNOTHERAPY

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BACKGROUND:
Human natural killer (NK) cell lines serve as an attractive source for adoptive immunotherapy, but NK-92remains the only cell line being assessed in the clinic. Here, we established a novel NK cell line, NK101, from a patient with extra-nodal natural killer/T-cell lymphoma and examined its phenotypic, genomic and functional characteristics.

METHODS:
Single cell suspensions from lymphoma tissue were expanded with anti-NKp46/ anti-CD2-coated beads in the presence of IL-2. A continuously growing CD56+ cell clone was selected and designated as NK101. Flow cytometry and RNA sequencing were used to characterize phenotypic and genomic features of NK101. In vitro cytotoxicity and IFN-gamma/TNF-alpha secretion were measured by flow cytometry-based cytotoxicity assay and enzyme-linked immunosorbent assay, respectively, after direct co-culture with tumor cells. Immunomodulatory potential of NK101 was assessed in an indirect co-culture system using conditioned medium. Finally, in vivo antitumor efficacy was evaluated in an immunocompetent, syngeneic 4T1 mammary tumor model.

RESULTS:
NK101 displayed features of CD56dimCD62L+ intermediate stage NK subset with the potential to simultaneously act as a cytokine producer and a cytotoxic effector. Comparative analysis of NK101 and NK-92 revealed that NK101 expressed lower levels of perforin and granzyme B that correlated with weaker cytotoxicity, but produced higher levels of pro-inflammatory cytokines including IFN-gamma and TNF-alpha. Contrarily, NK-92 produced greater amounts of anti-inflammatory cytokines, IL-1 receptor antagonist and IL-10. Genome-wide analysis revealed that genes associated with positive regulation of leukocyte proliferation were overexpressed in NK101, while those with opposite function were highly enriched in NK-92. The consequence of such expressional and functional discrepancies was well-represented in (i) indirect co-culture system where conditioned medium derived from NK101 induced greater proliferation of human peripheral blood mononuclear cells and (ii) immunocompetent 4T1 tumor model where peritumoral injections of NK101 displayed stronger anti-tumor activities by inducing higher tumor-specific immune responses. In a manufacturing context, NK101 not only required shorter recovery time after thawing, but also exhibited faster growth profile than NK-92, yielding more than 200-fold higher cell numbers after 20-day culture.

CONCLUSION:
NK101 is a unique NK cell line bearing strong immunostimulatory potential and substantial scalability, providing an attractive source for adoptive cancer immunotherapy.

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DONOR-DERIVED NK CELLS AND TRASTUZUMAB AS COMBINATION THERAPY FOR BREAST CANCER

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NK cell therapy is a promising treatment option for cancer. We previously showed that alloreactive mouse NK cells can cure mice with 4T1 mouse breast cancer (BC). Here, we investigated the anti-tumor potential of human NK cells in human BC models and whether it can be enhanced by 1) mismatching HLA ligands with the inhibitory receptors KIR and NKG2A and 2) Trastuzumab, an anti-HER2 antibody that triggers antibody-dependent cellular cytotoxicity (ADCC).

NK cells were isolated from blood of healthy C1+/C2+/Bw4+ donors and IL-2 activated overnight or expanded with PM21 particles and IL-2. BC target cells were the human BC lines MCF-7 and SKBR3 or dissociated primary BC cells. The antitumor potential was assessed in flow cytometry-based cytotoxicity and CD107a-degranulation assays, with or without Trastuzumab (anti-HER2), and at 21% or 0.2% oxygen. KIR-ligand matched NK cells were identified as NKG2A- KIR2DL2/3+ (SKBR3) or KIR2DL1+ (MCF7); KIR-ligand mismatched NK cells as NKG2A- KIR2DL1+, KIR3DL1+, KIR2DL1+ KIR3DL1+ (SKBR3) or KIR2DL2/3+, KIR3DL1+, KIR2DL2/3+ KIR3DL1+ (MCF7).

Human NK cells degranulated against SKBR3 and MCF7 cells in vitro. In all conditions of both cell lines, KIR-HLA ligand mismatched NK cells degranulated better than matched NK cell subsets. To consider the 3D tumorenvironment, MCF7 cells were grown in immunodeficient mice and KIR-ligand mismatched NK cells also degranulated better against these MCF7 cells ex vivo. In combination with the ADCC-triggering antibody Trastuzumab, all NK cells degranulated very vigorously against HER2-overexpressing SKBR3 cells (20% without vs. 80% with Trastuzumab on average). Surprisingly, Trastuzumab did not enhance or only slightly enhanced the cytotoxic potential of NK cells against SKBR3. Addition of Trastuzumab to MCF7, expressing normal levels of HER2, slightly enhanced NK cell degranulation (35% without vs. 48% with Trastuzumab on average) and did not alter cytotoxicity levels. To further consider BC heterogenicity, primary BC cells were obtained. NK cells killed these primary BC cells in 4 of 8 patients and degranulation assays are ongoing.

In summary, donor NK cells have the potential to mediate anti-BC effects in preclinical models, while Trastuzumab strongly enhanced NK cell degranulation but did not enhance cytotoxicity of BC cells.

EFFECT OF ALLIIN ON FUNCTIONAL ACTIVITY OF NK CELLS IN LUMINAL A AND TRIPLE NEGATIVE BREAST CANCER DERIVED CELL LINES

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INTRODUCTION:
In breast cancer, dysregulations on the function and activity of NK cells has been observed. This is commonly reported with respect to inhibition of the production of IFN-γ, TNF-α and the cytotoxic activity in the aforementioned cells. Research and use of compounds that can enhance the antitumor activity of NKs and reduce the side effects of conventional chemotherapy represents a promising alternative in the treatment of breast cancer. In our study, we evaluated the effect of the Alliin, an organosulfur component of garlic, on the expression of CD107a, granzyme B, perforin, IFN-γ and TNF-α markers in NK cells in coculture with luminal A and triple negative breast cancer derived cell lines.

MATERIAL AND METHODS:
Breast cancer cell lines MCF-7 and HCC70 were seeded and subsequently the NK92 cells were added in a ratio 1:8 (target/effector) together with the Alliin (10 µM and 1mM). The cocultures were incubated overnight and the expression of CD107a, granzyme B, perforin, IFN-γ, and TNF-α was assessed via intracellular labeling by flow cytometry. We use a K562 coculture as a positive control group for the activation of the NK cells.

RESULTS:
The K562 and MCF-7 cocultures significantly increased the expression of CD107a and decreased the expression of granzyme B within the cell ($p < 0.05$). In contrast, the HCC70 coculture showed the lowest levels of CD107a expression, however, it significantly decreased the expression of granzyme B ($p < 0.05$). Nevertheless, the Alliin treatment did not have a direct effect on the expression of granzyme B, perforin, IFN-γ and TNF-α in the K562, MCF-7 or HCC70 cocultures in comparison with the basal group.

CONCLUSION: The NK92 line showed less cytotoxic activity on triple negative breast cancer cells HCC70 compared to the K562 and MCF-7 lines. Furthermore, the treatment with Alliin does not increase the activity of the NK cells against these tumor cell lines.

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ELOTUZUMAB ENHANCES SLAMF7 INTERACTIONS BETWEEN NK AND MULTIPLE MYELOMA CELLS TO CO-STIMULATE CYTOTOXICITY

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Elotuzumab (Elo) is a therapeutic IgG1 monoclonal antibody targeting SLAMF7 (CS1, CRACC, CD319), which is highly expressed on multiple myeloma (MM) cells, NK cells, and subsets of other leukocytes. Elo promotes potent NK cell–mediated ADCC via Fc RIIIA (CD16) toward SLAMF7+ MM cells. Since NK cells express the adaptor protein EAT-2, which facilitates activation signaling through SLAMF7, we hypothesized that Elo may also activate NK cells upon directly engaging with the SLAMF7 expressed on the surface of NK cells. Consistent with this hypothesis, we showed that Elo can enhance calcium signaling responses in primary human NK cells via a co-stimulation mechanism, independent of CD16 binding. We further found that Elo, but not other SLAMF7 antibodies, uniquely enhances cytotoxicity mediated by CD16-negative NK-92 cells toward SLAMF7+ target cells. The CD16-independent basis for the enhanced cytotoxicity was further confirmed with F(ab')2 and Fc-mutant forms of Elo that cannot bind CD16. The effect required expression of SLAMF7 on both NK-92 cells and target cells, but was abrogated if the NK cells expressed a naturally-occurring variant of SLAMF7 lacking most of the cytoplasmic domain, thereby implicating co-stimulatory signaling. The CD16-independent co-stimulation by Elo was associated with increased expression of CD69, CD25, NKG2D, ICAM-1, and activated LFA-1 on NK-92 cells, and enhanced cytotoxicity was partially reduced by NKG2D blocking antibodies. In addition, the Fc mutant form of Elo promoted cytotoxicity of SLAMF7+ target cells by primary NK cells from most healthy donors, especially if previously cultured in IL-2. Enhanced responsiveness of IL- 2-activated NK cells to Fc-mutant Elo is consistent with therapeutic efficacy of Elo in combination with lenalidomide, which is known to stimulate IL-2 production by T cells that is presumed to be the primary basis for enhanced NK cell function. We conclude that in addition to promoting NK cell-mediated ADCC (CD16-dependent) responses, Elo has unique capacity to promote co-stimulatory SLAMF7-SLAMF7 interactions to enhance NK cytotoxicity toward MM target cells in a CD16-independent manner.

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ENGINEERED NK92-CD64 CELLS FOR THE TREATMENT OF PROSTATE CANCER

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Targeted natural killer (NK) cell therapeutics have the potential to revolutionize solid tumor treatment. Solid tumors adopt an immunosuppressive microenvironment to evade immune detection and augment progression of the disease. Targeted NK cell therapeutics may overcome these defenses through multiple native mechanisms of cancer detection, potent cytotoxicity, and stimulation of anti-tumor immune responses. Several strategies are currently under exploration to target NK cells to solid tumors. NK cells have been engineered to express chimeric antigen receptors (CARs) that redirect the effector cells to tumor-associated antigens or chemokine receptors that home NK cells to the tumor site. Another strategy is engineering NK cells to express CD64 (FcγR1), a high affinity receptor for human immunoglobulin G (IgG) Fc expressed by myeloid cells, to enhance antibody-dependent cell cytotoxicity (ADCC) in combination with therapeutic monoclonal antibodies (mAbs). In our study, we stably expressed CD64 in NK92 cells (NK92-CD64) and showed that CD64 can capture soluble mAbs with two to three orders of magnitude higher affinity than the native NK cell IgG Fc receptor CD16A (FcγIIIA). Engineered NK92-CD64 cells enhance targeting of tumor cells compared to CD16A-expressing NK92 cells and can function as a “ready-made CAR” when mAb is pre-docked before treatment. This system allows us to substitute the therapeutic mAb and effectively redirect the NK92 cells to multiple tumor-associated antigens without needing to design additional CAR constructs. Having established proof of concept, we are now testing this NK cell platform for therapeutic efficacy using in vitro and in vivo models of prostate cancer. We are targeting several relevant prostate cancer antigens including TROP2, a transmembrane glycoprotein that is overexpressed in castration-resistant prostate cancer, and FAP, a membrane bound serine protease expressed by cancer-associated fibroblasts in the tumor microenvironment.

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ENHANCEMENT OF NK CELL CYTOTOXIC ACTIVITY AGAINST SOLID TUMORS BY KNOCKOUT OF NKG2A

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INTRODUCTION:
Natural Killer (NK) cells have the inherent ability to kill infected and malignant cells and thus represent an attractive cell population for cell-based immunotherapy against tumors. However, their therapeutic efficacy has been of limited efficacy in clinic. We posit that engineering NK cells, either with chimeric antigen receptors (CAR) and inhibitory gene-invalidation, could improve their efficacy by targeting the tumor cells and overcoming the inhibitory environment, such as expression of HLA-E.

AIM:
We therefore proposed to invalidate, by CRISPR, the gene coding for NKG2A in NK cells and/or to express CAR against HER-2 to target more efficiently breast cancer cells.

METHODS:
Feldan Therapeutics developed an innovative and novel peptide-based technology, named Feldan shuttle, that allows the direct administration of gRNA-CRISPR nuclease complexes in human amplified NK cells, without any viral integration into the host genome. We then tested the efficacy of this strategy on a breast cancer cell line (MDA-MB231) on which we forced the expression HLA-E and its peptide.

RESULTS:
We were able to obtain a ~40% of NKG2A negative cells among the NK cell population. This NK cell population could kill HLA-E+ as efficiently as HLA-E- breast cancer cells, contrary to control NK cells which displayed a reduced cytotoxic activity toward HLA-E+ cells. When sorted, unmodified NKG2Anegative cell population were spontaneously re-expressing ~40% of NKG2A among the population. In contrast, sorted CRISPR-NK cell one were only re-expressing ~15% of NKG2A. We observed a correlation between the lytic activity against HLA-E+ target cells and the percentage of NKG2Anegative NK cells ($p=0,0081$, $r2=0,2782$, $n=4$) but not against HLA-E- target cells. In addition, we noted that this correlation was observed under the ratio of 40% of NKG2Anegative cells while a plateau was reached after 40%, suggesting that a pure population of NKG2Anegative may not be necessary to observe the maximum lytic capacity.

CONCLUSION:
NKG2A shut down by CRISPR technology via a new method of shuttling in NK cells was associated with an increased cytotoxicity towards HLA-E+ cancer cells. Moreover, our data suggest that a pure NKG2Anegative NK cell population is not needed to bypass the HLA-E inhibitory signal from tumor microenvironment. We are currently aiming to combine this strategy with a chimeric antigen receptor targeting HER-2 on breast cancer cells (ongoing experiments). Together, this data could open new avenue in cancer cellular immunotherapy with NK cells.

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EXPRESSION OF RECOMBINANT FC RECEPTORS BY ENGINEERED NK CELLS TO ENHANCE CANCER CELL KILLING BY ADCC

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A key mechanism of action of anti-tumor mAbs is antibody-dependent cell-mediated cytotoxicity (ADCC) by natural killer (NK) cells. Human NK cells exclusively recognize tumor-targeting therapeutic mAbs by CD16A (FcγRIIIA). A limitation of this IgG Fc receptor is that it binds to antibodies with low affinity. Studies in animal models and patients show that increasing its binding affinity to anti-tumor mAbs improves cancer killing and survival. The primary focus on enhancing this process has been through antibody engineering. Our goal is generating higher affinity versions of FcγR expressed by engineered NK cells for universal antigen targeting by anti-tumor mAbs. To this end, we have generated a recombinant FcγR that includes the transmembrane and cytoplasmic regions of CD16A, required for NK cell signal transduction, and the extracellular region of CD64 (FcγRI), the only high affinity FcγR family member and is expressed by certain myeloid cells. The recombinant FcγR is referred to as CD64/16A. CD16A and CD64/16A were expressed in the human NK cell line NK92, which lacks endogenous FcγRs, as well as in human induced pluripotent stem cells (iPSC), which are highly-stable, genetically-modifiable, clonal cell lines. The engineered iPSCs were then differentiated into NK cells. CD64/16A in NK cells facilitated conjugation to antibody-treated tumor cells, ADCC, and cytokine production, demonstrating functional activity by its two components. Unlike NK cells expressing CD16A, CD64/16A captured soluble therapeutic mAbs and the modified NK cells mediated potent tumor cell killing. Thus, CD64/16A can stably bind to soluble anti-tumor mAbs and function as a docking platform for NK cell arming with switchable and mixable targeting elements. Its expression in iPSC-derived NK cells overcomes issues of donor and patient-sourced NK cells and provides an “off-the-shelf” option for allogeneic adoptive cell therapy.

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HUMAN CD96 CORRELATES TO NK CELL EXHAUSTION AND PREDICTS THE PROGNOSIS OF HUMAN HEPATOCELLULAR CARCINOMA

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Immune checkpoint blockade has become a promising therapeutic approach to reverse immune cell exhaustion. Co-inhibitory CD96 and TIGIT, together with co-stimulatory CD226, bind to common ligand CD155. The balancing between three receptors fine tunes the immune responses against tumors. In this study, we investigated the expression of CD96, TIGIT and CD226 in 55 fresh human hepatocellular carcinoma (HCC) samples, 236 paraffin-embedded HCC samples, and 20 normal human livers. The cumulative percentage, absolute count, and MFI of CD96+ NK cells are significantly increased in the intratumoral tissues of HCC, and break the balance between three receptors. Human CD96+ NK cells are functionally exhausted with impaired IFN-γ and TNF-α production, high gene expression of IL-10 and TGF-β1, and low gene expression of T-bet, IL-15, perforin and granzyme B. In addition, blocking CD96-CD155 interaction specifically increases lysis of HepG2 cells by NK cells. HCC patients with high level of CD96 or CD155 expression within tumor are strongly associated with deteriorating disease condition, shorter disease-free survival (DFS) and overall survival (OS) times. Patients with higher cumulative percentage of CD96+ NK cells within tumor also exhibit shorter DFS. High plasma level of TGF-β1 in HCC patients up-regulates CD96 expression and dynamically shifts the balance between CD96, TIGIT and CD226 in NK cells. Blocking TGF-β1 specifically restores normal CD96 expression and reverses the dysfunction of NK cells. Conclusion: these findings indicate that human intratumoral CD96+ NK cells are functionally exhausted and patients with higher intratumoral CD96 expression exhibit poorer clinical outcomes. Blocking CD96-CD155 interaction or TGF-β1 restores NK cell immunity against tumors by reversing NK cell exhaustion, suggesting a possible therapeutic role of CD96 in fighting liver cancer.

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IDENTIFICATION OF SPECIFIC GENE REGULATION DURING ACTIVATION OF ONKORD® NATURAL KILLER CELLS BY TRANSCRIPTOME ANALYSIS AND COMPARISON WITH CYTOTOXIC POTENTIAL

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Glycostem Therapeutics (Oss, the Netherlands) investigates a Natural Killer (NK)-cell based immunotherapy (oNKord®) for the treatment of haematological malignancies and solid tumours. oNKord® is produced in a multi-step process, starting from fresh umbilical cord blood-derived CD34+ hematopoietic stem cells, isolated and expanded ex vivo and then differentiated into functional NK cells in a feeder cell-free culture system. oNKord® is committed as an off-the-shelf, allogeneic cancer immunotherapy and Phase I/II clinical trial in acute myeloid leukaemia (AML) and in multiple myeloma (MM) will begin in Q1 2020.

oNKord® batches are each generated from a single CD34+ cells donor and show similar patterns of expression for cell surface receptors and potent in vitro cytotoxicity against various tumour cell lines. Nevertheless, the interindividual variability between CD34+ cells donors can result in heterogeneous batches with dissimilar growth and functionality, that cannot be predicted beforehand. Therefore, we investigated the biological variability between oNKord® batches by comparing in vitro cytotoxicity against tumour cell lines with transcriptome profiling of several oNKord® products (n=6). Hence, we generated 6 oNKord® batches and we monitored relevant parameters as cell expansion, differentiation progression, cell purity, impurity and receptor phenotype. Next, we challenged each batch with tumour cells and analysed the cytotoxic potential. In parallel, we performed total RNA isolation from challenged and unchallenged oNKord® cells and we performed transcriptome analysis with RNA-sequencing, using an Illumina HiSeq 4000 platform.

Using differential expression analysis, we compared the profiles of challenged vs unchallenged cells and we identified specific gene expression signatures for challenged oNKord®. Furthermore, the comparison of the transcriptome profile with the cytotoxicity potential of the 6 oNKord® products allowed us to identify gene expression differences between ‘excellent’ and ‘neutral’ killers.

It is crucial for NK cell immunotherapy to get an understanding of the molecular pathways activated during the killing process and their correlation with the product phenotype and cytotoxic potential. This has significantly improved our knowledge about oNKord® and its batch-related functionality. From such data sets, we will provide statistic rationales to prove the criteria to be used to identify the ‘excellent’ products and to support strategies to be used to improve the homogeneity and the performances of all oNKord® batches.

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IMPACT OF IL33 ON NK CELLS

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Interleukin-33 (IL-33) is a member of the IL-1 family and interacts with membrane-bound suppression of tumorigenicity 2 (ST2)L receptor (IL-1RL1) and coreceptor IL1RAP. IL-33 is mostly localized in the nucleus and expressed by endothelial, epithelial and fibroblast-like cells, during homeostasis and inflammation. Surface-bound ST2 ligation leads to signal induction of MyD88/Traf6/IRAK4 pathway which in turn leads to proliferation and activation of the target cell. Natural Killer (NK) cells have been shown to be among the targets of IL-33 in vivo. Various methods have been developed and varieties of cytokines (IL2, IL-12, IL-15, IL-18, IL-21) have been used for large scale NK cell expansion. Defining the optimal regimen that does not contain any feeder cells, is an important issue to empower the clinical use of NK cells. Our aim with this study is to utilize IL-33 during NK cell expansion, observe and analyze the NK cell fraction after NK cell expansion. For this aim, feeder-free autologous peripheral blood mononuclear cells (PBMCs) from healthy donors were incubated with increasing concentrations (5ng/ml, 10ng/ml, 15ng/ml) of recombinant human IL-33, with or without recombinant human IL-2 (500 IU/ml) addition and cultured for 21 days for NK cell expansion. Cells collected from each condition at different time points were evaluated for functional and phenotypic characterization. Cells were examined for proliferation, cytotoxicity and IFN-g secretion. Responses are variable between donors, but we observed a general trend that IL-33 addition together with IL-2 induced NK cell fold expansion and the percentage of NK cells with lower T cell contamination and an augmented cytotoxicity of primary NK cells.

In this study, we observed that IL-33 can be used as a costimulatory factor for NK cell expansion, however IL-33 by itself is not enough to activate and expand NK cells specifically. Interestingly IL-33 showed this effect independently of surface ST2L expression. Furthermore, we observed no difference in the expression of the activation markers CD69, CD25 and NKp44. We are currently assessing the transcriptomic profile of the cells with and without IL-33 stimulation.

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NATURAL KILLER CELLS GENETICALLY MODIFIED TO OVER-EXPRESS DNAM-1 EXERT ENHANCED ANTI-TUMOR RESPONSES AGAINST CD112/CD155+ SARCOMAS AND OTHER MALIGNANCIES

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Sarcomas are a broad category of cancers of mesenchymal origin that occur in bone and soft tissues. With conventional treatment approaches 25 to 50% of the patients relapse with a median survival of only 10 to 15 months. Due to the vast heterogeneity across the different subtypes of sarcomas and the lack of efficient treatment alternatives, there is an imminent need for the development of novel targeted treatment strategies such as cell-based immunotherapy.

Natural Killer (NK) cells exert tumoricidal activity upon detection of a complex array of tumor ligands without the need for prior activation, which makes them promising candidates for cancer immunotherapy. We investigated the potential role of NK cells in sarcoma surveillance and identified specific receptor-ligand interactions that can be targeted for the development of novel sarcoma-tailored NK cell-based therapies. For this purpose, i) we investigated the NK cell receptor-ligand immune profile of 23 freshly isolated and short-term in vitro propagated sarcoma explants ii) developed a screening platform that identifies which activating receptors equip NK cells to efficiently respond against sarcomas.

Our results identified a common expression signature comprising the proliferative marker PCNA along with the DNAM-1 ligands CD112 and/or CD155. These markers enabled the distinction of sarcoma cells in freshly isolated tumor homogenates. This made it possible to comparatively analyze the NK cell ligand expression profiles of freshly isolated sarcoma cells and matched corresponding explants. Detailed characterizations of tumor-infiltrating leukocytes from freshly dissociated sarcomas and matched peripheral blood revealed a general decrease in NK cells in the tumor compared to the periphery.

To further investigate the DNAM-1/CD112 and/or CD155 activation axis, as well as other NK cell-sarcoma interactions, we generated a screening platform where genetically modified NK-92 cells over-express one receptor at a time These NK-92 variants were tested for their capacity to degranulate against different primary human sarcoma explants, While WT NK-92 degranulation responses were surprisingly low, GM NK-92 cells that over-express DNAM-1 efficiently degranulated against all tested sarcoma explants, which was in line with the strong presence of DNAM-1 activating ligands CD112 and/or CD155.

Overexpression of DNAM-1 provides an efficient and novel way of arming NK cells against tumor targets expressing CD112 and/or CD115. This approach utilizes the well-studied and clinically used model NK cell line NK-92, which would allow the development of a standardized and well-characterized off-the-shelf therapeutic, but could also be adapted to the use of primary NK cells.

