Comparative analysis of CD1d-restricted natural killer T cells in people who inject drugs with chronic or spontaneously resolved hepatitis C

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Background and aims:
Natural killer T (NKT) cells represent a subset of immune cells that share characteristics of the innate and adaptive immunity. Invariant NKT cells recognize glycolipid antigens such as α galactosylceramide (αGalCer) presented by the non classical MHC molecule CD1d. Decreased NKT cell frequencies have been reported in chronically HCV infected patients, however, contradicting reports exist. We therefore aimed to comparatively study NKT cell frequencies and function in people who inject drugs (PWID) with chronic and spontaneously resolved HCV infection.

Patients and Methods:
CD1d-restricted NKT (CD1d NKT) cells of chronically HCV infected PWID (n=28) and PWID with resolved HCV infection (n=33) were analyzed by flow cytometry utilizing a CD1d-tetramer complexed with αGalCer.

Results:
CD1d NKT cell frequencies did not differ between PWID with resolved HCV infection and PWID with chronic HCV infection. Interestingly, phenotypic analysis of CD1d NKT cells of chronically infected PWID showed significantly higher expression of the activation marker CD38 ($p=0.0008$), however, this was not associated with apparent differences in PMA/ionomycin-stimulated effector functions (IFNγ, IL 2, TNFα, IL 4 and CD107a). Treatment of PBMCs with αGalCer induced robust CD1d NKT cell expansion associated with upregulation of CD38 independent of the HCV status. While no effect of IFNα stimulation on CD1d NKT cell activation could be detected, treatment with a combination of IL 12/15 and 18, which are described to activate NK cells, leads to a significant increase in CD38+ CD1d NKT cells ($p<0.01$). In peripheral blood, monocytes were the cell type with the highest level of CD1d expression. Although CD1d was further upregulated by IFNα treatment ($p=0.012$), there was no difference in CD1d expression on peripheral monocytes between PWID with chronic and resolved HCV infection. Nevertheless, presence of monocytes promoted NKT cell activation by αGalCer in vitro as depletion of monocytes significantly reduced the frequency of CD38+ CD1d NKT cells ($p<0.05$).

Conclusion:
Chronic HCV infection in PWID is associated with increased expression of the activation marker CD38 on CD1d NKT cells, possibly via type I interferon-induced upregulation of CD1d on monocytes or other liver resident cells or via direct activation by pro-inflammatory cytokines. The functional consequences of increased activation of CD1d NKT cells are not fully defined.

Domain 1-Negative NKp46 Splice Variants Dominate Human Nasal Lavage Following Respiratory Viral Infection and Manifest Higher Activity

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Natural Killer (NK) cell activating receptor NKp46/NCR1 is a key receptor that is involved in elimination of virus infected and tumour cells. NCR1 gene is transcribed into five different splice variants. Functional importance and physiological distribution of NKp46 isoforms are yet not fully understood. Here we shed light on differential expression of NKp46 splice variants in viral respiratory tract infections and their functional difference at cellular level. NKp46 was the most predominantly expressed NCR in the nasal lavage of patients infected with respiratory virus respectively respiratory syncytia virus, adenovirus, human metapneumovirus, or influenza A. A noticeable correlation between NKp46 and IFN-γ as well as NKp46 and TNF-α proved strong infiltration of NK cells within respiratory tract after viral infection. Expression of NKp30 was very less and NKp44 was absent in all of the patients. Domain 1-negative NKp46 splice variants i.e. NKp46 isoform d was the highly expressed NKp46 isoform in the nasal lavage following viral infections. Unique property of our laboratory generated anti NKp46 mAb, D2-9A5 which recognize D2 domain of NKp46 and commercial anti NKp46 mAb, 9E2 which recognize D1 domain of NKp46 helped us to identify a small subset of NKp46 D 1 negative splice variant within cultured human primary NK cells. This NKp46 D 1 negative subset also showed higher degranulation efficiency in term of CD107a surface expression. NK92 cell lines expressing NKp46 D1 negative and NKp46 D 1 positive splice variants also showed functional difference when interacting with cancer cell. NKp46 D 1 negative isoform expressing NK92 cell line showed more degranulation activity. To our knowledge, for the first time we bring in front the conclusive evidence showing the physiological distribution and functional importance of NKp46 splice variants in pathological condition.