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NK CELL DEFICIENCY IN ATOPIC DERMATITIS REVEALS A NOVEL IMMUNOTHERAPY STRATEGY

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Atopic dermatitis (AD) is a chronic, inflammatory skin disease characterized by itchy, red, scaly rashes. In the United States, AD affects 6-10% of the population and costs $5.3 billion dollars annually in medical treatment. AD-associated inflammation is driven by a type 2 inflammatory module involving T helper type 2 cells, group 2 innate lymphoid cells, and the type 2 cytokines. Therapeutic approaches currently focus directly on barrier restoration and immunosuppression. However, whether some aspect of the immune system might be impaired or required to regulate the aberrant inflammatory response observed in AD is less clear. We demonstrate that AD patients harbor a deficiency in blood natural killer (NK) cells. Low numbers of blood NK cells have both diagnostic value for AD and improved along with clinical parameters and biomarkers in response to therapy. We undertook multidimensional mass cytometry analysis and RNA profiling of blood NK cells from AD and control patients. These analyses revealed aberrant activation and susceptibility to cell death that was associated with a loss of mature NK cells. Taken together, these findings indicate that NK cell reduction is a central feature of AD that may serve as a diagnostic and treatment-responsive biomarker. In addition, we hypothesized that NK cells may provide inhibitory signals that are lost in the setting of AD-associated inflammation. Previous studies have indicated that NK cells and NK cell-derived cytokines can limit type 2 inflammation in other systems. In agreement with this, we found that NK cell deficiency in a murine model of AD was associated with enhanced type 2 inflammation in the skin, suggesting that NK cells play a critical immunoregulatory role. Based on these findings, we tested an NK cell-boosting immunotherapy treatment and found marked improvement in AD-like disease in mice. These findings implicate a systemic NK cell reduction in AD pathology and reveal a new treatment paradigm in which NK cells can be enhanced in order to restrain pathogenic type 2 inflammation and improve disease.

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NK CELL FUNCTIONS ARE NOT AFFECTED BY IVIG; INDIVIDUAL DIFFERENCES ARE NOT FULLY EXPLAINED BY VARIATIONS OF THE FCGR LOCUS

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Intravenous Ig (IVIg) are used to treat inflammatory/autoimmune diseases and immunodeficiencies. Numerous mechanisms of action are reported, yet their therapeutic effects are not completely understood in inflammatory/autoimmune diseases. Controversy remains concerning the effect of IVIg on NK cells including function and cell numbers after therapy. Human NK cells express the IgG receptor CD16 (FcγRIIIa). Furthermore, a growing list of variations in the FCGR gene locus has been associated with several diseases and response to monoclonal antibody therapeutics. Therefore, the aim of the current study was to study the effect of IVIg on NK cell function in patients treated with IVIg.

To this end, NK cells were isolated prior and three days post IVIg administration from the peripheral blood of three groups of patients, treated for antibody immunodeficiencies (11), inflammatory myopathies (11), and autoimmune neuropathies (11). The levels of surface-bound IgG, CD56 and CD16 cell surface expression, and circulating levels of soluble CD56 and CD16 were evaluated by flow cytometry and ELISA, respectively. NK cell function (ADCC and cytotoxic capacity) was analyzed ex vivo using a non-radioactive cytotoxicity assay, whereas intracellular interferon-gamma (IFNγ) expression and degranulation (CD107a) by flow cytometry. The most clinical relevant single nucleotide polymorphisms (SNP) and copy number variations (CNV) of FCGR polymorphisms were investigated by next generation sequencing.

Overall, IVIg treatment had no effect, neither on NK cell numbers nor on NK cell function. Direct NK cytotoxicity and degranulation remained intact after IVIg therapy. In addition, IFNγ induction upon stimulation of NK cells was not affected. IgG bound to CD16 on NK cells was not modified by IVIg infusion. Furthermore, no correlation was found between CD16 and CD56 surface expression levels on NK cells and serum levels of soluble CD56 and CD16 post IVIg therapy. CNVs were altered in 10% of the patients; one patient with a low CNV for FCGR3A also exhibited lower levels of ADCC; FCGR3A V158F gene polymorphism correlated with ADCC in some patients whereas other clinically relevant SNPs for FCGR3A and FCGR2B/C were not associated with NK activity.

In conclusion, IVIg therapy did not influence NK cytotoxicity when analyzed three days after administration. Individual differences of NK cell responses were not fully explained by genetic variations of the FCGR locus in patients with immunodeficiencies and inflammatory/autoimmune disorders. Clinically, these findings are of relevance, as NK cells are important players to control viral infections, and for the elimination of malignant cells.

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NKG2C EXPRESSING T CELLS HAVE NATURAL KILLER-LIKE FEATURES AND EXHIBIT POTENT ANTI-LEUKEMIA AND ANTI-HCMV FUNCTIONS

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The distinction between innate and adaptive immunity is become increasingly blurred. In particular, T cells have been shown to acquire “innate-like” properties during certain conditions, including HCMV infection, that can drive the expansion of NKG2C+ NK and NKG2C+ T cells. It is unclear what factors contribute to the generation of NKG2C+ T cells and how and when these cells deviate from conventional clonotypic T cells.

We evaluated T and NK cell populations in 331 healthy donors by flow cytometry. Our results demonstrated that NKG2C was detectable in both TCRαβ CD8+ T cells (hereafter referred to as IL-CD8) and TCRγδ Vδ1+ T cells, but only in individuals with prior CMV exposure.

Additionally, our high-resolution phenotype analysis revealed that IL-CD8 are oligoclonal for their TCR, express CD56, inhibitory KIR and are effector memory TEMRA cells (CD45RA+CD45RO-CD57+/-CCR7−CD27−CD127−CD62L+/- CD95+). Furthermore, IL-CD8 are negative for the checkpoint molecule PD1 and for the co-stimulatory molecule CD28.

We performed RNA-seq analysis on IL-CD8 population from 5 different individuals. Strikingly, transcription factor Bcl11b, which initiates commitment to the T cell lineage pathway and represses the innate genetic program, was significantly down-modulated compared to NKG2C-negative CD8 T cells. We confirmed the increased gene expression of CD57 (B3GAT1) and decreased expression of PD-1 (PDCD1). We also observed increased expression of granzyme B (GZMB), perforin (PRF1) and granulysin (GNLY). DAP12 (TYROBP) was significantly overexpressed, suggesting that IL-CD8 may signal through NKG2C, independent of TCR-MHC interactions.

Importantly, compared to the NKG2C negative counterpart from the same individual, IL-CD8 cells exhibit high effector function against leukemia cells, solid tumors as well as HCMV infected fibroblasts. We also observed that IL-CD8 cells expand in transplant recipients after CMV reactivation.

The novel finding that IL-CD8 cells have cytotoxic response to leukemia and virally infected target cells has immediate translation to the allogeneic hematopoietic cell transplantation (HCT) setting, in which patients with acute leukemia or pre-leukemic conditions, such as myelodysplastic syndrome, are transplanted with curative intent. On the basis of the higher anti-leukemia response of IL-CD8 cells, allograft-associated protection from relapse may be partly attributable to the IL-CD8 population. Given their itrinsic capacity to recognize diseased cells and the absence of CD28 costimulatory receptor, IL-CD8 present and attractive alternative for allogeneic cellular therapy, including CAR-T based therapies, for solid and hematopoietic tumor treatment.

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OPTIMIZATION OF LARGE-SCALE EXPANSION AND CRYOPRESERVATION OF HUMAN NATURAL KILLER CELLS FOR ANTI-TUMOR THERAPY

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Natural killer (NK) cells are in charge of as members of the innate immune system and play a key roles of viral infection, other infectious disease and cancer. In current setting, autologous NK cells have shown their safety, however, they fail to prove efficacy because of inhibition by self-MHC I. Allogeneic NK cell therapy has been developed for the treatment of cancer patients because NK cells with KIR-major histocompatibility incompatibility could induce a graft-versus-tumor response without causing GVHD. We established an expansion method for large-scale production of highly purified and functionally active NK cells, as well as a freezing medium for the expanded NK cells. In the present study, we assessed the effect of cryopreservation on the expanded NK cells in regards to viability, phenotype, and anti-tumor activity. NK cells were enormously expanded (about 15,000-fold expansion) with high viability and purity by stimulating CD3+ T cell-depleted peripheral blood mononuclear cells (PBMCs) with irradiated autologous PBMCs in the presence of IL-2 and OKT3 for 3 weeks. Cell viability was slightly reduced after freezing and thawing, but cytotoxicity and cytokine secretion were not significantly different. In a xenograft mouse model of hepatocellular carcinoma cells, cryopreserved NK cells had slightly lower anti-tumor efficacy than freshly expanded NK cells, but this was overcome by a 2-fold increased dose of cryopreserved NK cells. In vivo antibody-dependent cell cytotoxicity (ADCC) activity of cryopreserved NK cells was also demonstrated in a SCID mouse model injected with Raji cells with rituximab co-administration. Therefore, we demonstrated that expanded/frozen NK cells maintain viability, phenotype, and anti-tumor activity immediately after thawing, indicating that expanded/frozen NK cells can provide ‘ready-to-use’ cell therapy for cancer patients.

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PD-L1-SPECIFIC IMMUNOCYTOKINES CARRYING AN IL-15 SUPERAGONIST AUGMENT FUNCTIONALITY AND ANTITUMOR ACTIVITY OF CAR-ENGINEERED NK CELLS

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Natural killer cells play an important role in cancer immunosurveillance, with their cytotoxicity triggered rapidly upon stimulation through germline-encoded cell surface receptors. In addition, NK cells modulate adaptive antitumor immunity by maintaining the quality of dendritic cells and improving presentation of tumor antigens. Genetic engineering of NK cells with chimeric antigen receptors (CARs) can enhance specific recognition and selective elimination of tumor cells. Nevertheless, expression of programmed death receptor-ligand 1 (PD-L1) by tumor cells may dampen the CAR NK cells' direct and indirect antitumor activity. To overcome such immunosuppressive effects in the tumor microenvironment, we aim to develop advanced CAR-NK cells which secrete PD-L1-specific antibodycytokine fusions that carry pro-inflammatory IL-15. Binding of these immunocytokines to PD-L1 on the surface of tumor cells is intended to restrict IL-15 to the tumor site for local activation of innate and adaptive bystander immune cells, while simultaneously blocking the PD-1/PD-L1 immune checkpoint. For initial testing of their functionality, antibody-cytokine fusion proteins composed of a single chain fragment variable (scFv) domain of PD-L1-specific antibody atezolizumab and human IL-15, or an IL-15 superagonist sequence (RD-IL15) encompassing the sushi domain of IL-15Rα (amino acid residues 31-107), a flexible linker and affinity-optimized IL-15N72D were expressed in HEK293 cells and purified from culture supernatants. Binding of the recombinant proteins to PD-L1 and functionality of their IL-15 domains were verified by flow cytometry and in bioactivity assays demonstrating activation of the IL-15 receptor pathway. Thereby fusion proteins carrying the IL-15 superagonist proved more effective than the molecule harboring unmodified IL-15. The recombinant immunocytokines also enhanced T-cell activation in mixed lymphocyte reactions, and increased cytotoxicity of NK cells against tumor cells. Introduction of the immunocytokine sequences into ErbB2 (HER2)-specific CAR-NK-92 cells by lentiviral transduction resulted in secretion of the fusion proteins into the culture supernatant, and growth and effective CAR-mediated cytotoxicity of the producer cells in the absence of exogenous IL-2. In transwell assays, the immunocytokines secreted by CAR-NK-92 improved expansion of co-cultured CD8+ T cells, and enhanced cell killing activity of co-cultured lymphocytes from peripheral blood. Our results demonstrate that PD-L1-specific IL-15 immunocytokines expressed in CAR-NK cells are functional, modulating the producer cells' growth and direct antitumor activity as well as enhancing their stimulatory activity towards bystander immune cells.

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POLY(I:C) IN IMMUNOTHERAPY FOR GLIOBLASTOMA: WHAT'S IN IT FOR NATURAL KILLER CELLS?

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Glioblastoma (GBM) is the most common malignant primary brain tumor with a devastating prognosis. Since novel treatment options are urgently required, immunotherapy is intensively being investigated following remarkable recent successes in other cancer types. While we are currently running a dendritic cell (DC) vaccination trial in GBM – with promising preliminary results – we attempt preclinically to further enhance this treatment by employing poly(I:C) to potentiate DC and to unlock high-potential combination strategies. Here we focus on the interplay between GBM and natural killer (NK) cells.

Primary GBM cells were derived from tumor specimens obtained following standard surgery of GBM patients and were treated with 10µg/ml poly(I:C). Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors and cocultured with GBM cells. Flow cytometry was used for phenotyping of GBM/NK cells and analysis of activation status and migration of NK cells in coculture or transwell migration assays, respectively. Cytokine secretion by GBM cells was assessed by electrochemiluminescence, activation of PBMC in cocultures by IFN-γ and granzyme B ELISA. Poly(I:C) treatment significantly upregulates/induces secretion of CCL4, CCL5, CXCL9 and CXCL10 by GBM cells, but NK cell attraction retained unaltered. Nonetheless, significant increases of CD69+ and CD107a+ NK cells in cocultures with poly(I:C)-treated versus untreated GBM cells demonstrate elevated NK cell activation. Furthermore, significant increases in IFN-γ and granzyme B release by total PBMC were observed. Poly(I:C) treatment generates an immunostimulatory secretome by GBM cells, possibly explaining the increased NK cell activation: significant induction of IFN-α and IFN-β, significant reduction of TGF-β, and significant upregulation of IL-15. Indeed, IL-15 activates NK cells, as witnessed by significant upregulation of NKG2D and TIM-3 expression and K562 killing. Intriguingly, poly(I:C) also creates an NK-cell hostile GBM cell membrane proteome: significant upregulation of HLA-E and immune checkpoints PD-L1, PD-L2, CD70, and CD73, while HLA-ABC and ULBP-2/5/6 show a trend towards up- /downregulation, respectively. Combining PD-L1 blockade with poly(I:C) significantly upregulated immune activation (IFN-γ).

Poly(I:C)-treated GBM cells activate NK cells, despite manifold inhibitory signals on the GBM cells, although these present an opportunity as combination strategy targets. Since NK cell migration towards GBM cells is not elevated likewise, establishing the actual value of NK cells in this GBM therapy requires in vivo investigation. Possibly, drugmediated NK cell attraction or emphasis on T cells rather than NK cells are warranted. In general, we show poly(I:C) also bears potential as adjuvant to immune checkpoint blockade in GBM.

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PRECLINICAL ASSESSMENT OF SUITABLE NK CELL SOURCES FOR CHIMERIC ANTIGEN RECEPTOR NK-BASED "OFF-THE-SHELF" AML IMMUNOTHERAPIES

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The introduction of chimeric antigen receptors (CARs) to augment the anticancer activity of immune cells represents one of the major clinical advances in recent years. To test the hypotheses that NK cells engineered to express a CAR specific for antigens associated with AML show enhanced anti-leukemia activity, and, furthermore, whether primary NK cells could be a powerful alternative NK cell source compared to the established CAR-NK-92 cell line, both NK cell types were sorted after CAR transduction, and relevant parameters of effector-relevant functional cytotoxicity were assessed. Therefore, an alpha-retroviral vector system was used to generate anti-CD123-CAR-NK cells. Stable expression of the anti-CD123-CAR is demonstrated in NK-92 cells and primary human donor NK (dNK) cells. EGFPsorted CAR NK cells have improved anti-leukemia activity compared to control NK cells that lack a functional CARs. However, in terms of viability, effectiveness, risk of side effects, and clinical practicality and applicability, an important question is whether gene-modified NK cell lines represent better CAR effector cells than primary human donor CARNK (CAR-dNK) cells. Comparison of the functional activities of sorted CAR-NK cells generated using the NK-92 cell line with those generated from primary human dNK cells demonstrated that CAR-NK-92 cells had stronger cytotoxic activity against leukemia cells compared to CAR-dNK cells. CAR-NK-92 and CAR-dNK cells had similar CD107a surface expression upon co-incubation with leukemia cells. However, CAR-NK-92 cells secreted higher granzyme A and interleukin-17A levels, while CAR-dNK cells secreted more tumour necrosis factor alpha, interferon gamma, and granulysin. In addition, CAR-NK-92 cells revealed a significantly higher potential for adverse side effects against nonmalignant cells. In short, this work shows the feasibility for further development of CAR-NK strategies to treat leukemia.

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PRECLINICAL DEVELOPMENT OF CAR NK CELLS

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Following the successes of CD19 CAR T cells in clinical trials, commercialization of these therapies revealed a number of practical limitations. In particular, the dependency on an autologous setting required the therapeutic cells to be generated individually for each patient resulting in high costs and increased susceptibility to manufacturing issues. In an attempt to further optimize this unique therapeutic strategy, we developed an approach for large-scale production of CAR-expressing NK cells. As NK cells lack the potential to cause GvHD and display a differential cytokine response than T cells, their offer the advantages of being an allogenic source and a reduced risk of life-threatening cytokine storms during therapy.

In this study, we used a Baboon endogenous virus(BaEV)-pseudotyped lentiviral vector system to efficiently transduce NK cells with various CARs and routinely obtained transduction efficiencies between 30-70%. The transduction protocol was implemented into the CliniMACS Prodigy, a closed and automated cell manufacturing system which allows large-scale generation of therapeutic cells under GMP-compliant conditions, currently the leading automatic manufacturing system for cellular therapies. Following cell expansion, antigen engagement by CAR expressing NK cells induced CAR-specific activation as characterized by NK cell degranulation, secretion of inflammatory cytokines and an effective killing of antigen expressing target cells that are resistant to natural cytotoxicity. Having assessed the performance of different CAR constructs in vitro, current efforts are now focusing on evaluating the in vivo functionality using mouse xenograft models.

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PROFILING OF KINASE ACTIVITY CHANGES IN ONKORD® NATURAL KILLER CELLS IN RESPONSE TO CHRONIC MYELOGENOUS LEUKEMIA

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Glycostem Therapeutics (Oss, the Netherlands) developed a Natural Killer (NK) cell-based therapy for cancer, which is based on differentiating hematopoietic stem cells derived from umbilical cord blood (UCB) into NK cells (oNKord®). From previous studies, it appeared that these oNKord® cells are functionally different from peripheral blood NK (PBNK) cells, as they show different receptor expression, and show high cytotoxic capacity against tumour cells.

NK cell cytotoxic capacity is dependent on the engagement of activating receptors, leading to a downstream signaling which results in cytokine release and cytotoxic lysis of the tumour cell. This downstream signaling is highly dependent on activity of kinases, for example the protein tyrosine kinases of the Src family and Syk family. However, the exact activation mechanism of oNKord® cells to lyse tumour cells is unknown. Therefore, we performed a kinase activity profiling of oNKord® cells with the aim to explore the differences in kinase activity of oNKord® cells upon co-culture with tumour cells and identify the underlying activating mechanisms of oNKord® cells.

To obtain a challenged condition, oNKord® cells were co-cultured with the chronic myelogenous leukemia (CML) cell line K562 at an effector/target ratio of 1:1 (n=6). To obtain an unchallenged condition as a control, oNKord® cells were treated in the same way as the co-culture condition, except for the addition of K562. After 0 or 15 minutes of incubation, samples were collected and proteins were immediately isolated. Using the PamGene PamChip® microarray, differences of activity of serine-threonine kinases (STK) or protein tyrosine kinases (PTK) between unchallenged and challenged conditions were determined. Briefly, the chip is coated with peptide sequences (144 peptides for STK and 196 peptides for PTK) containing phosphorylation sites which correlate with upstream kinases. To detect kinase activity, fluorescently labelled anti-phospho antibodies were used. Data analysis and interpretation was performed using the PamGene BioNavigator® software. Time point 0 minutes was used to normalize the data. Results show clear upregulation of activity of STK kinases between the conditions unchallenged vs. challenged oNKord® cells while activity of PTK kinases showed a more variable outcome of activity.

In conclusion, this project will improve the understanding of the downstream signaling pathways activated in oNKord® during target cell killing and can lead to the identification of new molecular targets to be further investigated for the clinical application of oNKord®.

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PROPORTION OF KIR-POSITIVE NK CELLS, EXPANDED UNDER STIMULATION WITH IL-2 AND FEEDER CELLS, INCREASE MODERATELY AFTER RETROVIRAL GENE TRANSDUCTION PROCEDURE

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Genetic modification of NK cells may further improve their anti-tumor efficacy. However, the viral gene transduction may affect viability of NK cells and the expression of non-targeted molecules including activating and inhibitory receptors. The aim of this work was to analyze changes in the phenotypic profile and viability of NK cells after the procedure of retrovitral gene transduction.We used retroviral particles expressing CD2 to induce the overexpression of the costimulatory molecule in NK cells in order to improve their cytotoxic capabilities. The retrovitral particles were constructed using pBABE-hCD2 vector expressing CD2 and the pseudotyping construct expressing RD114 envelope glycoprotein. Primary NK cells were preliminary stimulated with IL-2 (100 units/ml) and engineered K562 feeder cells expressing membrane-bound IL-21 (K562-mbIL21). Cytometric analysis of the stimulated NK cells revealed high expression of HLA-DR indicating that they were activated (P<0,001, compared to initial HLA-DR proportion). Most of cells had significantly lower proportion of CD57+ cells and higher proportion of NKG2A+ cells in culture as compared to fresh un-manipulated NK cells (P<0,001 and P=0,012, respectively). The cultured NK cells also expressed activating receptors CD16, NKG2D, NKp30. We have shown that the decrease in CD57+ cell proportion was associated with the low proliferative activity of these cells during cultivation. Retroviral particles were shown to effectively transduce the activated NK cells resulting in an increase in CD2 expression whereas surface levels of NKG2A, CD16, HLA-DR, did not change significantly. This suggests that, after transduction, the cells retained the activated state. At the same time, KIR2DL2/DL3 and CD57 levels (both MFI and positive cell proportion) increased inNK cell cultures subjected to retroviral gene transduction procedure. This argues in favor of the survival cells with a more differentiated phenotype. The proportion of apoptotic and necrotic cells after transduction did not change and amounted to about 5%, which indicates a high cell viability. It is possible that KIR2DL2/DL3-positive NK cells have an advantage in survival after retroviral transduction.

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PROVIDING A HOMING RECEPTOR FOR CD19 CAR ENGINEERED NK CELLS – IMPROVING CELLULAR IMMUNOTHERAPY FOR B-CELL LYMPHOMA

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A key factor for the efficacy of cellular immunotherapies is biodistribution, which affects the local effector to target ratio. The inability of therapeutic immune cells to reach the tumor cells can dramatically affect clinical outcomes. The CCR7 chemokine receptor drives recruitment of immune cells to secondary lymphoid organs that express the cognate ligands CCL19 and CCL21. Expanded CAR-T cells often express low level of CCR7, which may decrease their ability to home to secondary lymphoid organs. Engineering immune cells to express the CCR7 receptor, particularly in CD19 CAR-expressing cytotoxic cells, can therefore improve their efficacy by increasing their targeted migration to lymphoma tumor sites. Despite robust initial responses, CD19 CAR-T cell therapy can be associated with significant toxicities. CAR-engineered natural killer (NK) cells potentially provide a safer alternative while maintaining efficacy. aNK™ and CD16-expressing haNK™ cells are clinical grade cell lines derived from the NK-92 cell line that have demonstrated potent cytotoxicity against malignant cell lines, as well as safety and efficacy in phase I trials. We have established a clonal aNK-derived cell line, R7-19.1, that stably co-expresses the CCR7 receptor, a CD19 CAR, and the high affinity CD16 receptor (158V) via a non-viral transfection method.

In vitro, R7-19.1 cells showed increased migration towards human CCL19-engineered K562 cells (K-19) in a modified Boyden Chamber assay. R7-19.1 cells displayed high cytotoxicity against K562 cells (92.4 ± 2.4% at 5:1 E:T ratio) and were able to efficiently kill the CD19+ NK-resistant cell line SUP-B15 (97 ± 0.6% at 5:1 E:T ratio). In addition, R7- 19.1 mediated potent ADCC in combination with rituximab (83.2 ± 2.8% at 5:1 E:T ratio against a CD19-, CD20+ subline of SUP-B15). In vivo, intravenously (i.v.) administered R7-19.1 cells migrated towards subcutaneous (s.c.) K-19 tumors more efficiently than non-CCR7-expressing NK cells. In NSG mice that were injected i.v. with human CCL19- expressing Raji lymphoma cells (Raji-19.5), treatment with R7-19.1 significantly prolonged survival ($p < 0.0001$). Further, in a s.c. model of Raji-19.5, R7-19.1 cells were able to slow tumor progression to a greater extent than CD19CAR+/CD16+ aNK cells.

In conclusion, we showed that the expression of CCR7 in an “off-the-shelf” CD19 CAR engineered aNK cell line improved homing towards lymph node chemokine CCL19 both in vitro and in vivo, and that such an improved homing resulted in greater anti-tumor activity in vivo.

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REAL-TIME TRACKING OF ADMINISTERED HIGHLY ACTIVATED NK CELLS IN AN ORTHOTOPIC HUMAN HEPATOCELLULAR CARCINOMA XENOGRAFT MODEL

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BACKGROUND & PURPOSE:
Adoptive transfer of expanded natural killer (NK) cells is a promising cancer therapy. However, the biodistribution of ex vivo-expanded NK cells in hepatocellular carcinoma is not fully understood, to lead hinder the development of an effective NK cell transfer therapy.

MATERIALS & METHODS:
Peripheral blood mononuclear cells from healthy donors were co-cultured with irradiated K562 myelogenous leukemia cells for NK cells ex vivo expansion. NK cells were labeled with a near-infrared (NIR) lipophilic cytoplasmic dye (XenoLight DiR fluorescent dye;1,1'-dioctadecyltetramethyl indotricarbo cyanine iodide). The proliferation and cytotoxicity activity of NK cells were performed using the WST-8 assay. Surface receptor expression levels of NK cells was assessed using flow cytometry. To evaluate the migrations patterns of NK cells between both non-tumors bearing and liver carcinoma orthotopic NSG mice model, NK cells labeled with DiR dye were tracking with the fluorescence-labeled organism bioimaging instrument (FOBI) imaging system.

RESULTS:
The dye XenoLight was nontoxic and did not affect proliferation or surface receptors expression levels of NK cells. In vivo, intravenously administered NK cells stained with DiR dye mostly accumulated in the lungs of non-tumor– bearing mice immediately after NK cell injection. The fluorescent signals increased in the liver and spleen, beginning 4 hours after injection and gradually reduced thereafter until 2 weeks, whereas the signal disappeared from the lungs after 7 days. In early stages and late stages of HCC orthotopic mice models, NK cells migrated faster to liver 1-hour post injection compared to normal mice. In early stage of HCC orthotopic model, the signal in liver was increased until 24-48 h post-injection and rapidly decreased at 1 week after NK injection. The signal of NK cells was dominant in tumor-bearing liver of late stage HCC mice until 1week after NK cells injection and persistent until 2 weeks.

CONCLUSION:
Optical imaging with NK cells stained with DiR dye was successfully performed for real-time biodistribution of ex vivo-expanded NK cells in orthotopic human hepatocellular carcinoma model. NK cells stained with DiR dye migrated rapidly to tumor-bearing liver and persisted until 2 weeks after administration. Administration of NK cells in different stage of tumor progression had different patterns in both migration and persistence of ex vivo-expanded NK cells in hepatocellular carcinoma orthotopic models.

KEY WORDS:
NK cells, In vivo tracking, HepG2 hepatocellular carcinoma orthotopic models, Adoptive transfer, Immunotherapy.

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REDIRECTED OPTIMIZED CELL KILLING (ROCK®): A NOVEL MULTISPECIFIC ANTIBODY PLATFORM FOR INNATE IMMUNE CELL ENGAGEMENT TO FIGHT CANCER

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Antibody-dependent cellular cytotoxicity (ADCC) has been described as a potent mechanism to destroy cells with high expression of antigen for a given IgG. Malignantly transformed cells downregulate tumor-specific/-associated antigens (TSA/TAA) or express these at low levels rendering ADCC of monoclonal or Fc-enhanced antibodies significantly less potent. To overcome these limitations, we have developed tetravalent, bispecific innate cell engagers (ICEs) which bind both the TSA/TAA on target cells and CD16A (Fc gamma receptor III) on NK cells and macrophages with high affinity, leading to significantly enhanced potency and efficacy to kill target cells compared to other ADCC-based approaches.

Recently, we have used our ROCK® platform to generate a gallery of tetravalent, bispecific ICEs based on different scaffolds and with desired properties. We show that these ICEs bind with high affinity and selectivity to CD16A, mediating killing of malignantly transformed cells with a detection threshold of <1000 copies of TSA/TAA per target cell in the absence of NK cell fratricide. Moreover, highlighting the differentiation of our approach, we provide molecular evidence for a unique epitope of these ICEs on CD16A preventing competition with plasma IgG binding.

An update on our product and development candidates to treat hematological and solid cancers will be provided. Results from phase 1 and 2 clinical studies of our lead product candidate AFM13 have demonstrated evidence of safety and clinical efficacy as both monotherapy and in combination with anti-PD-1 in patients with CD30+ lymphomas. Based on the ICEs' design limitations of other approaches can be overcome, through improved potency, efficacy, absence of plasma IgG competition and safety. Thus, our ROCK® based ICEs have the potential to become an integral part of a variety of treatment options for hematologic and solid tumors, both as monotherapy and in combination with other agents or adoptive NK cell transfer.

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REGULATION OF NK CELL FUNCTIONS BY E3 UBIQUITIN LIGASE COMPLEX CO-RECEPTOR CEREBLON

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Rearrangement of the actin cytoskeleton is a key cellular event regulating cytotoxic and immunoregulatory effector functions as well as migration of Natural Killer (NK) cells. However, dynamic reorganization of actin is a very complex process which remains largely unknown. In this study, we investigated the role of the protein Cereblon (CRBN), an E3 ubiquitin ligase complex co-receptor and the primary target of the Immunomodulatory drugs (IMiDs) in NK cells. Several studies described the capability of IMiDs to stimulate NK cell activity but the molecular mechanisms underlying these effects have never been investigated. Intriguingly, by confocal fluorescent microscopy, we observed that CRBN colocalizes with filamentous (F)-actin in chemokine-treated NK cells and is recruited to the lytic immunological synapse, thus suggesting a role for this protein in cytoskeleton reorganization. Accordingly, silencing of CRBN in NK cells results in a reduced cytotoxicity which correlates with a defect in conjugate and lytic synapse formation with target cells. Moreover, we found that treatment with Lenalidomide also induces CRBN co-localization with filamentous (F)-actin and enhances NK cell migration following different chemokine stimulation without affecting the expression of chemokine receptors. We observed that CRBN shRNA knockdown significantly impairs the ability of NK cells to migrate in response to a chemotactic gradient and reduces the enhancing effect of Lenalidomide on NK cell migration. Taken together, our data reveal a previously unrecognized role of CRBN as an important regulator of NK cell functions and suggest that Lenalidomide-mediated CRBN activation is responsible for the stimulatory effect on NK cells of this drug.

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SAFETY ANALYSIS OF EX VIVO EXPANDED CANINE NK CELLS IN XENOGENEIC MOUSE MODEL OF GRAFT-VERSUS-HOST DISEASE

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Adoptive transfer of allogeneic natural killer (NK) cells has arose as a promising immunotherapeutic strategy for cancer. Although canine NK cells are still defined as non-B, non-T large granular lymphocytes, it has been reported that canine NK cells share a part of the phenotypic characteristics of T lymphocytes. So, safety studies are crucial to use allogeneic canine NK cells for immunotherapy. But the safety of infusion of allogeneic NK cells has not been elucidated in dogs. In this study, we examined the safety of cultured canine NK cells using xenogeneic graft-versus-host disease (GVHD) mouse model. After injection of freshly isolated canine PBMCs, or cultured non-B, non-T NK cells for 2 weeks or 3 weeks, changes in body weight, disease severity scores, and survival rate of the mice were determined, and also histopathological and immunohistochemical evaluation were performed. The mice injected with canine PBMCs died within 30 days after the injection with gradual weight loss and severe clinical signs caused by GVHD, such as hunching, ruffling, decreased activities, and alopecia. However, there were no weight loss and any clinical abnormalities in the mice injected with 2-week or 3-week cultured cells, and all mouse were survived during the 90-day experimental period. Histopathological and immunohistochemical examination revealed that the mice injected with canine PBMCs showed multiple lesions including marked necrosis in the lung, liver, kidney, and stomach, and the injected canine immune cells infiltrated around the lesions. However, these changes were not observed in the mice injected with 2-week or 3-week cultured NK cells. The results of this study suggest that xenogeneic canine NK cells do not cause significant side effects such as GVHD, and allogeneic NK cells can be safely used for cancer immunotherapy in dogs.

SCREENING FOR ANTIBODIES THAT INDUCE NK CYTOTOXICITY

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Cancer immunotherapies have garnered a great deal of success over the last decade. Much of this success has been driven by the development of antibody-based therapeutics that unleash the cytotoxic potential of CD8+ T cells via immune checkpoint blockade or CD3/T cell receptor (TCR) complex stimulation. Like CD8+ T cells, natural killer (NK) cells are cytotoxic effector cells that are known to mediate anti-tumor responses. They play a key role in tumor immunosurveillance and are able to identify and remove target cells by recognizing stress-induced ligands that may be overexpressed on cancer cells. NK cells are also known to perform antibody-dependent cellular cytotoxicity (ADCC), a mechanism that is utilized by multiple, current therapeutic monoclonal antibodies (mAbs) to eradicate tumor cells. As such, NK cells are attractive candidates for new cancer immunotherapies. Whereas all T cells express the CD3/TCR complex that can be exploited by immunomodulatory molecules to redirect T cell activity, NK cells express multiple activating and inhibitory receptors that govern NK activity. Moreover, the NK cell repertoire is phenotypically heterogeneous and may vary among individuals. The heterogeneous expression of these receptors makes it difficult to identify which receptors should be targeted to recruit and stimulate NK cells. In order to determine the best target within such a heterogeneous population, we coupled mammalian display to a Next-Generation Sequencing (NGS) readout to characterize the ability of a curated set of antibodies to redirect NK cytotoxicity. Through this functional screen, we are able to identify antibodies that are not only able to bind to NK cells, but that are also able to elicit NK cell killing.

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SWITCHABLE CHIMERIC ANTIGEN RECEPTOR-ENGINEERED IPSC-DERIVED NATURAL KILLER CELLS

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Switchable chimeric antigen receptors (sCAR) provide an important strategy to precisely regulate CAR-mediated antitumor activity. The sCAR system combines a CAR with an injectable "switch" molecule (derived from an antibody Fab) for tumor antigen recognition. Previous studies have demonstrated that this sCAR system expressed in T cells provides tight control over T cell-mediated anti-tumor activity. Here, we translated this approach to engineer natural killer (NK) cells with the sCAR to provide a universal, targeted cell-therapy approach that does not have to be derived in a patient-specific manner. First, we expressed the sCAR scFV combined with our previously described NK cell-optimized CAR4 signaling motifs consisting of the NKG2D transmembrane domain, 2B4 co-stimulatory domain and the CD3 chain into NK92 cells. We then used both an anti-CD19 switch to target B cell malignancies and an anti- Frizzled7 (FZD7) switch to target solid tumors. Both systems demonstrated a switch-specific dose response in killing either CD19+ Raji B cell lymphoma cells or FZD7+ MA-148 ovarian cancer cells. Next, we engineered human induced pluripotent stem cells (iPSCs) with the sCAR and derived sCAR-expressing NK cells. Again, these sCAR-iPSC-NK cells killed the target tumor cells in a switch-dependent manner. Additionally, specificity of this system is demonstrated by only a basal level of tumor killing when a control switch lacking the sCAR recognition was used, despite of the increased dose of the switch. Similarly, there is no switch-mediated killing when Raji-CD19KO or MA148-FZD7KO cells lacking the specific antigen were used. Current studies are testing the iPSC-sCAR system in vivo. Together, this iPSC-sCAR strategy enables close control over CAR-mediated activity. Additionally, this system provides flexibility to target multiple antigens on tumor cells to prevent antigen-loss escape variants that can lead to relapsed disease.

THE CD30/CD16A INNATE CELL ENGAGER AFM13 ELICITS POLYFUNCTIONAL NK CELL RESPONSES EFFECTIVELY TRIGGERING MEMORY-LIKE (ML) NK CELLS AGAINST CD30+ TARGETS

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Natural killer (NK) cells are crucial innate immune effector cells that rapidly recognize and eliminate infected, stressed and malignant cells. One barrier to broadly applying NK cell therapy across many cancer types is inconsistent cancer cell recognition, which may be overcome by immune cell engagers. AFM13 is a tetravalent bispecific antibody based on the ROCK® platform characterized by bivalent binding to CD30 and CD16A, with clinical efficacy in CD30+ malignancies. Additionally, adoptively transferred memory-like (ML) NK cells have demonstrated enhanced anti-tumor activity that may be receptive to AFM13-based targeting to enhance target cell recognition. However, our understanding of NK cell phenotype of functional responses triggered via AFM13 remain incomplete. To address these questions, we analyzed healthy donor-derived conventional (cNK) and ML (IL-12/15/18-induced) NK cell functional responses to CD30+ lymphoma cells with AFM13. Primary cNK cells co-incubated with AFM13-labeled Hut-78 cells demonstrated increased IFN-γ, TNF, and degranulation, compared to Hut-78 cells or Raji (CD30-) targets + AFM13 as a negative control ($p<0.01$). ML NK cells also displayed enhanced cytokine production and killing of CD30+ tumor targets when co-incubated with Hut-78+AFM13 ($p<0.01$). To define the single-cell specificity of cNK cell responses to AFM13, similar assays were performed using mass cytometry assessing 39 lineage, maturation, activating and inhibitory receptors, and function-relevant NK cell markers. We then used CITRUS to define the NK cell subsets associated with increased functionality (SAM, FDR<0.05). CITRUS-based clustering divided the NK cell populations into two main groups associated with increased effector responses. Based on back-gating, we determined that both mature (NKG2A-CD57+) and immature (NKG2A+ CD57-) subsets displayed enhanced IFN-γ ($p<0.05$), Mip1a ($p<0.0001$), and CD107a ($p<0.0001$) in the presence of Hut-78+AFM13 compared to Hut-78+AFM12 (CD19/CD16A bispecific innate immune cell engager, negative control), with mature cells producing the most IFN-γ and MIP1α compared to immature NK cells ($p < 0.05$), or AFM12 triggered NK cells ($p<0.001$). AFM13 also resulted in increased frequencies of IFN-γ+CD107a+MIP1α+ multifunctional cells, in both mature and immature subsets, compared to AFM12 ($p<0.05$). Finally, within the NK cells stimulated with Hut-78+AFM13, we observed significantly increased CD57, KIR2DL1, KIR2DL2/2DL3, KIR3DL1 ($p<0.01$), NKp30, NKp44, and NKp80 ($p<0.01$) in the IFN-γ producing cells compared to IFN-γ-negative cells. Overall, AFM13 enhanced the magnitude and quality of NK cell responses against lymphoma targets. Collectively, these data indicate that target cell recognition of NK cells can be significantly enhanced by AFM13, yet influenced by inhibitory receptors expression, maturation state, and memory-like differentiation.

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THE ROLE OF GALECTIN-3 IN NK CELL INTERACTIONS WITH MYELOID CELLS

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The inflammatory process involves an intricate interplay between different innate and adaptive immune cells and soluble substances. Galectin-3, one out of 15 known endogenous β-galactoside-binding galectins, is an inflammatory mediator that is released in different settings by e.g. activated macrophages. As Galectin-3 binds to glycoprotein structures on the cell surface of myeloid cells, it may be present in both soluble and surface-bound forms. Recently, Galectin-3 was proposed to be a ligand to the natural cytotoxicity receptor NKp30. With this background, we set out to investigate the immunomodulatory role of Galectin-3 in interactions between NK cells and myeloid cells.

In a human in vivo skin chamber model of aseptic inflammation, we detect high levels of Galectin-3 (>10ng/ml) in cell-free exudates from the skin chambers, a level significantly higher as compared to serum controls. Using genetically engineered K562 cell variants, lacking specific ligands to NK cell receptors, such as B7-H6, PVR and Nectin-2 we here characterize the effect of Galectin-3 on NK cell cytotoxicity.

In co-culture experiments with myeloid cells, the presence of Galectin-3 triggered substantial cell death in NK cells. This phenomenon was dependent on Galectin-3-dependent activation of the myeloid enzyme NOX-2, triggering release of reactive oxygen species. The NK cell death could be partially prevented by addition of lactose, which binds Galectin-3, or catalase, which catalyzes the degradation of hydrogen peroxide.

Taken together, this study suggests a role for Galectin-3 as an immunomodulatory agent that impacts on NK cell function in multiple ways in an inflammatory setting, and sheds light on the role of NK cells in the complex regulation of the inflammatory response.

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VIRAL AND NON-VIRAL SLEEPING BEAUTY (SB) TRANSPOSITION-BASED ENGINEERING OF CAR NK CELLS FOR TREATMENT OF ACUTE LYMPHOBLASTIC LEUKEMIA

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Natural Killer (NK) cells are known for their target-independent cytotoxic potential without inducing T cell related graft versus-host-disease. NK cells equipped with a synthetic chimeric antigen receptor (CAR) targeting CD19 redirect specificity killing and can enhance the efficacy to treat acute lymphoblastic leukemia (ALL). Therefor the genetic information needs to be stably integrated into the host cell genome. Usually viral vectors are used to generate CARNK cells. However, since viral generation of CAR cells is limited by their genotoxicity, cost and regulatory demands, we developed a non-viral gene delivery protocol using the Sleeping Beauty (SB) transposon system for third party NK cells as a source to produce 'off the shelf' CAR-engineered cell products.

Human NK cells were isolated from peripheral blood mononuclear cells and equipped with CD19-CAR using viral or non-viral vectors. For non-viral gene delivery, minicircle DNA (MC) containing SB transposon construct carrying either a Venus fluorescent protein (MC-Venus) or a CD19-CAR expression cassette was used. Efficiency of gene transfer and viability of modified NK cells was analysed by flow cytometry. Cytotoxicity of CD19-CAR NK cells was addressed using CD19 positiv ALL cell line as target. NK cells transduced with the alpharetroviral CD19-CAR vector showed higher transduction rates than cells transduced with the lentiviral vector, even at low multiplicity of infection (MOI) (alpharetroviral: MOI1: 11%, MOI5: 49%, MOI10: 68%; lentiviral: MOI1: 1.5%, MOI5: 8%, MOI10: 14%). Additionally, alpharetrovirally transduced CD19-CAR NK cells displayed higher cell killing activity against ALL cells than lentivirally transduced or non-transduced NK cells (90% vs. 62% vs. 9%, E:T ratio of 1:1; 88% vs. 58% vs. 10%, E:T ratio of 0.5:1).

After SB transposition-mediated non-viral gene delivery, the proportion of Venus-positive NK cells increased from 25% (day 1 after electroporation) up to 37%, with stable expression of the marker (>29%) over two weeks. Viability after electroporation was over 90%, and comparable to that of non-electroporated NK cells. For NK cells electrotransfected with MC-CD19, we found an increasing CD19-CAR expression from 12% (day 2) up to a peak of 50% (day 7).

Our results show that viral generated CD19-CAR NK cells are highly cytotoxic against cancer cells, with highly effective cell killing achieved already at low E:T ratios. The Sleeping Beauty transposon system is a very promising non-viral alternative for gene transfer into NK cells for ALL therapy, and may be useful to generate genetically modified NK cells also for a broad range of other clinical applications.

SESSION 8
Tissue-associated NK cell Subsets

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30-COLOR FLOW CYTOMETRY: UNRAVELLING HUMAN NK CELL REPERTOIRE DIVERSITY IN BLOOD AND LIVER

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Recent findings have demonstrated extraordinary diversity in peripheral blood human natural killer (NK) cells and suggested environmental control of receptor expression patterns on distinct subsets of NK cells. However, tissue localisation may also influence NK cell differentiation to an even higher extent and less is known about the phenotypic and functional characteristics of various human tissue-resident NK cells. Additionally, understanding tissue-specific signals which regulate these characteristics is critical to determine how immune memory and homeostasis in the tissue are regulated and how tissue-resident cells can be harnessed in therapeutic settings. Advances in single-cell technologies have allowed a higher resolution study of these cells, but most are still limited by a high cost per cell or a requirement for a large number of cells. This cell number limitation is often the case with clinical samples. Here we harness the power of high parameter flow cytometry to unravel the complexity of NK cell repertoire diversity in liver and compare it to peripheral blood. We demonstrate how a 30-color panel for the simultaneous measurement of surface tissue-residency markers, including activating and inhibitory receptors and chemokine receptors, as well as transcription factors, can be used in combination with widely available computational algorithms to describe the diversity of liver NK cells. This is particularly important when working with rare clinical samples for which time and simplicity of experimental manipulation are of the essence. These findings represent a reproducible approach to diversity profiling of tissue-resident NK cells in various homeostatic and pathological conditions such as reproduction, infection, and cancer.

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A ROLE FOR ALLOSTERIC DISULFIDES IN REGULATING THE FUNCTION OF HUMAN KILLER CELL RECEPTOR KIR2DL4

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KIR2DL4 is an unusual member of the killer cell Ig-like receptor (KIR) family expressed in human NK cells. Unlike most other KIR that are at the cell surface, it resides mostly in endosomes and activates NK cells in response to soluble agonists, such as soluble HLA-G and a monoclonal antibody. KIR2DL4 signals from endosomes for a unique proinflammatory and proangiogenic response, which has relevance to placental development due to the restricted expression of its ligand, HLA-G, by fetal trophoblast cells during early pregnancy. To understand the nature and regulation of KIR2DL4–HLA-G interactions, we used an unbiased random mutagenesis approach and screened mutants of KIR2DL4 in a cellular assay for their ability to load soluble HLA-G into KIR2DL4+ endosomes. With this approach, amino acid residues in KIR2DL4 that are important for endosomal localization and HLA-G recognition were identified. Specifically, we have identified a putative allosteric cysteine in the extracellular domain of this receptor, which may be involved in a disulfide switching mechanism to control receptor internalization and ligand binding.

Protein disulfide isomerase (PDI) acting at the cell surface may be responsible for this switch as inhibition of PDI interfered with internalization of KIR2DL4 and uptake of HLA-G into endosomes. The configuration of the relevant disulfide bond is -RHstaple, which is a hallmark of many allosteric disulfides. Thus, KIR2DL4 localization and function is regulated by allosteric disulfide bonds at the cell surface. Studies are underway to explore the dynamic regulation of receptor structure by this allosteric disulfide switch to control ligand binding, localization and signaling by this receptor.

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ACCUMULATION OF TUMOR INFILTRATING CD49A+ NK CELLS CORRELATES TO POORPROGNOSIS OF HUMAN HEPATOCELLULAR CARCINOMA

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The discovery of CD49a+ liver-resident NK cells in mice not only changes the classic concept of NK cells but also opens a new window for the study of NK cells. Although compelling evidences have suggested important roles of NK cells in liver diseases, whether and how CD49a+ NK cells contribute to liver diseases remain unclear.

In this study, we identified the accumulation of CD49a+ tissue-resident NK cells in human hepatocellular carcinoma (HCC), which was significantly higher than the proportion found in peritumoral tissues. Furthermore, the exhausted and regulatory phenotypes of this tissue-resident NK cell population were revealed on both the protein level and mRNA level. The proportion of CD49a+ NK cells was positively correlated to the proportion of NK cells expressing inhibitory receptors, in addition, CD49a+ NK cells expressed higher levels of checkpoint molecules PD-1, CD96 and TIGIT. Global transcriptomic analysis showed a role of CD49a+ tissue-resident NK cells in the negative regulation of immune responses. Moreover, comparison between murine and human CD49a+ NK cells revealed their distinct characteristics and functions. Finally, accumulation of tissue-resident CD49a+ NK cells in the liver tumor was correlated to deteriorating disease condition and poor prognosis.

Our findings not only reveal the accumulation of CD49a+ NK cells in liver tumor but also suggest a possible role of this cell subset in the negative regulation of immune responses and the development of HCC. Accumulation of CD49a+ tissue-resident NK cells in human hepatocellular carcinoma (HCC) and its correlation with poor disease condition and prognosis suggest a possible role of this cell subset in the negative regulation of immune responses and the development of HCC.

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ARYL-HYDROCARBON RECEPTOR-DRIVEN REGULATION OF INNATE LYMPHOCYTE RESPONSES IN CHRONIC LIVER DAMAGE

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Natural Killer (NK) and Innate Lymphoid Cells (ILCs) play significant roles in the immune defence, as well as in the control of tissue damage and tissue repair. However, their dysregulated responses have been also involved in several pathologies, including aggravated inflammation and/or carcinogenesis. Understanding the contribution of innate lymphocytes and modules of their activation during disease progression is still incomplete, in particular with regards to their organ-, tissue- and microenvironment-specific functions. The Aryl-hydrocarbon Receptor (AhR) is a ligandinduced transcription factor that binds various exo- and endogenous polycyclic hydrocarbons and induces transcription of numerous target genes involved in development, cell differentiation and immune responses. Here, we show that NKp46-expressing innate lymphocytes, including NK cells and liver-resident ILC1s, express AhR and respond to stimulation with AhR ligands. Exposure of NK cells to the degradation product of tryphophan metabolism, Kynurenine, an endogenous AhR ligand, induced changes in the global gene expression and primed NK cells for enhanced effector responses, including cytokine secretion and migration. In the context of diet-induced chronic liver damage, NK cells and liver-resident ILC1s accumulated in the liver in an AhR-dependent manner. Conditional AhR deletion in NKp46-expressing cells abolished NK cell and ILC1 accumulation, which correlated with reduced numbers of inflammatory monocytes in the liver tissue and reduced liver damage. In conclusion, AhR is expressed by NK cells and liver-resident ILC1s and regulates their effector responses in the context of chronic liver damage, which might be of significance for the maintenance of liver tissue homeostasis, and clinical intervention in liver disease.