Functional analysis of soluble NKG2D ligand expression in umbilical cord blood plasma reveals activating potential of soluble MICA-129val variants

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We previously reported that cord blood plasma (CBP) contains significantly higher levels of soluble NKG2D ligands (sNKG2DLs), such as sMICB and sULBP1, compared to healthy adults. sNKG2DL engagement with its receptor NKG2D causes NK cells to become hyporesponsive and is thought to be an important mechanism leading to tumour or viral immune escape. As sNKG2DLs are present in CBP we hypothesise that they could represent an additional fetal-maternal tolerance mechanism.
The current work focuses on functional analysis of 181 CBP samples. We correlated potential to suppress production of IFN-gamma by stimulated NK cells to concentration of sNKG2DLs detected in CBP. Moreover, to detect possible allelic differences we have carried out typing of cord blood for MICA and MICB allelic and promoter types.
We found strongest correlations between sNKG2DLs and reduced NK cell function related to increasing concentration of exosomal sULBP1, which was present in all CBP samples tested with a possible maternal source. Our previous experiments indicated a possible fetal source of sMICA/B, which was not associated with exosomes. We found that common MICB alleles, such as MICB*005:02 result in the highest concentrations of sMICB. Interestingly, MICB*005:02 was the only allele found to have more than one type of promoter polymorphism and was associated with 7 different types of promoter. Among these promoter polymorphisms, P2 resulted in the highest expression of sMICB and most suppression of IFN-gamma and P9 the least sMICB expression and most IFN-gamma production, indicating that sMICB is also suppressing NK cell function. The overall contribution of sMICA in CBP to this mechanism was surprising and appeared counter-intuitive. Owing to a lack of MICA homozygotes, we examined the functional dimorphism encoding Met or Val at residue 129. We found most expression of sMICA associated with Val/Val, some with Met/Val but none with Met/Met. MICA molecules having Met or Val at position 129 associate with strong or weak NKG2D binding, respectively. However, expression of sMICA in CBP was associated with more production of IFN-gamma and therefore had the opposite effect to what was expected.
We propose a model for fetal-maternal tolerance whereby NK cell activity is limited by the presence of sULBP1 and sMICB in CBP. The release of 129val sMICA can reduce the overall net suppressive signal and break tolerance, allowing fetal NK cells to overcome immunological threats in utero.

Recognition of Ebola-GP expressing target cells by natural killer cells.

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Natural killer (NK) cells are lymphocytes of the innate immune system that deliver a selective cytotoxic effect against target cells such as virally infected and tumor cells. NK cell function involves cytokine secretion and tissue remodeling. NK cellular cytotoxicity is delicately balanced by signals mediated through inhibitory and activating receptors expressed by NK cells. The expression of ligands recognized by NK inhibitory receptors, such as HLA class I molecules, serves as a positive indicator of the integrity of cells, protecting against NK cell mediated cytolysis of normal healthy cells. Activating ligands, however, are usually stress-induced antigens signaling cellular distress caused by transformation or infection. Expression of such antigens will evoke NK cellular responses inducing lysis of the expressing cell and cytokine secretion by the NK cell to alarm the immune system.
The Ebola virus has been shown to possess viral evasion mechanisms that directly interfere with T Cell antiviral responses. By steric shielding over HLA-A,B,C, the viral glycoprotein GP can block TCR interaction thus leaving T Cells unable to attack infected cells. This mechanism could promote increased NK cell function against GP expressing cells; however, human primary NK cells were less reactive to GP-expressing HEK293 cells. This was demonstrated by reduced cytokine secretion, reduction in NK degranulation and reduced lysis of GP expressing target cells.
No direct interaction has been detected between Ebola GP and different NK cell receptors; however, we demonstrated that GP-expressing cells manifested a decrease in detection of GP expressing target cells by recombinant NKG2D and NKp30 receptors. MAb-based staining of NKG2D ligands (MICA, MICB, ULBPs) and NKp30 ligands (B7-H6) revealed the same effect. Moreover, digesting the GP on HEK293 by trypsin recovered the recognition by recombinant NKG2D receptor and by mAbs for NKG2D ligands. The cleaving of GP by trypsin also recovered the recognition of HLA class I molecules by W6/32 mAb. This data supports previous findings showing steric shielding of the Ebola glycoprotein over surface HLA class I and expands the observations to activating ligands for NK cells. Our results suggest a novel evasion mechanism employed by the Ebola virus to avoid NK cell immune response.

Adaptive NKG2C+ NK cells recognize HCMV strains in a peptide-specific manner

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Human Cytomegalovirus (HCMV) infection shapes the human NK-cell repertoire and, in a proportion of HCMV-seropositive individuals, is associated with the clonal-like expansion of an adaptive subset of NK cells expressing the activating receptor NKG2C. Adaptive NKG2C+ NK cells parallel memory CD4+ Th1 and CD8+ cytotoxic T lymphocytes in their global epigenetic landscape and display epigenetically imprinted cytokine memory as well as changes in the utilization of signaling molecules. Despite their clonal-like expansion and epigenetic signature, additional adaptive properties such as antigen specificity have not been characterized.
We have assessed the activation requirements of adaptive NKG2C+ NK cells and observed that NKG2C exhibits fine recognition of HCMV-encoded peptides. Engagement of NKG2C by peptides derived from different HCMV strains resulted in strikingly differential activation of effector functions and proliferation as well as in generation of adaptive signatures with different requirements for co-stimulation. We propose that peptide-specific recognition is a hallmark of adaptive NK cells and that differential activation by viral ligands controls the expansion and the phenotypic shift from conventional to adaptive NKG2C+ NK cells in HCMV-infected individuals in a strain-dependent manner.

HLA-Bw4 80(T) and multiple HLA-Bw4 copies combined with KIR3DL1 are associated with superior immune control of HCV infection in people who inject drugs

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Background and aims:
NK cell function is regulated by inhibitory and activating receptors including killer-cell immunoglobulin-like receptors (KIRs). Here, we analyzed the impact of different KIR/KIR-ligand genotypes on the outcome of HCV infection in people who inject drugs (PWID). KIR/KIR-ligand genotypes associated with spontaneous clearance of HCV infection were identified in a cohort of PWID from Germany (n=266) and further validated in a second anti-HCV positive cohort of PWID recruited in North America (n=342). Moreover, NK cells of PWID and healthy donors were functionally characterized according to their KIR/KIR-ligand genotype by flow cytometry.

Results:
Multivariate logistic regression analysis revealed that KIR3DL1/HLA-Bw4 80(T) was associated with spontaneous clearance of HCV infection in PWID, which was confirmed in the PWID cohort from North America. Moreover, compared with PWID with detectable HCV-RNA the frequency of individuals with multiple HLA-Bw4 alleles was significantly higher in anti-HCV positive PWID with resolved HCV infection (29.7% vs. 15.2%; p=0.0229) and in anti-HCV seronegative PWID (39.2%; p=0.0006). KIR3DL1+ NK cells from HLA-Bw4 80(T)-positive PWID showed superior functionality compared to HLA-Bw4 80(I)-positive PWID. This differential impact was not observed in healthy donors, however, here, the HLA-Bw4 copy number strongly correlated with the functionality of KIR3DL1+ NK cells.

Conclusion:
HLA-Bw4-80(T) and multiple HLA-Bw4 copies in combination with KIR3DL1 are associated with protection against chronic hepatitis C in PWID by distinct mechanisms. Better licensing of KIR3DL1+ NK cells in the presence of multiple HLA-Bw4 copies is beneficial prior to seroconversion whereas HLA-Bw4 80(T) may be beneficial during acute hepatitis C.

T cell regulation by NK cells via the activating receptor NCR1 (NKp46) during virus infections

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Natural killer (NK) cells are known for their function in recognizing and eliminating infected cells as well as "altered self" cells. By the expression of various inhibitory and activating receptors NK cells are able to sense sudden changes and exert their effector functions without further stimulation. There is raising evidence that NK cells have the ability to regulate T cell responses. It could be demonstrated that T cells lacking signal 3 during activation were killed by NK cells via the activating receptor Natural cytotoxicity receptor (NCR) 1 (NKp46) early during acute (Lymphocytic Choriomeningitis virus) LCMV infection. In this study, we examine the influence of NCR1 on T cells during acute and chronic LCMV infection.
Virus-specific CD8 T cells are increased and appear to be more activated in the absence of NCR1 which implicates a regulatory mechanism mediated via NCR1 engagement on NK cells during early acute and chronic LCMV infection. The increased presence of activated virus-specific CD8 T cells results in exacerbated immunopathology, implying that NCR1 and its ligands are involved in balancing antiviral immunity which is critical in the context of chronic infections. In line, early during chronic infections viral titers are reduced in absence of NCR1. Interestingly, numbers of virus-specific T cells decline later during chronic infection in absence of NCR1 showing that the increase of number and effector functions is temporary. In absence of NCR1, CD4 T cells differentiate preferably into TH1 cells and to a lower extent into CD4 follicular helper cells during chronic infection.
Abrupt changes during T cell activation might render overstimulated T cells or improperly activated T cells into NK cell targets which could be recognized via NCR1. We therefore propose that NK cells via NCR1 control highly activated T cells early during LCMV infection to protect the host from an overshooting T cell response.