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EXPRESSION OF CD49A IDENTIFIES A TISSUE-RESIDENT NK CELL SUBSET IN THE HUMAN LUNG WITH A DISTINCT TRANSCRIPTIONAL AND PROTEIN-SIGNATURE

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Human lung tissue-resident NK cells (trNK cells) are likely to play an important role in host responses towards viral infections, in inflammatory conditions, and in cancer. However, detailed insights into human trNK cells are still largely lacking.

Using RNA sequencing and 18-colour flow cytometry, we here show that human lung CD69+ NK cells co-expressing CD49a display hallmarks of tissue-resident lymphocytes, including high expression of CD103 and ZNF683, and reduced expression of SELL, S1PR5, and KLF2/3. CD49a+CD16- NK cells were functionally competent to a similar degree as CD49a-CD16- NK cells and produced IFN-, TNF, MIP-1, and GM-CSF. After stimulation with IL-15, CD49a+CD16- lung NK cells upregulated perforin, granzyme B, and Ki67, and expressed elevated levels of CCL5.

Comparison of RNA-seq datasets from trNK cells in human lung and bone marrow with tissue-resident memory CD8+ T cells in lung and spleen identified core genes co-regulated either by tissue-residency, cell-type, or location. In comparison to CD69+CD49a+CD16- and CD69-CD49a-CD103-CD16- NK cells, CD69+CD49a-CD103-CD16- (CD69- single positive) NK cells displayed intermediate expression levels of e.g. NKG2A, CCL5, RGS1, and SELL, low levels of CD57, CXCR3, and ZNF683, but also high levels of CCL3, CCL4, CXCR6, and S1PR1. Furthermore, very few genes were differentially expressed upon comparison to CD69- CD56brightCD16- NK cells. Hence, CD69-single positive CD16- NK cells might represent a NK cell population with a less distinct tissue-resident phenotype compared to CD69+CD16- NK cell co-expressing CD49a.

Together, our data indicate that human CD49a+CD16- lung trNK cells have distinct phenotypic and functional features, likely regulating their function in barrier immunity.

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HUMAN CD49+CD200R+T BET+EOMES- CELLS REPRESENT PUTATIVE NON-NK GROUP 1 INNATE LYMPHOID CELLS AND ARE EXPANDED IN LIVER DISEASE

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BACKGROUND:
Group 1 innate lymphoid cells (ILCs) comprise a family of cytokine-producing lymphocytes that includes cytotoxic natural killer (NK) cells and non-NK ILC1s. Whereas in mice the distinctions between NK cells and ILC1s have been well established, it is not yet clear how human NK cells and ILC1s can be distinguished.

MATERIAL AND METHODS:
Human tissue-infiltrating lymphocytes were isolated from 35 transplant livers, 20 liver perfusates (controls), and 5 liver tumor margin (controls). Lymphocytes were phenotypically characterized by multicolor flow cytometry and tested for cytolytic degranulation (CD107a) following PMA/ ionomycin stimulation. The cytokine profile of PMA/Ionomycin stimulated, sorted subsets (BD Aria fusion) was examined by CBA assay (Th1-cytokine kit, Biolegend). Single cell RNA sequencing was performed using 10X chromium platform followed by sequencing the libraries with an Illumina NovaSeq sequencer.

RESULTS:
Here we describe a population of ILC1-like cells that was enriched in human diseased livers (control vs diseased liver: 0.15% vs 0.39% of CD45+ lymphocytes; $p<0.01$) and that expressed CD49a, CD200R, and the transcription factor TBET. Markers for liver-tissue residency like CXCR6 and CD103 are only partially present on this subset. These human ILC1-like cells produced IFN-g, TNF-a, and IL-2 following stimulation ex vivo, and they could be further distinguished from NK cells by their lack of expression of NKp80, EOMES, and perforin, their low capacity for cytolytic degranulation (CD107a), and their unique transcriptional profiles as assessed by single cell RNA-sequencing analyses.

CONCLUSION:
Collectively, these data support the identification of a putative human ILC1 population that is selectively expanded in liver disease.

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IDENTIFICATION OF AN EOMES-HIGH, T-BET-LOW NK CELL POPULATION IN PORCINE LIVER THAT IS HIGHLY SIMILAR TO HUMAN LIVER RESIDENT NK CELLS

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Long-lived liver resident NK cells (lrNK) have been described in human and mouse, but display remarkable speciesspecific phenotypic differences. Most notably, opposite expression patterns of T-box transcription factors Eomes and T-bet have been reported in human (Eomes-high,T-bet-low) and mouse (Eomes-low,T-bet-high) lrNK cells. In addition, whereas CD49a is considered as a defining cell surface marker for mouse lrNK cells, human lrNK cells appear to be defined by the lack of CD49e surface expression (Peng et al., 2013, J Clin Invest, Aw Yeang et al., 2017, J Immunol).

Therefore, we set out to investigate whether the pig may harbour lrNK cells and, if so, whether these phenotypically resemble either human or mouse lrNK cells. Porcine NK cells are characterized by their CD3-CD8a+ expression profile (Talker et al., 2013, Dev Comp Immunol). Porcine liver leukocytes were obtained by flushing the liver, as described for human liver leukocytes (Aw Yeang et al., 2017, J Immunol). In comparison with porcine PBMC, which only contained conventional CD3-CD8a+ NK cells, porcine liver leukocytes contained conventional NK cells and a very substantial additional CD3-CD8adim NK population. FACS isolation of the different NK populations showed that blood NK cells and conventional NK cells in porcine liver were Eomes-low,T-bet-high whereas the CD3-CD8adim NK population showed a Eomes-high,T-bet-low profile, similar to human lrNK cells. In addition and further similar to human lrNK cells, the porcine Eomes-high,T-bet-low NK population was negative for CD49e (whereas blood NK and conventional liver NK cells were CD49e-positive) and showed strong expression of CXCR6 and NKp46. Cytolytic assays showed that, like their human counterpart, porcine Eomes-high,T-bet-low liver NK cells showed little cytotoxic activity against K562 cells (and also against herpesvirus-infected cells), but could be triggered to degranulate and had the ability to produce IFNγ upon cytokine stimulation.

In summary, we identified a porcine liver NK cell population that shows strong similarity to human liver resident NK cells. Therefore, the pig may represent a unique model to study the characteristics and function of this particular cell population in health and disease.

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IDENTIFICATION OF PLZFHICD56BRIGHT NK CELLS IN HUMAN PERIPHERAL BLOOD SHARING PHENOTYPICAL AND FUNCTIONAL CHARACTERISTICS WITH LIVER-RESIDENT NK CELLS

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INTRODUCTION
In mice, the promyelocytic leukemia zinc finger protein (PLZF) is a key transcription factor for the development of liverresident NK (lrNK) cells and ILC1s. Human lrNK cells have been identified by their transcription factor profile to be EomeshiT-betlo and the expression of the surface molecules CXCR6, CD69 and CD56bright. However, the contribution of PLZF to the mechanisms regulating tissue-homing and tissue-residency of human NK cells remains unknown.

OBJECTIVES
The main objective of this study was to determine the association of PLZF with tissue-residency and characterize its expression in matched human peripheral blood-derived NK cells (pbNK cells) and intrahepatic NK cells (ihNK cells).

MATERIALS & METHODS
We obtained matched liver tissue and peripheral blood from individuals undergoing resection of metastases or liver transplantation. Samples were freshly phenotypically characterized using multiparameter flow cytometry. Functional response of ihNK and pbNK cells to K562 cells was measured by Granzyme K/B, IFN-γ- and TNF-α-production as well as degranulation (CD107a-expression) and compared to unstimulated cells.

RESULTS
CD56bright ihNK cells exhibited high expression of PLZF, while the majority of CD56bright pbNK cells expressed low levels of PLZF. CXCR6+CD69+CD56bright lrNK cells had a the highest expression of PLZF, compared to other subsets of CD56bright ihNK cells Remarkably, a small subset of CXCR6+CD69+CD56bright pbNK cells also expressed high levels of PLZF and shared functional characteristics with lrNK cells by exhibiting low expression of granzyme B and perforin and high expression of granzyme K and a poor IFN-γ- and TNF-α-response to K562 stimulation. However, CXCR6+CD69+CD-56bright pbNK cells did not display the typical liver-residency EomeshiTbetlo profile.

CONCLUSION
Our data indicate the involvement of PLZF in the regulation of liver-residency in human NK cells. PLZFhiCD56bright pbNK cells expressing liver-homing receptors might be part of circulating NK cells recruited to the liver, furthermore facilitating with the replenishment of lrNK cells.

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LIMITED RECIRCULATION OF CYTOTOXIC NK CELLS DURING NORMAL HOMEOSTASIS IN HUMANS

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Natural killer (NK) cells are well known for their cytolytic functions to directly kill virus-infected and malignant cells. Two major NK cell populations exist in humans: Cytotoxic CD56dim and immunoregulatory CD56bright NK cells. Although NK cells are distributed throughout the human body, little is known about which NK cell populations that traffic tissues and egress from tissues to recirculate back to blood. Here, we set out to determine the recirculation patterns of human NK cell subsets during normal homeostasis through access to multiple types of tissues, blood, and thoracic duct lymph (TDL). Whereas cytotoxic CD56dim NK cells were dominant in peripheral blood, non-lymphoid tissues (liver, uterus, duodenum, visceral adipose tissue) were enriched for immunoregulatory CD56bright NK cells expressing markers of tissue-residency. In line with this, only few cytotoxic CD56dim NK cells were present in secondary lymphoid organs and efferent lymph (TDL). Instead, afferent and efferent venous blood of human liver were similar in NK cell subset composition as peripheral blood, suggesting that cytotoxic CD56dim NK cells are retained in vasculature during steady-state. Finally, splenectomy led to lower expression of NK cell effector molecules suggesting a potential role for the spleen in arming NK cells for cellular cytotoxicity. Ongoing mechanistic efforts include assessment of NK cell subsets in patients before and after blocking cellular egress from tissues with FTY-720 (S1PR agonist) treatment. These results offer a revised model for NK cell subset trafficking behavior and suggests that cytotoxic CD56dim NK cells have limited access to peripheral tissues during normal homeostasis in human.

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LIVER-DERIVED TGF-β MAINTAINS THE EOMESHITBETLO PHENOTYPE OF LIVER RESIDENT NATURAL KILLER CELLS

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The human liver contains a diverse repertoire of tissue-resident immune cells, including large numbers of resident natural killer (NK) cells. Liver resident NK cells are CD56bright NK cells defined by a unique expression profile of transcription factors and cell surface markers (EomeshiTbetloCD69+CXCR6+CD49e-). Despite extensive characterisation of the phenotype of liver resident NK cells, it remains unclear how factors within the liver microenvironment induce and maintain this unique phenotype. To explore the phenotypic properties of these liverresident NK cells, we performed transcriptomics on sorted NK cell subpopulations from liver perfusate (n=5) obtained during liver transplantation. Gene set enrichment analysis identified a significant up-regulation of genes associated with tissue-residence of memory T cells, NKT and NK cells (NES = 1.67, FDR = 0.009) when compared with peripheral blood NK cells. Network analysis of the genes up-regulated in liver-resident NK cells versus peripheral CD56bright NK cells identified activation of the TGF-β signalling pathway (P < 0.001, FDR = 0.025), indicating a role for this cytokine in the induction of the liver-resident phenotype. Removal of healthy liver resident NK cells from donor liver perfusate and in vitro culture results in the gradual loss of the characteristic EomeshiTbetlo phenotype. This phenotypic loss could be halted through the dose-dependent addition of liver conditioned media (LCM), generated from the ex vivo culture of liver biopsies from healthy organ donors or recombinant TGF-β, but not IL-10 treatment. Blocking TGF-β receptor signalling using the inhibitor SB431542 or TGF-β1 blocking antibody, reversed the effect of LCM treatment on hepatic and peripheral NK cells. Our results describe the unique transcriptional landscape of liverresident CD56bright NK cells, and highlight the importance of TGF-β signalling in the induction and maintenance of this tissue-resident phenotype.

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MAPPING HUMAN UTERINE NK CELL DIFFERENTIATION IN SITU

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Uterine NK cells (uNK) cells are abundant endometrial and decidua immune cells that exhibit an important regulatory role at the maternal-fetal interface during pregnancy where they interact with fetal trophoblasts. However, uNK cells have for long been considered a homogeneous population of immune cells retaining fixed properties throughout their lifespan. This is distinct from circulating NK cells that first mature from CD56bright to CD56dim NK cells and then continuously specialize within the CD56dim NK cell compartment. A recent single-cell reconstruction of the human maternal-fetal interface suggested the presence of distinct subsets of uNK cells. Here, we aimed to perform a functional validation of this proposed heterogeneity, in space and time. First, a surface proteome screening of human decidual and endometrial-derived uNK cells as compared to conventional circulating NK cells was performed and integrated with transcriptomic data. The uNK cell compartment displayed vast heterogeneity distinct from circulating NK cells. Furthermore, a suggested linear differentiation process with stepwise acquisition of KIRs and CD39 was identified. This process occurred in situ in response to endometrial regeneration and subsequent decidualization but was absent post menopause. Furthermore, this process was dependent on cellular proliferation, could be recapitulated experimentally both in vitro and in vivo, and was associated with a distinct functional stratification of uNK cells. The importance of uNK cell differentiation in relation to maternal-fetal tolerance is currently being investigated. Taken together, our results identify a uNK cell differentiation pathway taking place in situ during endometrial regeneration.

KEY WORDS: tissue immunology, human immunology, uterine NK cells, immune cell differentiation, maternal-fetal interface.

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PULMONARY NATURAL KILLER CELLS CONTROL NEUTROPHIL INTRAVASCULAR MOTILITY AND RESPONSE TO ACUTE INFLAMMATION

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The lung has a unique structure allowing gas-exchange that requires an exceptionally thin barrier between blood andvair. That also makes it an ideal entrance point for many pathogens. Neutrophils, the most abundant immune cell in thevpulmonary vasculature, are critical to lung immunity yet excessive and sustained mobilization of neutrophils is avhallmark of several chronic inflammatory lung disorders. Natural Killer cells are implicated in immunological responses within the lung but relatively little is known about their localization, motility, or the specific mechanisms by which they contribute to local homeostasis. We interrogated the role of pulmonary NK cells in the regulation of neutrophils using lung-intravital microscopy in steady state mice and in response to inflammation.

This approach revealed that nearly all NK cells in the lung are intravascular showing an unexpected sessile behaviour as they remained stationary for extended periods of time within alveolar capillaries, with some cells remaining motionless for at least 60 minutes.

While neutrophils are able to move quickly, NK cells and neutrophils frequently interact for 5-10 minutes in capillaries. Interestingly, upon interaction with NK cells, neutrophils scanned the endothelium more slowly over larger distances.

NK cell depletion resulted in marked increase in neutrophil dynamics such as track length and duration (48.77±6 to 112.2±15µm and 2.50±0.3 to 4.77±0.7min respectively). Also, we observed an accumulation of neutrophils in LPSchallenged mice depleted of NK cells compared to control naïve mice. This finding represents a previously unappreciated behaviour and immunoregulatory function of natural killer cells in lung homeostasis by limiting potentially pathogenic neutrophil accumulation.

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THE HETEROGENEITY OF LIVER-RESIDENT NK CELLS REVEALED BY SINGLE-CELL RNA-SEQ

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Liver NK cells are heterogeneously composed of two distinct subsets, conventional NK (cNK) cells and liver-resident NK (LrNK) cells. Recent studies have reported that LrNK cells are able to mediate hapten-specific immune memory responses, confer the early protection of viral infection, regulate antiviral T cells that underlie liver tolerance and promote tumor immune evasion. Considering the diverse functions of LrNK cells, we reasoned that different subpopulations with specific phenotype and functions may exist in LrNK cells.

Here, we delineated the heterogeneity of mouse LrNK cells through single-cell RNA-seq of thousands of purified liver CD3-CD19-NK1.1+ NK cells. Unbiased transcriptional clustering revealed five distinct populations, corresponding to three cNK cell clusters with different maturation stages and two transcriptionally and functionally distinct subpopulations of LrNK cells. On the basis of these findings, we further confirmed that LrNK cells could be categorized into two subsets. These two subsets were distinct in terms of transcriptome profiles, phenotypes and functions. Specifically, one subset marked by higher expression of CD69, CD11c, EpCam and Gzms was highly cytotoxic, which was confirmed by a flow-based killing assay in vitro. In contrast, the other subset displayed weak cytotoxic functions, but it was enriched in genes encoding NK cell memory associated molecules, such as CD127, Thy1.2 and CD62L, raising a possibility that this subset might be the key player in LrNK-mediated memory responses.

Furthermore, there was no conversion between these two subsets indicating that they were both stable and mature subpopulations during homeostasis. Our results indicate that LrNK cells could be further divided into two distinct subsets with different functions.

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THE ROLE OF CHECKPOINT MOLECULES IN HUMAN INTRAHEPATIC NK CELLS

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INTRODUCTION
While NK cells have been shown to be involved in early control of hepatitis virus infections and protection against HCC, they have also been implicated in causing liver damage. The mechanisms by which the liver instructs a tolerogenic environment and controls NK cell function during steady state conditions is yet incompletely understood.

OBJECTIVES
The aim of this study was to investigate the role of checkpoint molecules in controlling the function of intrahepatic (ih) NK cells.

PATIENTS & METHODS
Peripheral blood (pb) and matched liver samples from individuals undergoing liver transplantation due to end stage liver diseases (n=12) and liver resection due to liver metastases or adenoma (n=8) as well as PBMC samples from healthy control individuals were used for this study. We performed detailed analysis of pb- and ih- derived NK cells in those samples using flow cytometry as well as single cell qPCR on flow-sorted NK cells. Additionally, we performed co-culture experiments with pbNK cells, isolated from healthy control individuals, with Huh7 cells, hepato cellular carcinoma cells, to study the inducibility of checkpoint molecules in vitro.

RESULTS
In line with previous results, the intrahepatic (ih) lymphocyte compartment was enriched for NK cells with a shift towards higher proportions of CD56bright NK cells, expressing markers of liver residency (CXCR6 and CD69). In addition, ihNK cells expressed a distinct pattern of checkpoint molecules, characterized by higher levels of TIGIT, TGFBR1, TGFBR2 (all $p<0.0001$), PD-1 ($p=0.0004$) and lower levels of DNAM-1, IL7R (all $p<0.0001$) as well as CD96 ($p=0.007$) compared to pbNK cells. Furthermore, liver-resident NK cells expressed higher levels of TIGIT, CD96 (all $p=0.005$), TGFBR2 ($p=0.02$), IL7R ($p=0.01$) and lower levels of DNAM-1 ($p=0.005$). These results were independent of the underlying liver disease and also observed in tumor-free liver resection samples. Furthermore, co-culture experiments with Huh7 cells led to a decline of DNAM-1 expression on NK cells, while other checkpoint molecules remained stable.

CONCLUSION
Taken together, our data show that peripheral, intrahepatic and liver-resident NK cells expressed distinct patterns of checkpoint molecules, suggesting these molecules might contribute to a tissue-specific control of NK cell function. A better understanding of the mechanisms involved in the control of NK cell function in tissues might allow for future NK cell-based therapeutic intervention strategies.

SESSION 9
NK Cells and infection

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A LEOPARD CAN CHANGE ITS SPOTS: HOW HCMV GENETIC VARIABILITY IMPACTS VIRAL FITNESS AND NK CELL LIGANDS

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Human cytomegalovirus (HCMV) is the leading cause of congenital infection resulting in severe morbidity and mortality among newborns worldwide; however, mechanisms and virulence factors contributing to HCMV pathogenesis and clinical outcomes remain unclear. To successfully establish a persistent infection, the virus evolved multiple mechanisms to avoid host immune recognition, including numerous immunomodulatory proteins conferring resistance to NK cell killing. Furthermore, HCMV demonstrates an exceptionally high degree of variability, particularly in viral genes contributing to immune evasion, contradicting the expectation that, being a large double stranded DNA virus, it should maintain high genome stability.

Thus, our aim was to determine whether and to what extent HCMV genomic differences affect viral fitness and its ability to modulate NK cell responses.

We enrolled a cohort of 15 congenitally infected children, and evaluated the degree of genetic polymorphism of HCMV isolates by high-throughput sequencing and phylogenetic analyses. Viral genes known to encode proteins with potent NK cell immunomodulatory functions were deeply investigated. The results showed extraordinary genetic and phenotypic diversity of the clinical isolates, reflected in both viral growth properties and ability to modulate NK cells. Growth analysis of isolates in several cellular models revealed different patterns of replication and dissemination that we grouped into three categories ("aggressiveness" of the strain): fast-, intermediate-, and slow-replicating strains.

We then evaluated the genetic polymorphism degree of the isolates, primarily focusing on viral genes encoding for potent NK immunomodulatory proteins (UL16, UL18, UL40, UL141 and UL142, US18-21, US9), and we determined whether genetic differences influenced NK cell activating ligand expression. FACS analysis of MICA, MICB, ULBP1, ULBP2/5/6, ULBP3, PVR/CD155, B7-H6, as well as HLA-I molecules, demonstrated that HCMV clinical strains affect NK cell ligands to different degrees. PVR/CD155 was strongly up-regulated with fast-replicative isolates, in contrast to the down-regulation observed with the laboratory strain Merlin. Conversely, ULBP2/5/6 was down-regulated independently from the strain, while other NK cell ligands, such as MICA, MICB, ULBP3, Nectin-2/CD112, B7-H6, were not significantly modulated by any of the HCMV isolates. Finally, to assess whether HCMV isolates affect NK cell effector functions, we performed co-culture experiments and analyzed IFN-γ expression. We observed a great variation between the clinical strains, indicating that the genetic variability actually reflects variability in NK cell effector functions.

Overall, our study contributes to understanding the impact of HCMV genetic variability on viral fitness and modulation of the immune response.

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A VIRAL INHIBITION ASSAY TO ASSESS THE ANTI-VIRAL EFFECT OF DIFFERENT NK CELL POPULATIONS DURING HCV INFECTION

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INTRODUCTION
Natural killer (NK) cells are able to recognize virus-infected and tumor cells and have been described to be important for the early control of HCV infections. They can be divided into different subpopulations, which display distinct antiviral potential, by the surface expression of activating and inhibitory receptors that tightly regulate NK cell activity. Killer cell immunoglobulin-like receptors (KIRs) are one of the main NK cell receptor families and have been associated with the resolution of HCV infection. Previous studies suggested a protective effect of the activating NK cell receptor KIR3DS1 as well as the inhibitory KIR2DL3 on the outcome of HCV infection.

OBJECTIVES
The aim of this project was to establish an in vitro viral inhibition assay to assess the anti-viral activity of different NK cell populations during HCV infection.

METHODS
In order to study the anti-viral potential of different NK cell populations in HCV infection, a luciferase-based inhibition assay in a HCV cell culture system was established. Huh7 cells were infected with a Jc1 reporter virus carrying a gaussia luciferase. Primary human NK cells from donors homozygous or heterozygous for the respective KIR of interest were either sorted for surface expression of a specific KIR or used in bulk and subsequently characterized in regards to their ability to suppress HCV replication. Furthermore, KIR-Fc fusion constructs were used in order to boost viral inhibition by NK cells towards Jc1-infected Huh7 cells.

RESULTS
The HCV inhibition assay enabled to analyze the anti-viral potentials of different NK cell populations. NK cells from KIR3DS1/KIR3DS1 homozygous donors as well as sorted KIR3DS1+ NK cells were able to significantly suppress viral replication by activating the anti-viral potential against HCV-infected Huh7 cells. In contrast, we did not observe a similar anti-viral effect of KIR2DL3+ NK cells. In addition, co incubation of HCV-infected cells with KIR3DS1-Fc constructs also enabled KIR3DS1- NK cells to inhibit viral replication more efficiently.

CONCLUSION
Taken together, the luciferase-based HCV replication assay provides a valuable tool to study NK cell mediated viral inhibition in cell culture, enabling us to investigate the role of different NK cell populations in HCV infection in more detail. The study demonstrates a high potential of KIR3DS1+ NK cells to suppress viral replication in vitro, as well as the ability to modulate anti-viral responses through the application of KIR-Fc constructs.