Upregulation of 2B4 (CD244) is associated with altered iNKT cell function during HIV infection

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Introduction: Invariant natural killer T (iNKT) cells are a specialized subset of T lymphocytes that expresses a highly restricted T cell receptor (TCR) to recognize self and foreign glycolipid and phospholipid of microbes presented by CD1d as cognate antigens. These cells have been implicated in innate immune responses against human immunodeficiency virus (HIV). However, the determinants of cellular dysfunction across the iNKT cell subsets are seldom defined in HIV disease progression.

Objective: Here we explore the role of negative check point regulatory molecule 2B4 on iNKT cell during HIV-1 infection.

Methods: We analyzed peripheral blood mono nuclear cells (PBMCs) by flow cytometry from 48 HIV-infected individuals, including antiretroviral therapy (ART) naïve (n=23), ART-treated (n=19), elite controllers (ECs, n=6) and healthy controls (n=15). To assess intracellular IFN-γ, 3x106 cells were stimulated with 100ng/ml α-galactosylceramide (GalCer) in a 24-well culture plate.

Results: Herein, we provide evidence for the involvement of the negative checkpoint regulator molecule 2B4 in iNKT cell alteration during HIV-1 infection in a well-defined cohort of HIV-untreated, ART-treated and elite controllers (ECs). We report an exaggerated 2B4 expression on peripheral blood iNKT cells in ART naïve subjects compared to healthy controls. Further, we observed a severe depletion of CD4+iNKT cell subset in ART naïve subjects. In sharp contrast to CD4-iNKT cells, 2B4 was predominantly expressed on CD4+iNKT cell subset in ART naïve subjects. Given the suppressive role of 2B4 in HIV-specific CD8+ T cells, we examined the levels of 2B4 expression on T-cell subsets including CD3+, CD4+ and CD8+ T cells. The expression of 2B4 was markedly increased on bulk CD3+ T cells of ART naïve subjects. In contrast to CD4+ T cells, CD8+ T cells displayed significantly higher levels of 2B4 in ART naïve individuals. Notably, an increased level of 2B4 on iNKT cells was strongly correlated with parameters associated with HIV disease progression (viral load, CD4+T cell counts and CD4/CD8 ratio). Further, iNKT cells from ART naïve individuals were lacking in their ability to produce intracellular IFN-γ after GalCer stimulation and IFN-γ+iNKT cells were directly correlated with viral load and inversely correlated with CD4+T cell counts.

Conclusion: Our results suggest that the levels of 2B4 expression and the downstream co-inhibitory signaling events potentially contribute to impaired iNKT cell responses. Hence, the 2B4 pathway could be a key target for prospective immunotherapeutic interventions against HIV infection.

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Effector functions of NK cells depend on the severity of retroviral infection

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Natural killer cells (NK cells) are part of the innate immunity. They exert a beneficial antiviral potential during mouse cytomegalovirus (MCMV) infection but display a more regulatory function during lymphocytic choriomeningitis virus (LCMV) infection. Furthermore, during LCMV or influenza infection, the outcome of the pathogenesis greatly depends on the viral dose.
Here, we used the well-established FV mouse model for the analysis of NK cell responses during early retroviral infection (3 days post infection) after infection with low and high viral doses.
The infection of mice with high viral doses did not result in elevated viral titers. Furthermore, high-dose FV infection significantly improved the maturation and activation of NK cells compared to low-dose infected mice. In vivo stimulation of NK cells using an IL-2/anti-IL-2 mAb complex significantly diminished viral loads in low-dose FV-infected animals but viral loads from high-dose infected animals did not change. Besides differences in the activation, NK cells from low-dose FV-infected animals revealed a decreased degranulation capacity and reduced FasL expression ex vivo. We also detected an augmented cytotoxic capacity of NK cells correlating with the severity of infection in vitro and also in vivo. Ablation of NK cells in high-dose FV infection increased viral loads whereas no difference in viral burden was detectable after low-dose infection. Moreover, we detected differences in the activation of dendritic cells and macrophages in high- and low-dose viral infections, which were associated with an altered secretion of cytokines such as IL-15 and IL-18.
The current study demonstrates that the severity of retroviral infection is important for the initialization of an efficient NK cells response.