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AN HIV-ENVELOPE SPECIFIC, IL-15 CONTAINING TRI-SPECIFIC KILLER ENGAGER (TRIKE) BOTH REACTIVATES AND DIRECTS NK CELL KILLING TOWARDS HIV-INFECTED T-CELLS

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While advancements in efficacy and use of anti-retroviral drugs have substantially ameliorated the health and longevity of HIV-infected individuals, these drugs are merely a stop-gap to prevent progression to AIDS and to limit further transmission of the virus. Despite the use of antiretrovirals to suppress HIV replication, infected individuals retain reservoirs of latently HIV-infected cells that, upon cessation of anti-retroviral therapy, could reactivate and reestablish an active infection. A curative solution necessitates the reactivation and subsequent destruction of these latently infected cells. The antibody response to HIV infection, while present, is generally ineffective due to the high rate of mutation of the virus which can rapidly eliminate epitopes recognized by the generated antibodies. However, in recent years a variety of HIV-specific antibodies have been identified in infected individuals which have strong neutralizing effects but a poor ability to elicit antibody dependent cell-mediated cytotoxicity (ADCC). Thus, we have designed bi- and tri-specific killer engagers (BiKE and TriKE) composed of a short-chain variable fragment derived from a broadly-neutralizing antibody (bnAb) against HIV-Env and a CD16 engager linked by an IL-15 molecule. The purpose of this tri-specific antibody construct is to utilize the broad specificity of these antibodies to target HIV while redirecting NK cell killing specifically to actively replicating infected cells though its recognition of membrane expressed Env and triggering NK cell degranulation though the low affinity Fc receptor, CD16. The addition of IL-15 as a linker should further activate NK cells thereby enhancing their response. IL-15 has also been identified as a potential reactivator of latently infected cells. Initial studies from our lab show enhanced NK cell cytokine production and killing of infected targets expressing HIV-Env when incubated with the HIV-specific constructs. PBMC from healthy donors incubated with the TriKE showed marked increases in immune cell activation in NK, CD4 and CD8 subsets, as well as inducing NK cell proliferation. Furthermore, IL-15, either monomeric or as part of the TriKE, demonstrates the ability to reactivate latently HIV-infected T-cells isolated from infected patients in vitro. A recent trial of an IL-15/IL-15Rα superagonist (Nant-803) in ART-treated HIV-infected patients also resulted in the detection of virus in the serum and immune activation. Together, these data indicate a potential role for an HIV-bnAb containing TriKE in the reactivation and elimination of the latently infected reservoir by harnessing NK cells ability to mediate ADCC.

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AN INNATE HELPING HAND FOR B CELLS ON THE MARGIN DURING CHRONIC VIRAL INFECTION

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Marginal zone B cells are an anatomically segregated population well-positioned to respond to blood borne pathogens and implicated in defense against encapsulated bacteria. These marginal zone B cells are also an important source of the anti-inflammatory cytokine, IL-10. As such, these cells are poised to prevent secondary bacterial infection or to limit immune pathology during chronic virus infection. Surprisingly, these marginal zone structures, including CD169+ macrophages and CD1d+CD21+CD23- B cells, were obliterated in mice chronically but not acutely infected with lymphocytic choriomeningitis virus (LCMV) in the absence of natural killer (NK) cells. As a result of marginal zone loss, these LCMV-infected mice became hypersusceptible to secondary infections with Streptococcus pneumoniae and Listeria monocytogenes. Immunoregulatory NK cells were previously demonstrated to kill activated CD4 T cells and indirectly suppress antiviral CD8 T cell responses in a CD4 T-cell dependent manner in the LCMV model, yet depletion of CD4 T cells did not restore the marginal zone in NK cell-deficient mice. Moreover, CD8 T cells are known to promote partial attrition of marginal zone B cells during chronic LCMV infection, but CD8 T-cell depletion only partially restored the marginal zone in infected, NK-cell deficient mice. Examination of Cd1d-deficient mice revealed that marginal zone decimation during virus infection in the absence of NK cells was also independent of NKT cells.

Importantly, neutralization of B-cell activating factor (BAFF, aka BLyS or Tnfsf13b) in NK-cell replete mice recapitulated the dramatic and selective loss of marginal zone B cells. During chronic LCMV infection, NK cells upregulate BAFF mRNA and protein expression, concomitant with virus-provoked loss of the prototypical sources of BAFF (e.g. fibroblastic reticular cells). As a result, splenic BAFF expression levels during LCMV infection are drastically reduced following depletion of NK cells. Thus, NK cells play an important role in curtailing harmful inflammation and preventing pathogenic bacterial diseases during chronic viral infection by producing BAFF to sustain the marginal zone. Of note, chronic infections with HIV or SIV are linked to both modulation of NK-cell function and depletion of marginal zone B cells. Therefore, this discovery has important clinical implications by highlighting a targetable mechanism to alleviate the burden of S. pneumoniae and other life-threatening secondary infections in post-viral pneumonia as well as in the context of AIDS.

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ANTIBODY-DEPENDENT NATURAL KILLER CELL ACTIVATION AFTER VACCINATION WITH THE 2-DOSE HETEROLOGOUS AD26.ZEBOV, MVA-BN-FILO EBOLA VACCINE REGIMEN

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BACKGROUND:
Antibody Fc-mediated functions such as antibody-dependent cellular cytotoxicity contribute to vaccineinduced protection against viral infections. Fc-mediated function of anti-Ebola glycoprotein antibodies suggest that Fc-dependent activation of effector cells, including NK cells, could play a role in vaccination against Ebola virus disease.

METHODS:
We analysed antibody-dependent NK cell responses in 75 U.K. based healthy adult (18-50 years of age) volunteers vaccinated in the phase 1 vaccine study EBL1001 (EBOVAC consortium, EU Innovative Medicines Initiative). The effect of anti-Ebola virus glycoprotein antibodies in the serum after vaccination with the novel 2-dose heterologous Ad26.ZEBOV (Ad26) followed by MVA-BN-Filo (MVA) with a 28 day or 56 day interval or the reverse directionality of these vectored vaccines with a 14, 28 or 56 day interval on primary human NK cell activation was tested (n=15 individuals per group). Degranulation (CD107a expression), CD16 downregulation and IFN-γ expression were used as readouts in flow cytometry based in vitro assays. The impact of NK cell differentiation on antibody-dependent activation was assessed in CD57 and NKG2C defined subsets.

RESULTS:
We demonstrate induction of CD107a and IFN-γ expression, combined with downregulation of CD16, in more differentiated NK cell subsets in response to in vitro stimulation with Ebola virus glycoprotein combined with post-dose 1 and post-dose 2 serum. These responses varied significantly with both MVA-Ad26 directionality and interval between vaccine doses. Using a uniform responder cell preparation, NK cell activation was found to be correlated with anti-glycoprotein antibody concentration, suggesting at least partial dependence on antibody concentration for optimal NK cell activation. Stimulation of NK cells from multiple individual donors with a standardised antigen/immune serum cocktail, revealed the impact of NK cell differentiation phenotype on antibody-dependent NK cell activation, with highly differentiated CD56dimCD57+ NK cells being the most responsive.

CONCLUSIONS:
This study thus highlights the dual importance of vaccine-induced anti-glycoprotein antibody concentration and NK cell differentiation status in promoting Fc-mediated activation of NK cells after vaccination with the 2 dose heterologous Ad26.ZEBOV, MVA-BN-Filo Ebola vaccine regimen.

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CD300A REDUCES NK CELL DEGRANULATION AND CYTOKINE PRODUCTION IN HIV-1 INFECTED PATIENTS

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INTRODUCTION:
CD300a is an inhibitory receptor expressed on lymphoid and myeloid cells. CD300a has the capacity to decrease NK cell-mediated killing of tumor cells and pseudorabies virus-infected cells through the interaction between the receptor and its ligands phosphatidylserine (PS) and phosphatidylethanolamine (PE). We have previously described that CD300a expression is modulated by HIV-1 infection on B cells and CD4+ T lymphocytes. Nevertheless, its expression and function on NK cells during HIV infection is still unknown.

METHODS:
Donors samples were obtained from HIV Biobank and clinical data from CoRIS. Using multiparametric flow cytometry, CD300a expression was determined on NK cell subsets from healthy donors (n = 13), untreated HIV-1 infected subjects (n = 14) and patients under combined antiretroviral therapy (cART) (n = 13). Redirected lysis assays were carried out with the P815 cell line as a target. Anti-CD16 monoclonal antibodies (mAb) were used to activate NK cells and anti-CD300a mAb were added to determine the inhibitory capacity of the receptor. The ability of NK cells to degranulate (CD107a) and produce cytokines (IFNγ, TNF and MIP1β) was analyzed.

RESULTS:
Different CD300a expression levels were associated to the expression of other NK cell markers such as CD57, NKG2A, NKG2C or NKp46 in all subjects. A higher percentage of CD300a+ cells was found in the CD56- NK cell subset from untreated HIV-1 infected patients than in healthy donors. Moreover, we demonstrated that CD300a reduces the capacity of NK cells to degranulate and produce cytokines. Importantly, we observed a higher percentage of CD300a-mediated inhibition in NK cells from HIV-1 infected patients.

CONCLUSION:
CD300a down-regulates CD16-mediated NK cell activation in healthy and HIV-1 infected subjects, mainly in patients under cART.

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CONTRIBUTION OF ADAPTIVE NKG2C+ NK CELLS TO THE CONTROL OF CYTOMEGALOVIRUS INFECTION IN KIDNEY TRANSPLANT RECIPIENTS

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Human cytomegalovirus (HCMV) infection has been related to an increased risk of graft loss and reduced survival in immunosuppressed kidney transplant recipients (KTR). HCMV infection promotes to a variable extent a reconfiguration of the NK cell compartment characterized by the adaptive differentiation and persistent expansion of an NKG2C+ NKG2A- NK cell subset. In addition to increased NKG2C expression levels, functionally mature adaptive NK cells develop other characteristic phenotypic and functional features, efficiently mediating specific antibodydependent effector functions.

In a previous report [1], pre-transplant NKG2C+ NK cells were associated with a reduced incidence of post-transplant HCMV viremia in KTR. The present study was carried out in cryopreserved peripheral blood mononuclear cells from the expanded KTR cohort (n=145) with homogeneous immunosuppression (tacrolimus, mycophenolate acid and steroids); cases receiving thymoglobulin, antiviral prophylaxis or with low risk of infection (D-R-) were excluded. A more detailed phenotypic profiling of NKG2C+ adaptive NK cells (i.e. NKG2A, CD57, ILT2 expression; FcεRIγ chain and PLZF loss) was carried out, and t-SNE analysis was performed on manually gated NK cells. In addition, NKG2C expression in T cells, including the Vδ2- subset involved in the response to HCMV, was also assessed. In parallel, the relation of NKG2C+ NK cells with T cells (CD4+ and CD8+) specific for HCMV immunodominant antigens (i.e. IE1 and pp65) was analyzed in samples from a subset of pre-transplant patients (n=29) and healthy controls (n=27).

A significant relation was observed between proportions of NKG2C+ NK cells co-expressing other adaptive markers and a reduced incidence of post-transplant HCMV viremia; no relation with NKG2C+ and Vδ2- T cells was detected (Wilcoxon). The association with NKG2C+ NKG2A- and NKG2C+ CD57+ NK cell subsets was confirmed by multivariate Cox regression analysis, including receptor age. No correlation between the proportions of NKG2C+ NK cells and T cells specific for HCMV antigens was observed. These results support that adaptive NKG2C+ NK cells contribute to the control of HCMV in KTR, together with the established role of T cells.

[1] Redondo-Pachón et al. 2017 J Immunol; 198:94-101

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DELINEATING THE ROLE OF FAM13A1 IN NATURAL KILLER CELLS

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Accumulative evidence from genome wide association studies (GWAS) reveals that family with sequence similarity 13, member A (FAM13A) gene is strongly associated with multiple complex diseases, such as chronic obstructive pulmonary disease (COPD), non-small cell lung cancer (NSCLC), asthma, pulmonary fibrosis, diabetes and others.

However, the molecular and cellular functions of FAM13A still largely remain elusive. Since the immune system is involved in most of these diseases that FAM13A is known to be associated with and our network-based computational prediction using gene correlation networks indicated that FAM13A might play a role in regulating the immune functions, we explored the immune functions of FAM13A. Here we observed that FAM13A1 depletion impaired the interferon gamma (INFG) production by NK cells in vitro. More strikingly, the lung metastasis induced by B16F10 melanoma cells in the FAM13A-knockout (KO) mice were more deteriorated compared with that in wild type (WT) controls. In the meantime, the in vivo murine cytomegalovirus (MCMV) infection data showed that FAM13A-KO mice had lower percentage of activated NK cells after the infection compared with the WT littermates. Our mouse RNA-Seq data indicated that FAM13A might modulate the Rho and Ras family signalling pathway, which might be one of the mechanisms through which FAM13A regulates cytokine production of NK cells. Altogether, our results demonstrate that loss of FAM13A leads to the dysfunction of NK cells. Our work provides important insights into the role of FAM13A in modulating NK-mediated immunosurveillance, which might consequently contribute to the pathogenesis of the diseases in which FAM13A is involved.

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DENGUE INDUCES THE EXPRESSION OF DNAM-1 AND NKG2D LIGANDS: POSSIBLE IMMUNE ESCAPE STRATEGY?

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Natural Killer (NK) cells are lymphocytes of the immune system whose activation during viral infection is dependent on the balance of the inhibitory and activating signals they receive from their various germline-encoded receptors. Once activated, NK cells not only participate in the initial anti-viral response but also influence the adaptive immune response through cytokine secretion. Dengue virus (DENV) is the most prevalent arbovirus in the world; prior studies have demonstrated that NK cells are activated during DENV infection. To identify the receptor-ligand interactions mediating this activation, we used cytometry by time of flight (CyTOF) to profile the expression of NK cell receptors and their known ligands in a cohort of acutely DENV-infected patients. Our analysis showed increased NK cell expression of perforin, a molecule associated with killing potential, in patients compared to healthy controls. Acute DENV infection is associated with significant alterations in the expression of ligands for the NK cell activating receptors NKG2D and DNAM-1, with an increase in expression of MICA/B, decreased expression of ULBP-1,2,5,6, and decreased expression of PVR (CD155) on monocytes. These data suggest a critical role for these activating receptors in the recognition of DENV-infected cells, particularly in light of prior data indicating that specific alleles of MICA and MICB are associated with disease progression. Finally, we show that DENV infection induces the soluble form of NKG2D ligands, MICA and MICB, but not soluble ULBP-1,2,3, in the sera of DENV-positive pediatric patients, as well as during in vitro DENV-infection of monocyte-derived immature dendritic cells (imDCs). Soluble ligands have been described in the context of cancer and have been shown to inhibit the NK cell response by competing with surface ligands and inducing the internalisation of the NKG2D receptor. This suggests a potential escape mechanism by which DENV evades the NK cell response. Future studies are necessary to determine the mechanism of DENV-mediated induction of the soluble form of the NKG2D ligands.

EARLY DONOR NK CELLS INFUSION A STRATEGY TO PREVENT HHV6 ENCEPHALITIS IN PEDIATRIC PATIENTS AFTER CD45RA-DEPLETED TRANSPLANTATION

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BACKGROUND
HHV-6 reactivation has been reported in 30-70% of patients after hematopoietic stem cell transplantation (HSCT) especially at engraftment and during the first month. Delayed immune reconstitution (IR) is a risk factor to develop HHV-6 encephalitis. The incidence of this complication is low (0.95 to 11.6%). We previously reported a unexpected 34% cumulative incidence of HHV-6 encephalitis in a serie of 25 pediatric patients receiving haploidentical HSCT with CD45RA-depletion. Simillar data were reported in a serie of 38 pediatric HSCT enrichment in CD4+ T cells and without Natural Killer cells in the graft. Because NK cells act against infected and transformed cells as part of innate immunity we demonstrated how in vitro co-culturing a 2:1 ratio of donor CD4+ T cells with CD56+ cells eliminated the virus, demonstrating the main role of donor NK cells in the antiviral immune response.

OBJECTIVES
NK cells infusion in could provide functional cells to protect against infections and to control HHV-6-infected cells in peripheral blood. We present our experience with NK cells infusion at day +7 post-HSCT after CD45RA-depleted transplantation to assess safety and infections rate focusing on HHV-6 reactivations and encephalitis.

METHODS.
A total of 10 patients received NK cells infusion on day +7 after CD45RA-depleted HSCT after nonmyeloablative conditioning. NK product was obtained performing CD3 depletion on donor non-mobilized leukapheresis product followed by CD56 enrichment using the CliniMACS® device. Protocol also included donor CD3+CD45RO+ lymphocyte infusion on a prophylactic regime on days +30, +60 and +90 (1x107/Kg) to boost immune reconstitution.

RESULTS
Ten pediatric patients median age 6 years (range 1-15) received CD45RA-depleted grafts from haploidentical (7) and match related donor (3). NK cells were infused on day +7 post-HSCT with a median dose of 1,05x107/Kg (range 1x106/Kg-1x108/Kg). There was only one infusion reaction. Patients presented full donor chimerism and were on complete remission at last follow up. Three patients presented acute GvHD≥ grade 2. There were 1 CMV and 1.

ADENOVIRUS REACTIVATION
Two patients presented Parvovirus infection. HHV-6 reactivation occurred in 2 patients, one of them was complicated with enteritis. No HHV-6 encephalitis was seen (0%).

CONCLUSIONS
Our preliminary data suggest that infusions of NK in the early post-HSCT (day +7) are a safe adoptive immunotherapy strategy to prevent HHV-6 encephalitis and other viral reactivations after CD45RA-depleted transplantation. However, to determine the real efficacy of this strategy, a larger number of patients and prospective studies are required.

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EXPRESSION OF THE GB GLYCOPROTEIN OF AN ALPHAHERPESVIRUS TRIGGERS NK CELL CYTOTOXICITY AND INCREASES BINDING OF THE ACTIVATING RECEPTOR PILRBETA

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Alphaherpesviruses represent the largest subfamily of the herpesviruses and include important pathogens of man and animal. These include e.g. herpes simplex virus (HSV) and varicella-zoster virus in man, pseudorabies virus (PRV) in pigs, bovine herpesvirus 1 in cattle and equine herpesvirus 1 in horses. Aggravated and sometimes life-threatening alphaherpesvirus-induced disease has been reported in patients with NK cell deficiencies.

We earlier identified the first known mechanisms how some alphaherpesviruses suppress cytotoxic activity by NK cells. These mechanisms include viral glycoprotein gD-mediated downregulation of the DNAM-1 ligand CD112 and viral kinase US3-induced upregulation of ligands for the inhibitory NK cell receptor CD300a (Grauwet et al., 2014, PNAS, Grauwet et al., 2016, J Virol).

We and others also showed that, despite these viral NK evasion mechanisms, NK cells are still able to recognize and kill alphaherpesvirus-infected cells.

In the current study, using the porcine alphaherpesvirus PRV and porcine NK cells as a model, we identified the conserved alphaherpesvirus gB glycoprotein as an important factor in activating NK cell activity.

We found that expression of the gB glycoprotein triggers NK cell-mediated cytotoxicity, both in PRV-infected and in gB-transfected cells. In addition, we report that, like their human and murine counterpart, porcine NK cells express the activating receptor paired immunoglobulin-like type 2 receptor beta (PILRbeta). We found that gB expression in PRV-infected or gB-transfected cells increases binding of recombinant porcine PILRbeta to the cell surface. The identification of gB as an NK cell activating protein may affect strategies to prevent or treat alphaherpesvirus infections.

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HIGHLY ACTIVATED NATURAL KILLER CELLS CONTRIBUTE TO THE DEFENSE AGAINST VIRAL RESPIRATORY INFECTIONS AFTER HAEMATOPOIETIC STEM CELL TRANSPLANTATION IN CHILDREN

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BACKGROUND:
Infection is the leading cause of non-relapse mortality after allogeneic haematopoietic stem cell transplantation (HSCT). Modified functions of immune cells in nasal secretions may play a role in post HSCT susceptibility to respiratory viral infections. We characterized immune cells in nasopharyngeal aspirate (NPAs) by flow cytometry in HSCT recipients along time and correlated it with the detection of respiratory viruses in the same samples.

METHODS:
In a prospective observational study, we determined T and NK cell number together with NK activation status in healthy controls and HSCT recipients by flow cytometry. We also determined by polymerase chain reaction (PCR) the presence of 16 respiratory viruses. Samples were collected pre HSCT, at day 0, +10, +20 and +30 after HSCT. Peripheral blood from the same patients and controls was also analyzed to determine T and NK cell numbers.

RESULTS:
A tptal of 27 HSCT recipients were enrolled and 16 had at least one viral detection in a NPA (60%). Rhinovirus was the most frequent pathogen (84% of positive NPAs). NPAs of patients contained fewer T and NK cells as compared with healthy controls (p=0.0074 and p=0.0216, respectively). Viral PCR+ patients showed higher NK cell numbers in their NPAs. Additionally, the activating receptors repertoire expressed by NK cells was higher in NPA samples than in peripheral blood samples, especially for NKp44 and NKp46 receptors. The outcome of HSCT recipients was not significantly correlated with the detection of respiratory viruses.

CONCLUSIONS:
Our study supports NK cells relevance for the immune defense against virus in the respiratory tract after an HSCT.

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HLA UPREGULATION DURING DENGUE VIRUS INFECTION SUPPRESSES THE NATURAL KILLER CELL RESPONSE

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Dengue virus (DENV) is the most prevalent mosquito-borne virus in the world and a major cause of morbidity in the tropics and subtropics. Upregulation of HLA class I molecules has long been considered a feature of DENV infection, yet this has not been evaluated in the setting of natural, human infection. Natural killer (NK) cells, an innate immune cell subset critical for mounting an early response to viral infection, are inhibited by self HLA class I, suggesting that upregulation of HLA class I during DENV infection could dampen the NK cell response. Here we addressed whether upregulation of HLA class I molecules occurs during in vivo DENV infection and, if so, whether this suppresses the NK cell response. We found that HLA class I expression was indeed upregulated during acute DENV infection across multiple cells lineages in vivo. To better understand the role of HLA class I upregulation, we infected primary human monocytes, a major target of DENV infection, in vitro. Upregulation of total HLA class I is dependent on active viral replication and is mediated largely by cytokines and other soluble factors induced by DENV infection, while upregulation of HLA-E occurs in the presence of UV-inactivated, replication-incompetent virus. Importantly, blocking DENV-infected monocytes with a pan-HLA class I Fab nearly doubles the frequency of degranulating NK cells, while blocking HLA-E does not significantly improve the NK cell response. These findings demonstrate that upregulation of HLA class I during DENV infection suppresses the NK cell response, potentially contributing to disease pathogenesis.

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HUMAN CYTOMEGALOVIRUS-INDUCED NK CELLS EXPRESS CD3 COMPLEX FOR ENHANCED ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY EFFECTOR FUNCTION

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Human natural killer (NK) cells are CD3-CD56+ lymphocytes that provide a rapid cytokine and cytotoxic response to virus-infected cells and tumor cells. Association of the gene-rearranged, antigen-specific T-cell receptor (TCR) with the CD3 co-receptor complex is critical for the development and functionality of each T cell. In contrast to T cells, which rely on gene rearrangement for receptor specificity, NK cells express an array of cell surface stimulatory and inhibitory receptors that are all germline-encoded.

HCMV infection expands in approximately 30-40% of exposed individuals an CD56dimCD57+NKG2C+NK cell population. By using integrated transcriptomic and epigenomic profiling HLA-E-recognizing NK populations from the same HCMV-infected individuals, we found HCMV-induced NK cells have distinct epigenetic signatures. CD3E, encodes CD3 epsilon, was prominent among the genes displaying increased accessibility within the promoter region.

We found CD3+ natural killer (NK) cells in healthy adults and umbilical cord blood transplantation recipients are induced by human cytomegalovirus (HCMV) infection. They express intracellular CD3ε, γ and δ. CD3+ NK cells were found with a mean frequency of 6.83% within the CD56dim population among 105 HCMV seropositive healthy donors (range 0.11% to 68%) compared to 2% among 48 HCMV seronegative donors (range 0.12% to 6.42%). CD3 positivity marks a distinct subset of human NK cells that exhibits the highest capacity for antibody-dependent function (ADCC).

Mechanistically, CD3+ NK cells express more CD247, which complexes with CD3ε, leading to an enhanced ADCC response. CD3+ NK cells selectively expand against HCMV-infected cells in the presence of HCMV-specific antibodies. In addition, Notch pathway signaling stabilizes CD3ε expression in CD3+ NK cells. Our findings indicate that HCMV infection epigenetically promotes CD3 complex expression among educated natural killer cells, revealing a potential clinical utility of CD3+ NK cells as adoptive cell therapy in antibody-based treatments

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HYPOXIA ENHANCES CYTOKINE-INDUCED NK CELL PRODUCTION OF IFNγ

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Natural Killer (NK) cells are acute responders to pathogen infection and potent mediators of inflammation through production of cytokines such as its signature cytokine, interferon-gamma (IFNγ). At inflammatory sites, activation of local and responding cells through receptor-ligand interactions requires an enormous amount of energy that is supplied through metabolic pathways that results in decreased ambient oxygen, creating a hypoxic milieu. Here we sought to determine how hypoxia affects NK cell responses after cytokine activation. We found that cytokines activated NK cells produce significantly higher amounts of IFNγ under hypoxic conditions compared to normal cell culture conditions however, plate-bound anti-NK1 stimulation did not have the same effect. The hypoxia-enhanced IFNγ production was a global effect occurring in all NK cells regardless of maturation state and effector status. The expression of IFNγ promoting transcription factors Tbet and Eomes was not altered during hypoxic stimulation, and Tbet was found to be dispensable for this affect. Furthermore, NFIL3-mTOR axis does not play a role in the increase of IFNγ response in NK cells. Strikingly, inhibitors of hypoxia-induced transcription factors (HIFs), HIF1α and HIF2α, showed that HIF1α is critical for the IFNγ response but this effect was primarily mediated through its impact on myeloid cells not NK cells. Furthermore, cytokine stimulated enriched NK cells alone did not show increased IFNγ production under hypoxic stimulation. Using depleting antibody for Gr-1+ positive cells, we showed this myeloid subset was required for enhanced IFNγ response in NK cells. Thus, hypoxia enhances cytokine-induced IFNγ production in NK cells through Gr-1+ myeloid cells.

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IMPAIRED RESPONSE TO ASPERGILLUS FUMIGATUS OF NK CELLS AFTER ALLOGENEIC STEM CELL TRANSPLANTATION BECAUSE OF ACTIN DYSFUNCTION AND CORTICOSTEROID THERAPY

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INTRODUCTION: Aspergillus (A.) fumigatus is a saprophytic mold, which can cause invasive aspergillosis (IA) in immuno-com-promised hosts. Stuehler et al. showed that patients after allogeneic stem cell transplantation (alloSCT) have a higher risk to develop IA when NK cell counts are lower and NK cell reconstitution time takes longer (Stuehler et al., 2015). Current NK cell models postulate dynamic NK cell movements between the human blood and the lung (Marquardt et al., 2017) which enables NK cell interactions with A. fumigatus in both locations of the hu-man body. We demonstrated in former studies that the neuronal cell adhesion molecule 1 (NCAM-1, CD56) mediates the interaction between NK cells and A. fumigatus hyphae in vitro, leading to NK cell activation and secretion of CC-chemokine ligands CCL3, 4, and 5 (Ziegler, Weiss, et al., 2017).

OBJECTIVES: We functionally characterized the interaction of NK cells isolated from alloSCT patients (60, 90, 120, and 180 days post transplantation) and healthy individuals with A. fumigatus hyphae in vitro.

MATERIAL & METHODS: NK cells were isolated by negative isolation from whole blood (Miltenyi Biotec). Cells were pre-stimulated with Proleukin (Novartis) overnight before NK – A. fumigatus co-cultures were set for 6 h. Cell culture supernatants were analyzed by multiplex immunoassays and analysis of surface marker expression was performed by flow cytometry. Actin dynamics were visualized by Structured Illumination Microscopy (SIM) and were further ana-lyzed by live cell staining and flow cytometry.

RESULTS: NK cell counts rapidly recover after alloSCT, however, anomalous NK cell subset distribution (CD56brightCD16- and CD56dimCD16+) prolongs for several months. While CD16 expression normalizes after 180 d post alloSCT, CD56 expression remains higher in patients compared to healthy individuals, leading to a more CD56bright phe-notype. NK cells obtained after alloSCT displayed functional deficiencies regarding cytotoxic release and chemokine secretion after fungal co-culture. Interestingly, CD56 binding to the fungus was inhibited when blood was collected during corticosteroid treatment, concluding reduced fungal recognition by NK cells. Fur-thermore, fungal mediated actin polymerization was inhibited in alloSCT patients compared to healthy individ-uals and normalized 180 d post alloSCT.

CONCLUSION: We hypothesize that the role of NK cells is even more relevant during fungal infections in absence of other immune cell types, e.g. neutrophils or T cells. By showing a long-term deficiency in fungal mediated actin polymerization, we postulate that NK cells obtained after alloSCT may display further defects in cell functions that are dependent on actin.

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LIVER NK CELL SUBSETS: HETEROGENEITY AND ROLES DURING VIRAL INFECTION

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Natural killer (NK) cells are important innate effectors, serving as the first line of defense against infection and tumors.

During the past decade, extensive research into NK cell heterogeneity has led to the discovery of new subsets of NK cells that are distinct from conventional NK (cNK) cells, which attracts much interest of many investigators. For example, the liver, an organ with a predisposition to persistent viral infection, contains two distinct NK cell subsets at steady state: CD49a−CD49b+ cNK cells and CD49a+CD49b− liver-resident NK (LrNK) cells (also referred to as liver ILC1s). Although much progress has been made in identifying the transcription factors controlling LrNK and cNK cell development, the dynamic composition and roles of liver NK cell subsets during infection are poorly understood.

Here, our study found that there were three subsets of liver NK cells in several mouse models of hepatotopic viral infection, including LrNK cells, cNK cells and CD49a+CD49b+ (DP) NK cells. All of them exhibited a dramactic increase in cell numbers during the first week of infection. Viral infection induced up-regulated expression of PD-L1 by LrNK cells, which could further inhibited hepatic T cell antiviral function via the PD-1/PD-L1 axis. In contrast, cNK cells could promote antiviral T cell responses and upregulate CD49a expression to become DP NK cells. DP NK cells peaked at day 5 post LCMV-Arm infection, and then gradually fell to a normal level. They displayed an activated and mature phenotype. Moreover, compared with LrNK cells and cNK cells, DP NK cells showed increased functional competence, as evidenced by higher amounts of IFN-γ production and stronger cytotoxic capabilities during viral infection. Therefore, our study reveals that the composition of liver NK cells changes dynamically in response to viral infection, and their roles in regulating antiviral T cell responses are different.

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NATURAL KILLER CELLS IN CHRONIC BACTERIAL LUNG INFECTION: ELUCIDATING MECHANISMS OF ACTION

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Natural Killer (NK) cells are the first line of immune defence and make up 10% of the lung resident lymphocytes. Their role against viral infections and in tumour immunology has been explored but their mechanisms of action is unknown in the host action against bacteria. In order to explore the role of NK cells in anti-bacterial defence, we will be using a Transporter associated with Antigen Processing (TAP) – deficiency model, where there is loss of function mutation in the TAP 1 or 2 subunit leading to a very low cell surface expression of HLA class I molecules. Patients with TAP deficency have recurrent bacterial infections of the respiratory tract and lung and NK cells showing no cytotoxic activities towards the HLA class I deficient targets. We believe that these non-functional NK cells contribute to an increased incidence of bacterial infection. To test this, we will establish a model of chronic lung inflammation in wildtype and TAP-KO C57BL/6 mice by infecting them with Pseudomonas aeruginosa RP73 strain. The samples acquired will be used for phenotyping and functional assays studying the role of NK cells in the microenvironment.

Mucosal-associated invariant T (MAIT) cells, a subset of T cells involved in anti-bacterial defence, will also be investigated using the same mouse model with MAIT CAST (increased MAIT frequency) and MR1-KO (MAIT deficent) mice. To understand the intercellular communications between the two cell types and their interaction with the pathogenic bacteria, we will look at the properties of the exosomes released by the cells using the mice samples and human NK cell lines. Our studies have already shown the cytotoxic role of NK-exosomes against target cell lines and we have characterized MAIT cell population along with the difference in NK cell education in the various mice strains.

Studying NK cells and MAIT cells using these models represents an opportunity to understand their development, education and activity in bacterial infections.

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NEF-DEPENDENT DOWNMODULATION OF THE NKG2A-RECEPTOR LIGAND HLA-E BY PRIMARY HIV-1 STRAINS

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Earlier studies demonstrated that NKG2A+ NK cells are an NK-cell subset with high functionality against HIV-1– infected cells and are sensitive to changes in HLA-E surface levels. To date, increased or unchanged HLA-E surface levels have been reported in HIV-1 infection, mostly studying cell-line adapted viruses. Here, a panel of eleven distinct HIV-1 strains was investigated with regard to their effect on HLA-E surface expression. No significant effects on HLAE levels were observed following infection with four T-cell line adapted HIV-1 strains (NL4-3, SF2, SF162 and LAI). In contrast, three primary HIV-1 strains (CH236, CH077 and CH198) induced significant downmodulation of HLA-E surface levels comparing the median fluorescence intensity of HLA-E between infected (p24pos, CD4dim) and uninfected bystander cells (p24neg, CD4pos). Infection of primary CD4+ T cells with CH077 and CH198 resulted in median relative changes in surface HLA-E of –35% (range –49% to –24%) and –40% (range –56% to –22%), respectively. Both viruses were chosen for further characterization. The Nef protein of CH077 or CH198 was sufficient to reduce HLA-E surface levels in Jurkat T cells (fold change of 0.67 [range 0.58 to 0.84]) and 0.80 [range 0.72 to 0.86], respectively). Furthermore, swapping the cytoplasmic tail of HLA-A2 to that of HLA-E showed that the resulting MHC molecule remained sensitive to Nef-mediated downregulation in Jurkat cells, albeit to a lesser degree compared to full-length HLA-A2. In contrast, the cytoplasmic tail of HLA-C*04 did not enable Nef-induced downregulation.

Finally, disruption of nef resulted in increased HLA-E surface levels in infected primary CD4+-T cells (median relative change of CH198: +11%; CH077: -25%) compared to wild type virus infection (CH198: –50%; CH077: –52%).

Intriguingly, an effect was also observed upon mutation of vpu (CH198: –28%; CH077: –29%). Taken together, these data show that some primary HIV-1 strains use their Nef and/or Vpu proteins to decrease HLAE surface levels. HIV-1–mediated HLA-E surface downregulation may have implications for recognition and killing of HIV-1–infected cells by the NKG2A+ NK-cell subset.

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NK CELL REACTIVITY AGAINST HEPATITIS INFECTIONS

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Hepatitis C virus (HCV) induces chronic infection in around 150 million people and leads to progression of liver cirrhosis, fibrosis and hepatocellular carcinoma. Early and strong Natural Killer (NK) cell activation is associated to the HCV clearance in Hepatitis patients. Using the well-established HCV replicon system, we showed that after co-culture of HCV replicon-carrying cells with peripheral blood mononuclear cells (PBMCs), NK cells increased expression of the IL-2 receptor alpha chain (CD25), proliferate more and produce IFN-γ. In a next step, we performed microarrays of sort-purified NK cell populations from these co-cultures. We observed that the integrin alpha-1 (CD49a) molecule and certain chemokine receptors involved in liver homing were upregulated in NK cells upon HCV replicon co-culture, associated with activation of the NF-kB and JAK-STAT signaling pathways. We confirmed increase of CD49a expression on protein level and are currently investigating underlying mechanisms as well as function of CD49a+ NK cells. Additionally, we are validating candidates from our microarray study and are in more detail exploring the importance of myeloid cell-NK cells axis in response to HCV infection.

So far, our co-cultures of PBMCs with HBV-infected hepatoma cells did not result in robust NK cell activation. Thus, we are now focussing on elucidating how NK cells respond to HDV-infected hepatocytes that occurs as co-infection with HBV. Hepatitis D virus (HDV) infects around 15-25 million people worldwide. HDV depends on the envelope proteins of Hepatitis B virus (HBV) and thus occurs as co-infection or superinfection of HBV-infected individuals, with increasing risk of more severe form of hepatitis, progression of liver cirrhosis and cancer. Studies have shown that HDV infection induces higher intrinsic innate responses compared to HBV monoinfection. Accordingly, our experiments revealed enhanced reactivity of human NK cells after co-culture of PBMCs with HDV-infected hepatoma cells stably expressing NTCP receptor. The pathways leading to NK cell activiaton in these co-cultures will be further dissected in our studies.

Our findings will in more detail explain regulation of distinct pathways in NK cells induced by different Hepatitis viruses. Overall, obtained data could provide novel targets for the treatment of virus infections.

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NK CELL RESPONSES TO MYCOBACTERIUM TUBERCULOSIS ARE FUNCTIONALLY IMPAIRED IN INDIVIDUALS WITH ACTIVE TB DISEASE

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Infection with Mycobacterium tuberculosis (Mtb) imposes a significant burden on global public health, with approximately 10 million new cases of active tuberculosis (TB) disease and 1.6 million deaths every year. Over 90% of individuals infected with Mtb control bacterial replication and do not develop active TB, although the immune correlates of protection against development of active TB disease have not been clearly defined. Natural killer (NK) cells are increasingly recognized as a key component of the innate immune response to Mtb and as a link between innate and adaptive immunity, however the relationship between Mtb bacterial load and the functional capacity of NK cells has not been defined. To test the hypothesis that NK cells are dysregulated in the setting of high bacterial load, we evaluated the phenotype and functional profiles of NK cells in South African adults before and after treatment for pulmonary TB disease and compared these responses with those of South African healthy adults with latent Mtb infection (LTBI). We found that NK cell populations in individuals with active TB are characterized by decreased proportions of the CD56bright subset, compared with LTBI. Moreover, CD56bright NK cells exhibit decreased expression of CD94, NKG2D, NKp30, and NKp46 in active TB, compared with LTBI. The generic functional capacity of NK cells was evaluated by stimulation with MHC class I-devoid cells and antibody-coated target cells. NK cell cytotoxicity and cytokine production to MHC class I-devoid cells was significantly decreased in active TB, compared with LTBI, although NK cell reactivity to antibody-coated target cells was similar in the two groups. To evaluate NK cell reactivity to Mtb, we stimulated NK cells with Mtb whole cell lysate and cell wall antigens and found markedly reduced IFN-gamma production in individuals with active TB, compared with LTBI. Importantly, this diminished NK cell reactivity to Mtb in active TB persisted for up to 6 months after chemotherapy-mediated clearance of Mtb. Taken together, these data indicate that NK cell subset distribution, phenotypic profiles and functional capacity, to both generic and Mtb antigen stimulation, are dysregulated in individuals who develop active TB, and that these perturbations in NK cells persist despite successful treatment for active TB.

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NK CELL – INTRINSIC FCERIγ LIMITS CD8+ T-CELL EXPANSION AND THEREBY TURNS AN ACUTE INTO A CHRONIC VIRAL INFECTION

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During viral infection, tight regulation of CD8+ T-cell functions determines the outcome of the disease. Recently, others and we determined that the natural killer (NK) cells kill hyperproliferative CD8+ T cells in the context of viral infection, but molecules that are involved in shaping the regulatory capability of NK cells remain virtually unknown.

Here we used mice lacking the Fc-receptor common gamma chain (FcRγ, FcεRIγ, Fcer1g–/– mice) to determine the role of Fc-receptor and NK-receptor signaling in the process of CD8+ T-cell regulation. We found that the lack of FcRγ on NK cells limits their ability to restrain virus-specific CD8+ T cells and that the lack of FcRγ in Fcer1g–/– mice leads to enhanced CD8+ T-cell responses and rapid control of the chronic docile strain of the lymphocytic choriomeningitis virus (LCMV). Mechanistically, FcRγ stabilized the expression of NKp46 but not that of other killer cell–activating receptors on NK cells. Although FcRγ did not influence the development or activation of NK cell during LCMV infection, it specifically limited their ability to modulate CD8+ T-cell functions. In conclusion, we determined that FcRγ plays an important role in regulating CD8+ T-cell functions during chronic LCMV infection.

NK CELLS PROMOTE REACTIVATION OF CHRONIC TOXOPLASMA GONDII INFECTION BY NEGATIVELY REGULATING T CELL FUNCTION

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ILCs including NK cells regulate the activation of CD4+ and CD8+ T cells in acute viral infection, vaccination and the tumor microenvironment. However, the mechanisms underlying the development of these ILC responses and their impact on adaptive immune responses are not clear. Previous studies in mice demonstrate that CD8+ T cell exhaustion develops during chronic Toxoplasma gondii (T. gondii) infection resulting in parasite reactivation and death. The factors that promote this CD8+ T cell fate are unknown. Here we demonstrate that NK cell depletion with anti-NK1.1 is therapeutic and rescues chronic T. gondii infected mice from CD8+ T cell exhaustion induced death and increases their survival after lethal secondary challenge of the parasite. Our studies suggest that long-term chronic T. gondii infection promotes the development of a newly generated, yet modified NK cell compartment, which does not exhibit normal NK cell behavior. This splenic CD49a-CD49b+NKp46+ NK cell population develops during the early chronic phase of infection and increases through the late chronic phase of infection. They are TRAIL, Ly49, and 2B4 negative and do not increase PD1 or LAG3. These NK cells are enriched for expression of CD94-NKG2A and KLRG1. They are unable to produce IFNγ regardless of stimulation, are IL-10 negative, but do have increased CD107a on their surface and develop in both chronically infected male and female mice. These cells also were not found in brain where chronic infection is maintained. Anti-NK1.1 treatment reduced CD8+ T cell apoptosis, increased polyfunctional CD8+ T cell responses in spleen and brain and prevented parasite reactivation. Based on the NK cell receptor phenotype we observed NKp46 and CD94-NKG2A cognate ligands were measured on total splenocytes and CD8+ T cells. NKp46 ligand, as measured by soluble NCR1-hIgFc and NKG2A ligand Qa-1b expression were altered to suggest that NKp46 could promote development of this NK cell population. In support blockade with anti-NKp46 also rescued the chronically infected mice from death. Immunization with a non-persistent 100% protective T. gondii vaccination did not induce this cell population in the spleen, suggesting that persistent infection is essential for their development. We hypothesize that the chronic T. gondii infection environment results in the development of an NKp46 dependent modified NK cell population that reduces functional CD8+ T cells to promote persistent parasite infection in the brain.

Understanding how these cells develop could improve therapies to targeting complications associated with chronic infections, chronic inflammation and cancer.

NKG2CPOS NK CELLS ASSOCIATED WITH CMV INFECTION INHIBIT EXPANSION OF ACTIVATED VIRUS-SPECIFIC CD8 T CELLS

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BACKGROUND AND AIMS:
Infection with the human Cytomegalovirus (CMV) results in the expansion of CMV-specific CD8 T cells and NKG2CposNK cells. Here, we analyzed the impact of CMV-associated changes in the NK cell compartment on antiviral CD8 T cells.

RESULTS:
PBMCs of CMV seronegative and seropositive donors were cultivated in the absence or presence of irradiated NK cell target cells (K562). Virus-specific CD8 T cells were identified with dextramers. In CMV seropositive but not seronegative donors, antigen-specific expansion of CMV- or IAV-specific memory CD8 T cells was inhibited in the presence of K562 cells. CMV infection is associated with high levels of NKG2Cpos NK cells. We therefor analyzed how HLA-E, the ligand for NKG2C was regulated on activated CD8 T cells. Expression of HLA-E was upregulated on activated CD8 T cells. Importantly, the upregulation of HLA-E on virus-specific CD8 T cells was decreased in the presence of K562 cells in CMV seropositive donors, suggesting that negative regulation of CD8 T cells by activated NK cells is promoted in CMV infected donors. To analyze the mechanism of this regulation we sorted NKG2C expressing NK cells and stimulated them with K562 cells expressing high or low levels of HLA-E and analyzed the expression of different cytokines. NKG2C expressing NK cells that were stimulated with HLA-E high expressing K562 cells produced high levels of IFN gamma and the pro apoptotic molecules Granulysin, Perforin.

CONCLUSION:
The CMV-associated expansion of NKG2Cpos NK cells promotes negative regulation of virus-specific CD8 T cells presumably via the interaction between NKG2C and HLA-E on activated CD8 T cells by inducing apoptosis.

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PARALYSIS OF NK CELL FUNCTION BY VIRAL EXPOSURE

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The role of NK cells as critical anti-viral immune effectors is a well-established phenomenon, and clinical evidence has indicated specifically a significant requirement for NK cell immunity in varicella zoster virus (VZV) infection. As a herpesvirus, VZV infects human hosts for the duration of their lifetime, providing ample opportunity for interaction with the immune system. We have previously demonstrated that VZV can productively infect human NK cells, yet it is unknown how, or if, VZV can directly affect NK cell function. In vitro exposure of NK cells to VZV and subsequent challenging of effector function revealed that VZV potently impairs the ability of NK cells to respond to target cell stimulation. Remarkably, not only were virally infected NK cells affected, but antigen negative NK cells that were exposed to virus in culture were also inhibited. This powerful impairment of function was dependent on direct contact between NK cells and virally infected inoculum cells. Profiling of the NK cell surface receptor phenotype by multiparameter flow cytometry revealed that functional receptor expression is predominantly stable. Furthermore, inhibited NK cells were still capable of releasing cytotoxic granules when the stimulation signal bypassed receptor/ligand interactions and early signalling, suggesting that the virus paralyses NK cells from responding. Phosflow examination of key components in the degranulation signalling cascade also demonstrated perturbation following culture with virus. In addition to inhibiting degranulation, IFN- and TNF production were also repressed by virus co-culture, which was most strongly regulated in infected NK cells. Interestingly, the closely related virus, herpes simplex virus type 1 (HSV-1), was also capable of efficiently infecting NK cells, and exhibited a similar capacity to render NK cells unresponsive to target cell stimulation. Our findings demonstrate targeting of multiple aspects of NK cell anti-viral function by two human herpesviruses, and progress a growing understanding of pathogen inhibition of NK cell function.

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POTENTIAL OF THE NKG2D PATHWAY IN NK CELL-MEDIATED CLEARANCE OF HIV-1 RESERVOIRS

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Viral persistency in latently infected CD4+ T cells despite antiretroviral therapy (ART) represents a major drawback in the fight against HIV-1. One strategy to HIV-1 eradication is to reactivate integrated provirus using latency-reversing agents (LRAs), which should lead to immune-mediated killing of infected T cells that exit viral latency. Our group demonstrated that some LRAs simultaneously revert HIV-1 latency and induce expression of ligands for the NKG2D receptor (NKG2Ls), hence sensitizing T cells harboring reactivated virus to NKG2D-mediated killing by NK cells. In the attempt to boost NK cell-mediated clearance of HIV-1 reservoirs, we tested distinct LRAs either alone or in combination for their impact on the expression of NKG2DLs and latent HIV-1 as well as on the cytotoxicity of NK cells.

We found that, among NKG2DLs, ULBP2 is consistently up-regulated on those primary CD4+ T lymphocytes latently infected with HIV-1 that become p24-Gag+ upon virus reactivation. Combination of two LRAs with different mechanisms of action, such the hexamethylene bisacetamide (HMBA) pTEFb activator and the Prostratin PKC agonist, resulted in higher ULBP2 up-modulation on p24-Gag+ cells. In addition, LRA-reactivated p24-Gag+ cells were killed efficiently and in an NKG2D-dependent manner by NK cells that have been activated by IL-15 or, in some instances, by the LRA itself (e.g. Prostratin). On the other hand, we found that some LRAs have deleterious effects on NK cell function. For instance, HMBA reduced the expression of NKG2D and its DAP10 adaptor in NK cells, hence impairing NKG2D-mediated cytotoxicity and DAP10-dependent response to IL-15 stimulation. Furthermore, HMBA dampened NK cell-mediated clearance of Prostratin-reactivated HIV+ cells, notwithstanding their high ULBP2 expression.

We propose that the NKG2D/NKG2DLs axis could be exploited to allow NK-cell mediated clearance of HIV-1 reservoirs based on the fact that latent virus and NKG2D ligands are under the control of common regulatory pathways that can be simultaneously induced by LRAs. Yet, LRAs being taken forwards in HIV-1 eradication trials should be systematically tested for their impact on the effector function of NK cells.

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ROLE OF CXCR3 IN ANTI-MCMV EFFECTOR AND MEMORY RESPONSES

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Robust immune control and continued protection against infectious pathogens typically require generation of effector and memory lymphocyte populations, and heterogeneity in activation processes can support diversity in effector/memory cell fate. For example, in addition to antigen recognition, co-stimulatory signaling and exposure to pro-inflammatory cytokines and growth factors promote rapid proliferation and antiviral function of effector CD8+ T cells. In contrast, activated CD8+ T cells that have limited exposure to an inflammatory milieu are thought to preferentially contribute to the emerging pool of memory T cells. Although these processes were originally characterized in adaptive lymphocytes such as T cells, it is now appreciated that NK cells can similarly undergo clonal expansion and formation of long-lived memory cells following infection of herpesvirus family members such as cytomegalovirus (CMV). How microenvironmental niches in infected tissues influence NK cell, as well as CD8+ T cell, effector and memory cell generation, however, remains poorly understood. Chemokine receptors such as CXCR3 enable immune cells to access inflammatory sites and engage in stable contact interactions with other activated cells and/or target cells. We thus investigated the role of CXCR3 in the generation of NK cell and CD8+ T cell effector and memory responses following mouse CMV infection and were intrigued to find that CXCR3 is differentially required for MCMV-responsive NK cell and CD8+ T cell expansion and memory cell formation. These data suggest that MCMV-responsive NK cells and CD8+ T cells may utilize CXCR3 for distinct processes and may provide insight into how NK cell and CD8+ T cell activation is coordinated during infection and how to improve vaccination strategies. With future work we aim to determine if NK cell and T cell activation niches differ and if CXCR3 mediates divergent transcriptional programs within NK cell and T cell populations.