NCAM1 (CD56) on human blood natural killer (NK) cells has a functional role in recognizing the fungal mold A. fumigatus

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Introduction: Aspergillus fumigatus is a saprophytic fungal pathogen recognized and cleared by the innate and adaptive immune system (Cramer et al. 2011). The clearance of the mold is ineffective under immunosuppression and A. fumigatus causes severe infections in those patients. A study from Stuehler et al. (2015) showed that NK cells influence the outcome of invasive aspergillosis (IA) in patients after allogenic stem cell transplantation (HSCT) since lower cell counts and later cell reconstitutions contributed to a worsened outcome of IA. Former studies showed that NK cells act against the fungus by the secretion of IFN-gamma and perforin (Bouzani et al. 2011, Schmidt et al. 2011).
Objectives: We investigated the direct interaction of human blood NK cells with A. fumigatus.

Material & Methods: NK cells were isolated from healthy donors and were pre-stimulated with Pro-Leukine overnight. Cells were co-cultured with fungal germ tubes (MOI 0.5) for different time points. NCAM-1 interaction with A. fumigatus was investigated by real-time PCR, flow cytometry, western blot analysis, confocal laser scanning microscopy (CLSM) and direct stochastical reconstruction microscopy (dSTORM). Cytoskeletal analysis were performed by treatment with cytochalasin D, colchicine and staining with phalloidin. Functional NCAM1 analyses were performed by blocking of NCAM1 with monoclonal anti-CD56 antibody (Daniela Pende and Alessandro Moretta, Università di Genova, Italy).

Results: Flow cytometric analyses showed that NCAM-1 fluorescence intensity was decreased after co-culture with A. fumigatus while NK cell activation measured by CD69 fluorescence intensity increased to the same amount. By microscopy, we proved that NCAM-1 relocates to the fungal interface and that NCAM1 binding to A. fumigatus is specific since soluble NCAM1 binds fungal morphologies. NCAM1 re-localization was not associated with apoptosis, internalization or shedding of NCAM1, nor did we see changes on gene expression level. We further showed that this re-localization is an actin dependent mechanism since NCAM1 fluorescence intensities did not change when NK cells were pre-treated with cytochalasin D compared to untreated NK cells co-cultured with the fungus. Blocking of NCAM1 diminished re-localization to the contact side and inhibited fungus- mediated NK cell activation, indicating a functional role of NCAM1.

Conclusion: We conclude that NCAM1 plays a functional role in the early fungal recognition. Further studies have to clear the question which pathways are activated upon NCAM1 – A. fumigatus interaction.

Polarized macrophages use veiled surface structures to ingest HIV particles as well as NK-effectors.

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We established a culture system to characterize the inflammatory immune response of monocytes and dendritic cells in various diseases such as severe sepsis, diabetes type 2, autoinflammatory disorders such as macrophage activation syndromes (MAS), and from conditions with major tissue repair activities following trauma. After in vivo exposure to local growth factors, pro-inflammatory cytokines, and microbial compounds, these cells differentiate into macrophages and dendritic cells (Tacke and Randolph 2006). While these monocytes play a key role in eliminating invading bacteria, viruses, fungi, and protozoans, they can also play a role in the pathogenesis of inflammatory and degenerative diseases. In the present investigation we studied the sensitivity of NK-mediated cytolysis as well as virus-uptake mechanisms using recombinant HIV-particles with and without serum albumin (HSA) pre-incubation to address the effect of a novel albumin-derived peptide, EPI-X4 (Zirafi et al. 2015). Methods: In vivo activated antigen presenting cells were enriched by cell 14-28days of culture. Cell populations were were characterized by flow cytometric analysis to distinguish inflammatory macrophages (designated M1, M2a-M2c) and anti-inflammatory macrophages termed M2d. After 72h, the cell cultures were inoculated with HEK derived HIV-1 particles. After 48h, the cell cultures were examined by fluorescence and electron microscopy. Results: Different macrophages and dendritic cell subtypes differed regarding virus uptake structures, persistence of virus particles and susceptibility to NK-mediated cytolysis. HSA-pre-incubation significantly reduced HIV-uptake and increased NK-cytolysis by impairing the ingestion of NK-effectors. Most pronounced effects by HSA-treatment were observed in M2 type macrophage cultures. Macropinoctosis of HIV and NK cells occured by membrane protrusions of the phagocte. Thus impaired NK function may be due to macropinocytosis of the effectors. The blockade by HSA, suggests the involvement of CXCR4 or CXCR4/CXCR7 hybrid receptors, binding the HSA-derived Peptide EPI-X4. Conclusion: The current investigations suggest a novel virus specific uptake mechanism in macrophages and dendritic cells by macropinocytosis involving newly defined surface structures. The HSA-derived peptide EPI-X4 appears to block macropinocytosis and implies the involvement of CXCR4 and/or CXCR4/CXCR7 hybrid receptors.
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O. Zirafi et al. 2015. Cell Reports 11:1-11

Intracellular immunodynamics of lentiviral gene delivery in human Natural Killer cells

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Natural Killer (NK) cells are members of the innate immune system that target tumors and infected cells. Cancer immunotherapy approaches using genetically modified NK cells continue to inspire clinical trials with promising results but the protocols for genetic modification of NK cells are suboptimal.
NK cells they show strikingly high resistance to lentiviral gene delivery when compared to other cells of the immune system. Previously, we have shown that the use of BX795, a small molecule inhibitor of TBK1/IKK complex downstream of Toll-like Receptors and RIG-I-like receptors, significantly enhances lentiviral gene delivery to NK cells.
In the current study, we demonstrate that that while viral vector entry to NK cells can take place without major problems, the activation of antiviral signaling pathways leads to intracellular elimination of the vector. To study roles of 20 candidate genes in this process, CRISPR/Cas9 system was used and single-gene disruptions were applied in 293FT and NK-92 cell lines.
Our results demonstrate that, capsid recognition by TRIM5 in 293FT cells and dsRNA-induced signaling through RIG-I and TRIM25 in NK-92 cells represent major players affecting lentiviral gene delivery. Additionally, viral vector exposure was shown to increase MAPK activity in host cells, specifically p38 and JNK phosphorylation in NK-92 cells, as observed in wildtype HIV infections.
Overall, this study confirms that lentiviral gene delivery evokes an innate immune response in NK cells through multiple PRRs and cellular restriction factors. Small molecule inhibitors help to overcome this obstacle for promising applications in immunotherapy using genetically modified NK cells.

HIV-1-mediated downmodulation of HLA-C impacts target-cell recognition and antiviral activity of NK cells

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Interaction of inhibitory receptors with their respective HLA class I ligands confers functional competence (licensing) to natural killer (NK) cells. In contrast, NK cells lacking inhibitory receptors for self HLA class I are rendered hyporesponsive. Licensed NK cells may sense HIV-1-mediated alterations of HLA class I expression through poor binding of self-inhibitory receptors, thus potentially influencing the control of HIV-1 replication. Here we show that HIV-1-mediated downmodulation of HLA-C was associated with reduced binding to its respective inhibitory receptors. Despite this binding difference, licensed NK cells expressing self-inhibitory receptors for HLA-C displayed reduced antiviral activity in vitro as compared to their unlicensed counterparts, potentially due to residual inhibitory KIR binding. Blocking of self-inhibitory receptors in turn improved the antiviral activity of licensed NK cells. Nevertheless, NK cells were able to sense alterations of HLA-C expression demonstrated by increased antiviral activity when exposed to a viral strain which robustly downmodulated HLA-C in contrast to a strain that lacked this ability. Thus, our results suggest that the capability of HLA-C-licensed NK cells to control HIV-1 replication is determined by the strength of KIR/HLA-C interactions and is thus dependent on both the host's genetic background and the extent of virus-mediated HLA downregulation.

NK cell heterogeneity, diversity, and function in chronic hepatitis C virus infection and during interferon-free treatment

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Chronic viral infections, such as chronic hepatitis C virus (HCV) infection, are known to cause immune cell exhaustion, including alterations in NK cell phenotype and function. Novel interferon-free treatment strategies against HCV, based on direct-acting antivirals (DAAs), offer the opportunity to study whether rapid elimination of a chronic viral infection leads to restoration of the NK cell compartment. Here, we longitudinally studied NK cells in 26 chronic HCV patients before, during, and after DAA treatment. Two-thirds of the patients cleared the virus whereas one-third experienced a viral relapse after treatment cessation. The high-dimensional analysis approaches stochastic neighbor embedding, donor-to-donor expression variation, and diversity analysis revealed chronic HCV infection to cause a significant imprint on the NK cell compartment. However, DAA treatment resulted in only minor phenotypic alterations. Compared to the clear phenotypic imprint caused by chronic HCV, NK cell function showed only minor alterations before treatment. Instead, DAA treatment and clearance of HCV modulated NK cell ADCC and cytokine responses. Taken together, the study illustrates how a chronic viral infection affects the human NK cell compartment and dissects which features that are reversed upon successful elimination of the virus.