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SELECTIVE TARGETING OF HLA-C MOLECULES BY THE HUMAN CYTOMEGALOVIRUS ENCODED GLYCOPROTEIN US10

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Classical MHC-I (HLA-A/B/C) present a multitude of peptide species to surveilling CD8+ T-cells. In addition, most of them, together with non-classical HLA-E/F/G, also interact with various inhibitory and/or activating NK cell receptors (NKR). Despite a specific and potent immune system the human cytomegalovirus (HCMV) persists in healthy hosts.

HCMV control of CD8+ T-cell activation through manipulation of classical MHC-I is established and broadly documented. However, how HCMV interferes with the MHC-I/NKR axis is not well understood. To elucidate this, we established a fast protocol for flow cytometric analysis of MHC-I regulation by various HCMV encoded proteins. The glycoprotein US10 strongly regulated HLA-G, as described previously, but also HLA-E, HLA-C and some HLA-B, but not HLA-A or -F. Some of these effects were tested and confirmed in HCMV infected cells. To better understand the US10 MHC-I specificity, co-immunoprecipitation experiments were performed, revealing interesting features: Whereas all tested MHC-I were able to interact with US10 in their non-assembled form, only sensitive molecules were found to be retained in the ER. Surprisingly, US10 stabilized the assembled form of HLA-C*05:01 (in complex with beta-2-microglobulin), whereas this was not observed for HLA-A/B/E, suggesting a specific role for the interaction with HLA-C. In line with this, mass spectrometry of the US10 interactome revealed an outstanding binding to HLA-C.

In conclusion, ectopically expressed US10 can bind to all tested (HLA-A/B/C/E/G) allotypes. However, in HCMV infected cells only few MHC-I are spared from control by the other HCMV-encoded immunoevasins, mostly HLA-C and -E. Remarkably, US10 showed among the highest specificities for these MHC-I molecules, demarcating its function from other MHC-I immunoevasins.

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TARGETING MITOCHONDRIAL DYSFUNCTION CAN RESTORE ANTIVIRAL ACTIVITY OF HIV(+) NK CELLS

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INTRODUCTION:
Hepatitis C virus (HCV) co-infection in HIV(+) persons remains a relevant health problem. Not only is it less likely that acute hepatitis C will heal spontaneously than in HIV(-) patients, but there is also a significantly faster progression of liver disease with faster development of liver fibrosis than in HCV monoinfected patients. Recently, we showed that ineffective IFNg production of HIV(+) NK cells may contribute to low spontaneous clearance rate and accelerated progression of HCV-associated liver disease in HIV(+) patients. However, the mechanisms underlying impaired function of HIV(+) NK cells remained unclear.

PATIENTS AND METHODS:
NK cell IFNg production and NK cell-mediated inhibition of HCV replication were studied in HIV(+) RNA (-) and HIV(+) RNA (+) patients (each group n = 20) and compared to healthy controls (n = 10). NK cell-mediated inhibition of HCV replication was analyzed using the HuH7HCVreplicon model. IFNg production of NK cells was analysed by flow cytometry. Metabolic NK cell activity was studied using Seahorse XF Analyzer. Gene expression was analysed by rt-PCR.

RESULTS:
Activation of HIV(+) NK cells with different stimuli (IL-2 + Jurkat cells, HuH7HCV replicon cells or IL-12/15) resulted in a significantly reduced IFNg production compared to healthy NK cells, suggesting a rather global impairment of NK cell activity in HIV infection. Given the essential role played by metabolic processes in the development and function of NK cells we next studied whether dysregulation of intracellular metabolic processes may be involved in HIV-induced dysfunction of NK cells. Indeed, we found HIV(+) NK cells to display an impaired reserve respiratory capacity and glycolytic capacity suggesting mitochondrial and metabolic dysfunction. Accordingly, we observed HIV infection to be associated with an altered expression of genes involved in regulating metabolic and mitochondrialy activity. These functional alterations were found in HIV(+) RNA(+) and RNA(-) patients and especially in patients with CD4+ T cell count below 400 cells/µl. Moreover, we could show that the disturbed IFNg production of NK cells in HIV(+) patients with low CD4+ T cell count can be restored by stimulation with a mitochondrion-targeted antioxidant (MitoTEMPO). Accordingly, we found MitoTEMPO significantly increase anti-HCV activity of HIV(+) NK cells.

CONCLUSIONS:
Taken together, our data suggest that mitochondria may represent promising targets for novel therapeutic approaches to reconstitute NK cell functions in HIV-infected patients.

TEMPORAL ANALYSIS OF THE PLASMA MEMBRANE PROTEOME AFTER VACCINIA VIRUS INFECTION SUGGESTS VIRUS STRATEGIES TO EVADE NATURAL KILLER CELLS RESPONSE

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Vaccinia virus (VACV) is a poxvirus and was used as the vaccine to eradicate smallpox. VACV is a useful vaccine vector candidate due to its capacity to express foreign proteins against which a strong and long-lasting adaptive immune response can be raised. There is, however, an incomplete understanding of how VACV evades the host immune response and which host factors are important to raise a protective immunological memory.

During VACV infection, natural killer (NK) cells proliferate, are activated, limit viral replication, recognise and kill VACV-infected cells and can display memory-like qualities (Bukowsky et al., 1989; Brookes et al., 2015; Chisholm and Reyburn, 2006; Gillard et al., 2011). Little is known, however, about how NK cells interact with VACV-infected cells and which ligands trigger NK activation. To identify the cell surface proteins whose expression is altered by VACV and could trigger NK activation we performed a quantitative proteomic analysis of membrane-associated proteins from VACV-infected cells. This analysis revealed temporal changes in the expression of host and viral proteins. About 40 viral proteins and 750 membrane-associated host proteins were detected using tandem mass-tag spectrometry.

These data showed that VACV infection rapidly alters the expression of proteins with immune functions including NK cell ligands. VACV substantially downregulated the surface expression of nectins, plexins and MHC-I related proteins.

Moreover, VACV prevented the upregulation of the stress-induced ligands MICA/B, ULBPs and death receptors. The expression profile of multiple NK ligands was validated by flow cytometry. Comparison of these data with a similar study of HCMV-infected cells, identified a subset of surface proteins altered similarly by the two viruses. Both VACV and HCMV downregulated the surface expression of many cadherins and protocadherins suggesting this might be a conserved immune evasion strategy.

Collectively, these data constitute a valuable resource for the study of VACV proteins expressed at the cell surface, provide insights into the interaction of NK cells with VACV and suggest novel VACV immune evasion mechanisms.

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THE DE NOVO DNA METHYLTRANSFERASE, DNMT3A CRITICALLY REGULATES ANTIVIRAL NK CELL RESPONSES

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NK cells are innate cytotoxic lymphocytes that provide crucial defense against cancerous and virally-infected cells. In certain immunological settings, such as cytomegalovirus (CMV) infection, NK cells can undergo robust antigen-specific, clonal-like expansion and differentiate into long-lived memory cells, in a manner analogous to adaptive CD8+ T cell responses. Expression of genes controlling immune responses by NK cells must be rapidly and tightly coordinated, particularly at the epigenetic level. Nevertheless, the role of epigenetic regulators in programming NK cell-mediated immunity remains largely understudied. To investigate a role for DNA methylation in the regulation of NK cell immunity, we generated NK cell-specific knockouts of the Dnmt3a gene, which encodes a de novo DNA methyltransferase. We find that Dnmt3a is largely dispensable for classical and tissue-resident NK cell development and maturation in naïve mice, but is essential for the expansion of Ly49H+ NK cells in response to mouse CMV (MCMV) infection. Additionally, we find that Dnmt3a-deficient NK cells exhibit defects in infection-induced memory cell differentiation. Consistent with these findings, we find that Dnmt3a expression is low in resting NK cells, but increases in response to MCMV infection. Ongoing studies employ an unbiased molecular genomics approach to identify the gene loci targeted by Dnmt3a in resting and activated NK cells. We expect these studies to shed light on the mechanisms by which Dnmt3a regulates MCMV-driven proliferation and memory cell differentiation in Ly49H+ NK cells. In summary, these studies advance our knowledge on the epigenetic mechanisms driving NK cell immunity and could provide critical insights in designing potential NK-cell based vaccinations against infectious disease.

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THE EFFECTS OF B-1,3-1,6 GLUCANS ON PORCINE NK CELL ACTIVITY

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Beta-glucans are naturally occurring polysaccharides present e.g. in the cell walls of fungi, yeast, bacteria and cereals. They belong to the so-called microbe-associated molecular patterns (MAMPs) and have been shown to possess important immunomodulatory properties, mainly modulated by Complement Receptor 3 (CR3) and Dectin-1. Although Dectin-1 is absent on porcine natural killer (NK) cells, they do express CR3 on their surface. It has been claimed that NK cells can be activated by β-glucans, but clear evidence is lacking. Therefore, we set out to investigate whether or not β-1,3-1,6-glucans may modulate NK cell responses in vitro and if so, how these effects are mediated.

We investigated the effects of two types of β-glucans, differing in solubility and structure; Macrogard and Curdlan, at 10µg/ml. Cytotoxic assays with the NK-susceptible cell line K562 were performed and showed no direct effects of β- glucans on the cytotoxic capacity of FACS-purified porcine NK cells. However, when using PBMC instead of purified NK cell populations, addition of β-glucans resulted in significantly increased cytotoxicity towards K562 cells. NK cell depletion from the PBMC cytotoxicity assay resulted in lack of cytotoxicity against K562 cells, confirming that the increased cytotoxicity observed by addition of β-glucans was indeed due to increased activity of NK cells.

To better understand the mechanism underlying activation of NK cells by addition of β-glucans to PBMC, cytotoxic assays were performed with FACS-purified NK cells in the presence or absence of supernatant of β-glucan-stimulated PBMC. Supernatant of β-glucan-stimulated PBMC was able to increase the cytotoxicity of NK cells against K562 cells. We are currently investigating which cells and factors are responsible for this indirect activation of NK cells upon β-glucan priming. Preliminary results of depletion assays point towards CD172a+ cells (monocytes and/or dendritic cells) as a source of NK cell activating factors upon β-glucan stimulation.

These results show that β-glucans can indirectly stimulate porcine NK cell cytotoxicity, probably by triggering the production of NK cell-activating cytokines by myeloid cells. Since administration of β-glucans is generally not accompanied with side effects, further insights into immune modulation and NK cell activation by these molecules may pave the way towards new strategies in vaccine development, anti-viral therapies and possibly cancer therapies

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THE HUMAN NK CELL RECEPTOR KIR2DS4 DETECTS A CONSERVED BACTERIAL EPITOPE PRESENTED BY HLA-C

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Natural killer (NK) cells have an important role in immune defense against viruses and cancer. Activation of human NK cell cytotoxicity toward infected or tumor cells is regulated by killer-cell Ig-like receptors (KIR) that bind to HLA class I. Combinations of KIR with HLA-I are genetically associated with susceptibility to disease. KIR2DS4, an activating member of the KIR family with poorly defined ligands, is a receptor of unknown function. Here we show that KIR2DS4 has a strong preference for rare peptides carrying a tryptophan at position 8 of 9-mer peptides bound to HLA-C*05:01. The complex of a Trp8 peptide bound to HLA-C*05:01 was sufficient for activation of primary KIR2DS4+ NK cells, independently of activation by other receptors and of prior NK cell licensing. HLA-C*05:01+ cells that expressed the peptide epitope triggered KIR2DS4+ NK cell degranulation. We identified a 'self' peptide with Trp8, previously eluted from HLA-C*05:01, stimulated KIR2DS4+ NK cells, but Trp8 was not sufficient to define KIR2DS4 binding peptides. We show an inverse correlation of the worldwide allele frequency of functional KIR2DS4 with that of HLA-C*05:01, indicative of functional interaction and balancing selection. We found a highly conserved peptide sequence motif for HLA-C*05:01-restricted activation of human KIR2DS4+ NK cells in bacterial recombinase A (RecA). KIR2DS4+ NK cells were stimulated by RecA epitopes from multiple human pathogens, including Helicobacter, Chlamydia, Brucella and Campylobacter. We predict that over a thousand bacterial species could activate NK cells through KIR2DS4, and propose that human NK cells contribute also to immune defense against bacteria through recognition of a conserved RecA epitope presented by HLA-C*05:01.

THE HYPOXIC RESPONSE IN NATURAL KILLER CELLS CONTRIBUTES TO THE CONTROL OF BACTERIAL SKIN INFECTIONS

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The control of bacterial skin infections critically depends on the coordinated interaction of different immune cell subsets and Natural Killer (NK) cells crucially contribute to the orchestration of such inflammatory responses. Since bacterial skin infections are associated with severe hypoxia (low oxygen), NK cells within such bacterial lesions need to adapt rapidly to this harsh environment. Exposure of cells to low oxygen requires activation of Hypoxia-inducible factors (HIFs). HIFs are a family of oxygen-sensitive transcription factors that, in the presence of oxygen, are subject to proteasomal degradation via interaction with their negative regulator von Hippel-Lindau (VHL) protein. When oxygen levels decrease, HIFs induce the transcription of hundreds of genes involved in cellular and whole body homeostasis. We have recently shown that HIF-1α deficiency in NK cells impairs effector function but accelerates vascular remodeling in hypoxic tumors. However, the contribution of the HIF pathway in NK cells to the containment of bacterial skin lesions and the overall inflammatory response is not known.

To this end, we subcutaneously injected gram-positive group A streptococci (GAS) into the back skin of mice with a targeted deletion of HIF-1 (gain of cunction) or VHL (loss of function) in NKp46-expressing cells and their corresponding wildtype (WT) littermates. Subsequently, skin lesions, bacterial dissemination and immune cell infiltration were analyzed.

Skin lesions from mice with a deletion of HIF-1α in NK cells were significantly larger and showed increased vascular density along. This was associated with enhanced dissemination of GAS across the endothelial barrier to the spleen and sepsis but no differences in immune cell infiltration across genotypes. Moreover, RNA sequencing of activated NK cells revealed HIF-1-dependent expression of the powerful antimicrobial peptide cathelicidin, suggesting that HIF-1 in NK cells is required to control bacterial infections locally as well as systemically. In line with this, constitutive HIF expression via deletion of VHL in NK cells led to increased expression of cathelicidin, smaller bacterial skin lesions as well as a reduction of systemic dissemination of GAS and, therefore, an overall improved antimicrobial defence.

In summary, we show that the hypoxic response in NK cells critically contributes to the control of bacterial skin infections at the local and systemic level.

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TIGIT INHIBITION OF ADCC PARALLELS NK CELL ADAPTATION TO HCMV INFECTION

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INTRODUCTION:
Human immunodeficiency virus (HIV) persists in latently infected reservoirs despite effective antiretroviral treatment. Infected individuals suffer a greater risk for age-related morbidities, further increased by human cytomegalovirus (HCMV) co-infection. As HCMV infection modifies NK cell phenotype/function, we considered the role of exhaustion markers and their effect on NK cell-based strategies to purge HIV reservoirs.

METHODS AND RESULTS:
To evaluate functional and phenotypic impacts of HCMV-driven NK cell adaptation in HIV infection, we compared three groups distinguished by either HCMV-seronegative status or by high (> 20) versus low (< 6) percentages of NKG2C-expressing NK cells. Despite a hierarchy of phenotypic adaptation, antibody-dependent cellular cytotoxicity (ADCC) did not differ significantly between groups. Adapted NK cells did not display a classical exhausted phenotype as there was low LAG-3, PD-1 (<1%), and TIM-3 (<10%) expression, however, TIGIT was present on 20-80% of NK cells. T cells latently infected with HIV upregulate PVR (CD155), a ligand for TIGIT, therefore we investigated TIGIT modulation of NK cell functions. Blocking TIGIT/PVR interactions increased NK cell cytotoxicity proportional to percent TIGITpos NK cells ($p = 0.0055$). A greater increase in NK cell cytotoxicity occurred in HCMV co-infected individuals, which correlated with loss of FcεRIγ ($p = 0.002$). HIV-specific ADCC was measured against P815 cells infected with a recombinant vaccinia virus expressing gp160 (vPE16) using pooled HIV Ig to direct NK cells to gp120/gp41-expressing P815 cells. Engaging TIGIT reduced levels of HIV-specific ADCC with the extent of reduction reflecting the extent of NK cell adaptation to HCMV ($p = 0.0333$).

IMPLICATIONS:
These results suggest that in HIV infection, NK cell adaptation to HCMV infection relates to development of TIGIT-mediated NK cell inhibition. Blocking TIGIT can invigorate ADCC against HIV-infected CD4pos T cells in cure or treatment strategies.

VARIANTS OF THE IMMUNODOMINANT HCV NS5B2841 EPITOPE PRESENTED BY HLA-B*27:05 IMPACT THE FUNCTION OF KIR3DL1+ NK CELLS

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INTRODUCTION:
The HLA class I allele, HLA-B*27 has been reproducibly linked to spontaneous resolution of HCV GT1 infection. This protective effect is associated with an immunodominant CD8+ T cell epitope in NS5B2841 and multiple escape mutations within the presented epitope are needed to evade the virus specific CD8+ T cell response and consequently leading to viral persistence. Besides T cells, NK cells have been associated with HCV infection outcome. Interestingly HLA-B*27 harbours a Bw4 motif which is the ligand for the inhibitory NK cell receptor KIR3DL1 and the genetic combination of KIR3DL1 and HLA-Bw4 80(T) is associated with spontaneous immune control of HCV infection in a high-risk cohort of people who inject drugs (PWID).

OBJECTIVES:
Here we aimed to determine whether the protective effect of HLA-B*27 is only associated with HCV GT1 or can be extended to GT3. Since KIR3DL1 does not only bind the Bw4 motif, but also makes contact with residue p7, the residue under selection in the HLA-B*27 restricted NS5B2841 epitope, it is of interest to elucidate if viral peptide variants of the GT1 (ARMILMTHF) or GT3 (VRMVMMTHF) epitope presented by a HLA-B*27 allele that harbours a Bw4 80(T) motif might modulate NK cell function.

PATIENTS AND METHODS:
We utilised a cohort of 616 PWID, including 311 HCV-RNA positive PWID (54% GT1, 37% GT3 and 9% other GTs), 174 patients spontaneously resolving HCV infection (HCV-RNA negative) and 131 PWID remaining anti HCV seronegative (anti-HCV negative). TAP deficient T2 cells stably expressing HLA-B*27:05 were used to assess the ability of NS5B2481 peptide variants to stabilize HLA-B*27:05 and as NK cell targets in degranulation assays.

RESULTS:
HLA-B*27 was associated with a protective effect against GT1 in our PWID cohort. The prototype and escape variants of the ARMILMTHF epitope stabilized HLA-B*27:05 equally. Interestingly, T cell escape variants with a proline at position 7 were identified as strong inhibitory peptides and reduced the CD107a production of KIR3DL1+ NK cells. Notably, this inhibitory effect was effect was mediated by low expressing KIR3DL1 alleles.

CONCLUSION:
HLA-B*27 is protective against HCV GT1 in a high risk cohort of PWID. Our data suggests that HCV sequence variations within the HLA-B*27 restricted ARMILMTHF epitope might not only impair T cell recognition but also modulate NK cell function and potentially mediate NK escape.

SESSION 10
NK cells in clinics

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ALARMINS INDUCE NATURAL KILLER CELLS FOR REGULATORY FUNCTIONS

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Damage-associated molecular patterns (DAMPs), or alarmins, are most commonly associated with inciting inflammation among immune subsets, but their impact on NK cell function has not been determined. DAMPs of mitochondrial origin (mitoDAMPs) are highly prevalent consequent to tissue damage, ischemia-reperfusion injury, infections and cancer. We hypothesized that mitoDAMPs would signal for inflammatory responses in NK cells, but surprisingly, found the opposite effect: NK cells regulate ongoing inflammation. In response to stimulation with mitoDAMPs from an array of sources and species, both human and mouse NK splenic/peripheral blood NK cells produce the immune-regulatory cytokine, IL-10. IFN-γ production following stimulation of NK cells using TLR ligands, pro-inflammatory cytokines or PMA/ionomycin is dominantly diminished by NK cells treated with mitoDAMPs in a dose-dependent manner. Moreover, NK cells exhibit diminishing expression of CD69 and increasing expression of the regulatory hallmark CD73 with increasing doses of mitoDAMP stimulation. Furthermore, ongoing proliferation of T cells stimulated by CD3 and CD28 cross-linking is halted by NK cells treated with mitoDAMPs, but not in the presence of equivalent doses of mitoDAMPs alone. Together, these findings imply that NK cells act as regulatory cells to limit the extent of inflammation caused by mitoDAMPs. Therefore, NK cells may play an important role for controlling autoinflammation to maintain normal homeostasis, but may also contribute to immune evasion by limiting immune reactivity. Ongoing studies are investigating the features and specific subsets of NK cells important for driving these regulatory features.

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AN OFF THE SHELF, GMP COMPLIANT,FULLY CLOSED AND SEMI-AUTOMATED LARGE-SCALE PRODUCTION SYSTEM FOR ALLOGENEIC NK CELLS

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Natural killer cells (NK) have been on the mainstay in cancer treatment for over two decades. Most of the clinical trials till date, have either used autologous or allogeneic NK cells that were often produced in limited quantities to tailor the needs of a single patient. With the highly unmet need to treat large cohort of patients and with multiple doses, a true off the shelf approach is the only way. Glycostem began to envision this, by setting up a world's first completely closed manufacturing platform for allogeneic NK cells (oNKord®) from fresh umbilical cord blood stem cells. A closed system is a process where the cells are never exposed to the open environment to avoid a potential risk for contamination. Glycostem manufactures oNKord® at its own state of the art Class C/D GMP clean room, using PVC Sterile Connection Device and Sealer (Terumo), C-Flex Biowelder and Sealer (Sartorius), to connect and seal two tubes aseptically and keeping the system closed. These equipments are key elements in the routine functioning of the closed system, while cells are transferred, or medium is added. In our process, fresh cord blood is used as a starting material, using fully automated CliniMACS Prodigy to select CD34+ cells, which are then expanded using static cultures in incubators and further differentiated into mature NK cells in Xuri bioreactors. After 40 days of culture, NK cells are harvested, washed and aliquoted into cryobags and cryopreserved in controlled rate freezing device. Optimised freezing and thawing conditions, which result in high recovery and product functionality, enabled cell banking of multiple infusion doses produced from one manufacturing batch. Frozen doses are shipped to clinical centres in temperature controlled dry shippers and thawed using waterbath at the time for infusion. The NK cell product is scalable resulting in a 2000 - 3000-fold expansion, averaging up to 11 billion NK cells from a single cord blood unit (n=3). Validated QC testing methods, in process controls using flow cytometry assays and standardised potency assays are in place to address product safety, identity and functionality for release. Setting up of a completely closed system faces many challenges, and during this journey over the last two years, Glycostem has successfully resolved several bottlenecks and worked closely with the regulatory authorities in achieving an "universal off the shelf" NK cell therapy product.

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ANALYSIS OF NK CELL FUNCTIONS IN PATIENTS WITH HEREDITARY HEMOCHROMATOSIS

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Hereditary Hemochromatosis (HH) is an autosomal recessive disorder of the iron metabolism. The typical systemic iron overload in this disease can cause dysfunction of several organs by iron accumulation. Iron is crucial for cell function including immune responses, but on the other hand, it produces reactive oxygen species (ROS) by the catalysis of important chemical reactions. Since ROS are known to cause oxidative stress and cellular damage, a precise regulation of iron within cells is necessary. The identification of the HFE gene was a major breakthrough for the understanding of HH. This gene encodes for a novel major histocompatibility complex class 1-related molecule. Due to the fact that in preliminary studies some HH patients showed aberrant NK cell activities, we wanted to examine whether NK cells could be influenced by iron overload in HH patients.