NK cell frequency and function in SIV-infected macaques controlling viral replication

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Within the last years studies tried to reveal the role of NK cells in HIV infection. These multifunctional effector cells exert direct and indirect antiviral and immunomodulatory effects. Simian immunodeficiency virus (SIV)-infected rhesus macaques represent the most utilized non-human primate model for HIV research. As known for HIV patients, a rare group of SIV-infected rhesus macaques is capable of controlling infection in the absence of anti-retroviral therapy. Still, some animals lose their controller-status and progress to AIDS. The underlying mechanisms are not fully understood.
We aim at analyzing NK cell frequencies, phenotype and function in this special group to elucidate the role NK cells may play in disease control or progression.
We have access to a group of 17 controllers, which according to their plasma viral load were divided into elite controllers (n= 8, <100 copies/ml plasma), viremic controllers (n= 5, 102-104 copies/ml plasma) and failing controllers with increasing viral loads after a longer period of effective viral suppression (n= 4, >104 copies/ml plasma). Using flow cytometry we analyzed frequencies and absolute cell numbers of CD3- CD8a+ CD20-/dim CD159a+ NK cells in our controller cohort and compared the data to those of eight healthy controls. Furthermore, we performed functional assays to assess NK cell degranulation (CD107a expression) as well as granzyme B and perforin secretion in response to stimulation with target cells.
Highest NK cell frequencies and absolute numbers were detected in failing controllers (6.3% ± 2.4%; 47 ± 24), while elite controllers showed significantly lower frequencies (2.5% ± 0.7%; 29 ± 9), also compared to healthy controls (4.9% ± 2.4%; 51 ± 27; viremic controllers: (3.9% ± 2.7%; 32 ± 19). Overall, NK cell frequencies correlated with plasma viral load.
Despite varying NK cell frequencies, expression of CD107a did not differ between the different controller groups and in comparison to healthy animals. Nonetheless, failing controllers exhibited the highest perforin and granzyme B expression reaching significance when compared with healthy controls.
These preliminary results require more functional analysis to verify the possible role of NK cells in long-term SIV control. Upcoming studies will focus on analyzing the phenotype of NK cells in blood, lymph nodes and colonic biopsies. Additionally, the killing capacity as well as well as ADCC (antibody-dependent cellular cytotoxicity) will be analyzed in the different controller groups.

Peripheral and hepatic NK cells from HCV-infected patients express high amounts of CD39 and display an immunoregulatory phenotype

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It has been widely accepted that Natural Killer (NK) cells play a pivotal role in the control of viral infections such as HCV and HIV. The ectonucleotidase CD39 is able to degrade pro-inflammatory extracellular ATP to AMP and, together with CD73, to anti-inflammatory Adenosine. This pathway enables the limitation of inflammatory responses. A contribution of this regulatory pathway to dysfunctional immune responses during viral infection and liver disease has been described.

We investigated the expression of CD39 on NK cell populations derived from peripheral blood (PBMCs) via multicolor flow cytometry in a cohort (n=56) of HCV mono- and HIV/HCV co-infected patients compared to healthy controls. Furthermore we evaluated CD39 expression levels on NK cells derived from liver samples including HCV-infected samples (n=11).

HCV mono- and HIV/HCV coinfected PBMC samples contained higher numbers of CD39-positive NK cells in comparison to healthy controls. Furthermore, we observed a differentiation-associated expression pattern of CD39-positive NK cells. In addition, we found the highest frequencies of CD39 on NK cells derived from liver samples. Paired samples (n=5) reveal a higher expression of CD39 on hepatic NK cells compared to peripheral NK cells. Moreover, the expression of CD39 correlated positively with the modified histologic activity index (mHAI), a clinical score used to classify liver inflammation. Functionally, CD39-positive NK cells showed a higher proliferative capacity and produced larger amounts of Interferon-gamma upon stimulation compared to CD39-negative NK cells.
In this study we showed for the first time that CD39 expression is increased on peripheral and hepatic NK cells in chronic viral infection and that this pathway seems to be relevant for the regulation of NK cell function during viral infection and liver disease in general. Further experiments are required to address the question to what extent these cells are dysfunctional and contribute to a immunotolerant microenvironment in the liver.