In order to investigate the properties of HH NK cells, PBMC of hemochromatosis patients and age-matched controls were used for immunophenotyping and functional assays such as degranulation and chromium-release assays. In addition, a cytometric bead array and a ferritin ELISA were performed. We observed increased basal and stimulated production of pro-inflammatory cytokines, assuming a distinct functionality of HH PBMC compared to controls. In addition we did not find aberrant NK cell phenotypes, but a general decrease of total granulocyte numbers. These data demonstrate that NK cells are not changed in HH patients, which is a result of their personalized treatment of venesection, and underline the complexity and sensitivity of the immune system to systemic influences

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B7H6 IS A TUMOR PROGRESSION MARKER IN CERVICAL CANCER AND HIGH GRADE INTRA EPITHELIAL LESIONS

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INTRODUCTION

Cervical cancer is the third most frequent cause of female cancer mortality in Mexico. NK cells play an important role in tumor elimination and have been reported to be mediated by natural cytotoxic receptors, such as NKp30 receptor, present in the surface of NK cells and associated with signaling protein CD247 or ζ-chain. The NK receptor NKp30 has three isoforms: a, b and c. The first two are reported to have anti-tumoral effects, the third is immunoregulatory. The cell stress associated ligand of NKp30, B7H6, has been documented to lead to the activation of NK cells, and cytotoxicity against tumor cells. Tumor surface B7H6 has been reported upregulated in different diseases, such as melanoma, glioma, breast cancer, neuroblastoma, and sepsis. Similar to other activating ligands that play inhibitory roles when soluble, it is possible that B7H6 could be released via proteolytic cleavage or via exosomes, where it may be implicated as a marker for cancer progression.

OBJECTIVE

To reveal the presence of the NKp30 ligand, B7H6 in cervical biopsies of women with precursor lesions (low-grade and high-grade squamous intraepithelial lesions) and cervical cancer (squamous cervical cancer and uterine cervical adenocarcinoma).

RESULTS AND CONCLUSIONS

We found, for the first time, the in situ presence of B7H6 in biopsies from patients with dysplastic cervical tissue. Transformed keratinocytes in high-grade lesions and cervical cancer expressed increasing levels of B7H6. Additionally, B7H6 was found elevated in plasma B cells and stromal infiltrating mononuclear cells. Staining was notably higher, based on immunoreactive scores, in cervical adenocarcinoma versus squamous cell cervical carcinoma.

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CONSTITUTIVE DYSFUNCTION OF NATURAL KILLER CELLS IN THE AUTOIMMUNE DISEASE PRIMARY BILIARY CHOLANGITIS

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BACKGROUND
Primary Biliary Cholangitis (PBC) is an autoimmune disease leading to liver failure and accounts for approximately one fifth of liver transplants performed in the USA. PBC has a strong genetic component, especially polymorphisms associated with the IL-12/STAT4 pathway and is characterized by elevated Th1 cytokines. Natural killer (NK) make up 40% of the hepatic lymphocyte population and can lyse biliary epithelial cells, suggestive of an involvement in PBC. We investigated the role of NK cell dysfunction in PBC.

METHODS
Peripheral blood mononuclear cells (PBMCs) from individuals with PBC, the non-autoimmune liver disease haemochromatosis (HFe) and Healthy Controls (HC) were isolated. NK cells were characterised phenotypically and functionally by flow cytometry and RNA sequencing.

RESULTS
Circulating NK cells from individuals with PBC expressed significantly higher levels of the "liver-homing" marker CXCR6 and "memory" marker CD49a compared to patients with HFe (CXCR6 3.4% vs 2.4%, p=0.015; CD49a 2.2% vs 1.3%, p=0.006) and HCs (CXCR6 3.4% vs 2.0%, p=0.001; CD49a 2.2% vs 0.9%, p=0.009). Stimulation with minimal amounts of IL-12 (0.005ng/ml) led to up-regulation of CXCR6 on peripheral NK cells from PBC patients (2.9% to 4.8%, p<0.001), and enhanced IFNγ production (1.1% to 2.9%, p<0.001) not observed in the control groups. IFNγ secretion was most marked on CD49a+ NK cells (CD49a+ 8.6% vs 2.5% CD49a- NK cells, p<0.001), consistent with their priming in vivo. In RNAseq studies, resting NK cells from PBC patients had a constitutively activated transcriptional profile and upregulation of genes associated with IL-12/STAT4 signalling. Transcriptomic analysis also demonstrated upregulation of the amino acid transporter SLC7A5 in the CD49a+ NK cell subset in PBC, which is thought to be important for c-myc mediated metabolic reprogramming. Consistent with these findings, resting NK cells from PBC patients expressed significantly higher baseline levels of pSTAT4 compared to control groups (PBC 4.3%, HFe 2.3%, HCs 0.5%; p<0.04 vs HFe, p<0.001 vs HC), indicating in vivo activation.

CONCLUSION
This data suggest a novel model for NK cells in autoimmunity, in which exaggerated peripheral activation leads to expression of tissue homing markers thus contributing to disease pathogenesis.

DIFFERENT PATTERN EXPRESSION OF NKP30 ISOFORMS AND SOLUBLE LIGAND B7H6 FROM HEALTHY WOMEN COMPARED WITH CERVICAL CANCER AND PRECURSOR LESIONS

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INTRODUCTION
Cervical cancer is the third most frequent cause of female cancer mortality in Mexico. NK cells play an important role in tumor elimination. The NK receptor NKp30 has three isoforms: a, b and c. The first two are reported to have anti-tumoral effects, the third is immunoregulatory. The cell stress associated ligand of NKp30, B7H6, has been documented to lead to the activation of NK cells, and cytotoxicity against tumor cells. Tumor surface B7H6 has been reported upregulated in different diseases, such as melanoma, glioma, breast cancer, neuroblastoma, and sepsis. Similar to other activating ligands that play inhibitory roles when soluble, it is possible that B7H6 could be released via proteolytic cleavage or via exosomes, where it may be implicated as a marker for cancer progression.

OBJECTIVE
To reveal the distribution patterns of the NKp30 receptor and its isoforms in peripheral blood NK cells, and B7H6 in serum, in patients with cervical cancer and precursor lesions.

MATERIALS AND METHODS
PBMCs from patients with invasive cervical carcinoma (n=25) and precursor lesions (n=34), as well as healthy women (n=30), were recruited in the study. To characterize the NKp30 isoforms, RT-qPCR analysis (LightCycler© 96) was used; each isoform was determined using specific probes and primers and normalized to the β2-microglobulin gene. Multicolor flow cytometry was used to characterize the expression of NKp30 receptor within subclasses of NK cells: CD56dim, and CD56bright. The BD FACSCanto II® flow cytometer was used and analysis made with the FlowJo® software. Additionally, ELISA assays were used in order to quantify soluble B7H6.

RESULTS AND CONCLUSIONS
Our data provide the first evidence of increased soluble B7H6 in cervical cancer. While at the mRNA level NKp30 A and B were not changed, and immunoregulatory C was decreased, at the cell surface NKp30 percentage was decreased in NK cells from cervical cancer patients. A significant percentage of the NK cells appear to be inhibited, with perforin MFI decreased. As has been previously seen in other cancers, the balance between bright and dim NK cells was changed with dim cells being increased, however many of these dim cells seem to exhibit an exhausted phenotype.

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EARLY DONOR NK CELLS INFUSION A STRATEGY TO PREVENT HHV6 ENCEPHALITIS IN PEDIATRIC PATIENTS AFTER CD45RA-DEPLETED TRANSPLANTATION

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BACKGROUND

HHV-6 reactivation has been reported in 30-70% of patients after hematopoietic stem cell transplantation (HSCT) especially at engraftment and during the first month. Delayed immune reconstitution (IR) is a risk factor to develop HHV-6 encephalitis. The incidence of this complication is low (0.95 to 11.6%). We previously reported a unexpected 34% cumulative incidence of HHV-6 encephalitis in a serie of 25 pediatric patients receiving haploidentical HSCT with CD45RA-depletion. Simillar data were reported in a serie of 38 pediatric HSCT enrichment in CD4+ T cells and without Natural Killer cells in the graft. Because NK cells act against infected and transformed cells as part of innate immunity we demonstrated how in vitro co-culturing a 2:1 ratio of donor CD4+ T cells with CD56+ cells eliminated the virus, demonstrating the main role of donor NK cells in the antiviral immune response.

OBJECTIVES

NK cells infusion in could provide functional cells to protect against infections and to control HHV-6- infected cells in peripheral blood. We present our experience with NK cells infusion at day +7 post-HSCT after CD45RA-depleted transplantation to assess safety and infections rate focusing on HHV-6 reactivations and encephalitis.

METHODS

A total of 10 patients received NK cells infusion on day +7 after CD45RA-depleted HSCT after nonmyeloablative conditioning. NK product was obtained performing CD3 depletion on donor non-mobilized leukapheresis product followed by CD56 enrichment using the CliniMACS® device. Protocol also included donor CD3+CD45RO+ lymphocyte infusion on a prophylactic regime on days +30, +60 and +90 (1x107/Kg) to boost immune reconstitution.

RESULTS

Ten pediatric patients median age 6 years (range 1-15) received CD45RA-depleted grafts from haploidentical (7) and match related donor (3). NK cells were infused on day +7 post-HSCT with a median dose of 1,05x107/Kg (range 1x106/Kg-1x108/Kg). There was only one infusion reaction. Patients presented full donor chimerism and were on complete remission at last follow up. Three patients presented acute GvHD≥ grade 2. There were 1 CMV and 1 Adenovirus reactivation. Two patients presented Parvovirus infection. HHV-6 reactivation occurred in 2 patients, one of them was complicated with enteritis. No HHV-6 encephalitis was seen (0%).

CONCLUSIONS

Our preliminary data suggest that infusions of NK in the early post-HSCT (day +7) are a safe adoptive immunotherapy strategy to prevent HHV-6 encephalitis and other viral reactivations after CD45RA-depleted transplantation. However, to determine the real efficacy of this strategy, a larger number of patients and prospective studies are required.

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IL-15 RECEPTOR AGONIST ENHANCES CD8+ T CELL RESPONSES AGAINST DONOR ALLOGENIC NK CELLS FOLLOWING ADOPTIVE TRANSFER

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Allogeneic donor NK cells are a promising cellular immunotherapy for patients with hematologic malignancies. However, the optimal strategy to support transferred NK cells remains unclear. rhIL-2 has been used in clinical trials to support donor NK cell survival and activation, but may expand regulatory T cells. We hypothesized that an IL-15 receptor agonist would more effectively increase and expand adoptively transferred memory-like NK cells, compared to rhIL-2. To test this, we compared two cohorts of a clinical trial testing IL-12/15/18-differentiated donor memory-like (ML) NK cells for patients with rel/ref AML (NCT01898793). The first cohort was supported by low-dose rhIL-2, while the second cohort the IL-15 receptor superagonist complex N-803 (10 mcg/kg, SQ, q5days x 4 doses). Unexpectedly, reduced clinical responses were observed in the N-803 cohort [0/8 (0%) CR/CRi], compared to the IL-2 cohort ([6/11, (54%), CR/CRi], P<0.05. Furthermore, the first two patients treated experienced mild (grade 2) cytokine release syndrome (CRS), starting approximately 10 days following the NK cell infusion, which resolved within one week. Based on published clinical data demonstrating minimal CRS following allogeneic NK cell therapy, and knowing that IL-15 also stimulates CD8+ T cells, we hypothesized that N-803 was augmenting recipient CD8+ T cell allo-rejection of donor NK cells. Consistent with this, increased CD8+ T cell proliferation and activation were observed in the BM of N-803-treated, compared to IL-2-treated patients (57±9% vs. 24±7% Ki-67+ mean±SEM, p=0.03). PK data revealed that N-803 persisted at a high concentration (>500pg/ml) at day 10 post-injection in patients that received 2-4 doses of N-803. To directly test the ability of N-803 to stimulate CD8 T cell rejection in vitro, a mixed lymphocyte reaction (MLR) of normal donor PBMC against allogeneic ML NK cells was performed, supported by IL-2 (control) or IL-2+N-803. CD8+ T cells expanded more robustly in the presence of N-803 compared to controls, as determined by CFSE-dilution and cell counts (p<0.05). After a 10-day MLR, cytotoxicity assays using labeled ML NK cell targets revealed increased killing by T cells supported with N-803 compared to controls (p<0.001). Anti-MHC-I blocking mAb abrogated killing (p<0.001), confirming CD8+ T cells mediated the NK cell elimination. Compared to historical controls, a similar, independent clinical trial also demonstrated less-than-expected clinical responses, supporting these findings (NCT03050216). N-803 impacts on both NK and CD8 T cell proliferation and activation need to be considered in the allogeneic lymphocyte transfer setting.

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NK CELL RECONSTITUTION IN CHILDREN AND YOUNG ADULTS AFTER HLA-MISMATCHED T CELL REPLETE BONE MARROW TRANSPLANT WITH POST-TRANSPLANT CYCLOPHOSPHAMIDE

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INTRODUCTION:
Hematological malignancies account for about 30% of all childhood cancers, and treatment of high-risk disease frequently requires hematopoetic stem cell transplantation (HSCT). The use of post-transplant cyclophosphamide (PTCy) to prevent graft-vs-host disease has increased donor availability, but in adults, we previously showed a major impact on NK cell immune reconstitution by number, cytotoxicity, cytokine production, and phenotype. Children have improved reconstitution after HSCT, and the effect of PTCy on immune reconstitution in children is not known. A multicenter phase II clinical trial was designed to evaluate myeloablative halpoidentical HSCT in children and young adults with high risk hematological malignancies using PTCy. We present here the data on NK cell reconstitution following HSCT in these patients regarding number, function, and phenotype.

METHODS:
A total of 33/31 of patients were enrolled in the study. The inclusion criteria for the study was patients between ages 0.5-20 years who were then treated with busulfan and cyclophosphamide before being infused with partially HLA mismatch T cell replete bone marrow. Immune cell reconstitution, including NK cells, was evaluated on Days 30, 60, 100, 180 and 365 following the transplant.

RESULTS:
PBMCs isolated from patient samples were used in cytotoxicity experiments against K562 targets and showed 20-50% lysis, which slightly increased from Day 30 through Day 100, and then declined again through Day 365.

CONCLUSION:
Cytotoxic reconstitution of NK cells in children after haploHSCT with PTCy recapitulate our previous findings in adults. Phenotypic characterization using high-dimension mass cytometry (CYTOF) and further functional cytokine analysis are ongoing to determine whether children receiving haplo HSCT with PTCy for hematologic malignancies might benefit from donor NK cell adoptive transfer.

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NK GENE SIGNATURES AND SUBSETS IN AUTOIMMUNITY

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Multiple sclerosis (MS) and systemic lupus erythematosus (SLE) are autoimmune conditions that have been long considered to be T cell and B cell conditions respectively. Previously we found gene signatures in MS that are suggestive of a natural killer (NK) cell involvement, with expression of NK differentiation genes TBX21 and EOMES low in MS patients. Following on from this finding, we aimed to investigate whether NK cell gene signatures can be found in other autoimmune diseases such as SLE and to interrogate the NK cell subset that may drive these gene signatures in both MS and SLE. To investigate whether NK cell genes were aberrant in SLE, whole blood RNA was extracted from 46 SLE patients and 20 healthy controls. Gene expression was measured using a Nanostring nCounter mRNA expression assay incorporating over 500 immunological genes. Data was analysed using Partek Genomics Suite to identify differentially expressed genes. An NK signature was found in SLE with significantly lower expression of genes such as KLRC2, KLRC1, KLRB1, KLRF1, KLRG1, PRF1 and IL2RB. Using both NK gene signatures from MS (previously discovered) and SLE we designed and optimised a 21-marker flow panel to discern what cell subsets are driving these signatures. The NK signatures found in MS and SLE recognise a role for this arm of the innate immune response in the development and/or progression of these autoimmune diseases. The NK gene signatures also give us a clue to what needs to be investigated at the protein level to find NK subsets that may be aberrant in these conditions and could be targeted for treatment.

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ONCOGENIC FUNCTIONS OF MUTANT STAT5B IN INNATE(-LIKE) LYMPHOCYTES

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STAT5B is a master regulator of development, survival and function of innate and innate-like lymphocytes (including natural killer (NK) and NKT cells). The gain-of-function mutation N642H in the SH2 domain of human STAT5B is associated with aggressive forms of CD56+ T cell and NK cell lymphomas/leukemias. We aim to investigate the ability of STAT5B-N642H to act as an oncogenic driver in innate lymphocyte neoplasms. A mouse model expressing human (h)STAT5B-N642H under the Vav1 promoter develops severe CD8+ T cell neoplasia but no innate lymphocyte disease. We hypothesized that alterations in innate lymphocytes are masked by the T cell disease in the Vav1-driven mouse model. Using a transplantation model we demonstrated that in absence of classical T cells, Vav1-driven hSTAT5BN642H can give rise to an aggressive innate-like NKT cell leukemia, which was serially transplantable and responsive to JAK1/2 inhibitor treatment. Our NKT cell disease model, which closely resembles aggressive human CD56+ T-cell large granular lymphocyte (T-LGL) leukemia, will enable to investigate mechanisms of STAT5B-driven leukemogenesis and new therapeutic approaches. We detected that Vav1-driven hSTAT5B-N642H promotes a temporary expansion of NK cells but no NK cell leukemia development in this model. However, STAT5B-N642H enhances human NK cell proliferation and the mutation is found in patients with aggressive NK cell malignancies, indicating that it could be an oncogenic driver in NK cells. To test this idea, we will generate a novel mouse model in which expression of hSTAT5B-N642H is restricted to the NK cell lineage, thereby avoiding competition with more potently transformed cell types. This model will allow us to study whether and how mutant STAT5B may contributes to NK cell transformation. Mechanistic insights into mutant STAT5B-driven alterations during NK cell transformation might uncover new treatment options for currently untreatable NK cell malignancies.

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REGULATORY ROLE FOR NATURAL KILLER CELLS IN A MOUSE MODEL OF SYSTEMIC JUVENILE IDIOPATHIC ARTHRITIS

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OBJECTIVE
Mice deficient in interferon-gamma (IFN-γ KO mice) develop a systemic inflammatory syndrome in response to complete Freund's adjuvant (CFA). Symptoms resemble systemic juvenile idiopathic arthritis (sJIA), a childhood immune disorder of unknown etiology characterized by arthritis and serious systemic and hematological features, including spiking fever, hepatosplenomegaly, lymphadenopathy, anemia, thrombocytosis and neutrophilia. Wild-type (WT) mice experience a more subtle inflammation in response to CFA. Dysregulation of innate immune cells is considered to be important in the disease pathogenesis. In this study, we used this murine model to investigate the role of natural killer (NK) cells in the pathogenesis of sJIA.

RESULTS
NK cells of CFA-challenged IFN-γ KO mice displayed an aberrant balance of activating and inhibitory NK cell receptors, lower expression of cytotoxic proteins perforin and granzyme B, and a defective NK cell cytotoxicity. To investigate if the NK cell defects in IFN-γ KO mice explain their increased susceptibility to develop sJIA upon CFA challenge, NK cells were depleted by injection of anti-IL-2 receptor β- and anti-Asialo-GM1-antibodies in CFA-challenged WT mice. Depletion of NK cells resulted in increased severity of systemic inflammation and appearance of sJIA-like symptoms in WT mice. NK cells of CFA-challenged IFN-γ KO mice showed defective degranulation capacities towards activated autologous CD4+ T cells, dendritic cells and monocytes/macrophages. This is in line with the increased numbers and activation status (e.g. Rae-1, CD80, CD86, CD69…) of monocytes/macrophages and dendritic cells in these mice.

CONCLUSION
NK cells are defective in a mouse model of sJIA and impede disease development in CFA-challenged WT mice. Our findings point towards a regulatory role for NK cells in CFA-induced systemic inflammation possibly via control of activated monocytes/macrophages and dendritic cells.

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THE IMPACT OF DONOR KIR2DL1 ALLELE COMBINATIONS ON OUTCOME AFTER UNRELATED DONOR TRANSPLANTATION IN PATIENTS WITH ACUTE MYELOID LEUKEMIA

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To predict outcomes after allogeneic hematopoietic cell transplantation (alloHCT) using donor information on killer cell immunoglobulin-like receptor (KIR) gene polymorphisms is an ongoing challenge. With this study we focused on donor KIR2DL1 presence/absence, gene copy number, amino acid polymorphisms, allele content, phylogenetic tree-based clade assignment and patient C2-ligand in a model for relapse and mortality. We studied a large cohort of 2'222 patients with AML (80%) or MDS (20%) who received an alloHCT between 2005 and 2017. Donor samples were retrieved from the Collaborative Biobank and mapped to patient outcome data from the German Registry for Stem Cell Transplantation & the German Cooperative Transplant Study. The samples were genotyped for KIR and HLA with high-resolution amplicon-based Next Generation Sequencing (NGS).

No significant differences on cumulative incidence of relapse (CIR) or survival endpoints could be observed regarding presence/absence. A significantly lower CIR, however, was observed for two or three KIR2DL1 genes vs. none or one gene (Hazard Ratio (HR)=0.79 (95%-CI: 0.67-0.94), p=0.008, Wald test in adjusted Cox model). Presence or absence of at least one KIR2DL1 variant with a defined amino acid at position 16 (Proline/Arginine), 154 (Threonine/Proline), 182 (Histidine/Arginine), 216 (Glutamate/Lysine) and 245 (Arginine/Cysteine) showed no significant impact on patients outcome whereas for Leucine at position 114 we found a lower relapse incidence (HR=0.82 (0.69-0.97), p=0.021). When looking at presence or absence of KIR2DL1 alleles, partly data driven analyses showed that donors with a *003 + *001 genotype conveyed lower relapse incidences. Both alleles can be assigned to two phylogenetic clades (clade 1 + 2), both contain the same amino acids at position 154, 182, 216 and 245. Our data suggest that donors with at least two KIR2DL1 genes from clades 1 or 2 (47% of all donors) might reduce the risk of relapse (HR=0.82 (0.69-0.97), p=0.019) and improve Relapse-Free-Survival (RFS) (HR=0.88 (0.78-0.99), p=0.038). Intriguingly, this effect was most pronounced for C1/C1 patients pointing to the possibility that other KIRs determine this effect. We will further investigate genetic linkage and the functional differences between the KIR2DL1 clades. This outcome prediction model should be validated in independent cohorts to evaluate it's potential for improved donor selection.

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UNBIASED MASS CYTOMETRY ANALYSIS IN A MURINE MODEL OF ALLERGEN-SPECIFIC IMMUNOTHERAPY SUGGESTS A ROLE FOR NK CELLS TOLERANCE INDUCTION

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Allergen-specific immunotherapy (AIT) is currently the only curative treatment for recurrent and persistent allergic diseases. Based on recent evidence showing that high doses of the adjuvant CpG oligodeoxynucleotides (CpG) induce immune tolerance, we developed a high CpG-based AIT (CpG-AIT) in a murine model of asthma induced by the major cat allergen Fel d 1. CpG activates Toll-Like Receptor 9 (TLR9) expressing cells, among which are plasmacytoid dendritic cells (pDCs) B cells, and natural killer (NK) cells. Very little is known about the role of NK cells in AIT.

We focused our analysis on the early and late changes during the Fel d 1 CpG-AIT. BALB/c mice were sensitized by three i.p. injections using Fel d 1 and Al(OH)3 (days 9, 14, 28). Subsequently, mice received three i.p. courses of Fel d 1 + CpG-AIT (days 42, 56, 70). Finally, nasal instillation challenges using Fel d 1 were applied three consecutive days (once a day, days 83, 84 and 85). Immune cells were analysed at two time points: 24 hours after the first Fel d 1 CpGAIT injection (day 43), and after the final Fel d 1 challenge (day 86). Three groups of animals were investigated: i) allergic non-treated; ii) allergic, treated; and iii) non-allergic, non-treated. Cells from peritoneal cavity, mediastinal lymph nodes (MLN), spleen and lungs were acquired by mass cytometry. We analysed high-throughput single-cell mass cytometry data through a novel and unbiased method.

Results showed that as soon as 24 after the first treatment injection, pDC ratio rapidly increased at the injection site and in the analysed immune organs, suggesting a TLR9-dependent recruitment and migration. Concomitantly B cells showed signs of activation and early tolerance signatures. Unexpectedly, we found a significant increase in the ratio of Tbet+ NK cells in the peritoneal cavity (43.4%±11.65 vs. 58.1%±8.7) and the MLN (51.3%±9.6 to 72.4%±10.5) of treated mice. These Tbet+ NK cells showed a two-fold increase in the expression of tumour necrosis factor receptor 2 (TNFR2). NK cells from spleen of CpG-AIT-treated mice exhibited de novo expression of CD69 and TNFα. Upon completion of the treatment, at day 87, main allergic hallmarks were reduced, but no significant changes occurred in NK cells.

In summary, NK cells from PC and secondary immune organs showed an increased activation upon CpG-AIT treatment, suggesting a role for NK cells in early stages of CpG-AIT via TNF-α/TNFR2 pathway.

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