Comparative analysis of conventional natural killer cell responses to acute infection with Toxoplasma gondii strains of different virulence

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Toxoplasma gondii is a highly prevalent food-borne obligate intracellular parasitic protozoan present in 30% of humans and a significant health concern in immunocompromised people. Conventional Natural Killer Cells (cNK), members of group 1 innate lymphoid cells, are critical for early immunity to T. gondii via IFNγ production. However, little is understood about how cNK cell functionality, subpopulations, maturation and proliferation are affected by parasite infection and parasite virulence. Here we comprehensively performed this analysis with Type I virulent RH, Type II avirulent ME49 and fully attenuated Type I cps1-1 strains. In response to these three parasite strains, murine cNK cells produce IFNγ and become cytotoxic and polyfunctional (IFNγ+CD107a+) at the site of infection. In contrast to virulent RH and avirulent ME49 T. gondii strains, attenuated cps1-1 strain induced only local cNK cell response. Infections with RH and ME49 parasites significantly decreased cNK cell frequency and numbers in spleen 5 days post infection compared with cps1-1 parasites. cNK cell subsets expressing activating receptors (Ly49H and Ly49D) and inhibitory receptors (Ly49I and CD94/NKG2A) were similar when compared between the strains and at 5 days post infection. cNK cells were not proliferating (Ki67-) 5 days post infection with any of the strains. cNK cell maturation as measured by CD27, CD11b, and KLRG1 was affected after infection with different parasite strains. RH and ME49 infection significantly reduced mature cNK cell frequency and increased immature cNK cell populations compared with cps1-1 infection. Interestingly, KLRG1 was highly expressed on immature cNK cells after RH infection. Cytokine multiplex analysis indicated cNK cell responses correlated with proinflammatory cytokine levels, including IL-12. This study demonstrates infection with RH and ME49 parasites impacts cNK cell maturation during acute T. gondii infection. Different cNK cell responses could impact early immunity and susceptibility to these strains.

CD56dimCD16dim cells: a new human natural killer cell population

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Human natural killer cells can be subdivided in several subpopulations on the basis of the relative expression of the adhesion molecule CD56 and the activating receptor CD16. Whereas blood CD56brightCD16dim/- natural killer cells are classically viewed as immature precursors and cytokine producers, the numerically major CD56dimCD16bright subset is considered as the mostly cytotoxic one. In peripheral blood of healthy donors, we noticed the existence of a minor population of CD56dimCD16dim natural killer cells that was even considerably expanded in occasional control donors but also in two transporter associated with antigen processing-deficient patients, two familial haemophagocytic lymphohistiocytosis type II patients, and several common variable immunodeficiency patients. This population was detected but not expanded in three HIV-1-infected individuals. Among the multiple myeloma cases we investigated, one had an almost exclusive CD56dimCD16dim natural killer cell population. Phenotypically, the new subset contained a high percentage of relatively immature cells, as reflected by a significantly stronger representation of NKG2A+CD226-CD57- cells compared to their CD56dimCD16bright counterparts. From the functional point of view, sorted CD56dimCD16dim cells degranulated less than CD56dimCD16bright cells. The population was also identified in various organs of immunodeficient mice with a human immune system (“humanized” mice) reconstituted from human cord blood stem cells. In conclusion, we have identified a new NK cell subpopulation with distinct phenotypic and functional features, probably representing the immediate precursor cells of the CD56dimCD16bright subset.

Tumor-priming converts NK cells to memory-like NK cells

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Fascinating earlier evidence suggests an intrinsic capacity of human natural killer (NK) cells to acquire adaptive immune features in the context of cytomegalovirus (CMV) infection or pro-inflammatory cytokine stimulation. As the role of memory NK cells in cancer has so far remained elusive and adoptive natural killer (NK) cell transfer in relapsing pediatric acute B cell precursor leukemia (BCP-ALL) patients awaits improvement, we asked the question whether tumor-priming could promote the generation of memory NK cells with enhanced graft-versus-leukemia (GvL) reactivity. Here, we provide substantial evidence that priming of naive human NK cells with pediatric acute B cell leukemia specimens induces a functional conversion to tumor-induced memory-like (TIML)-NK cells displaying a heightened tumor-specific cytotoxicity and enhanced perforin synthesis. Cell cycles analyses reveal that tumor-priming sustainably alters the balance between NK cell activation and apoptosis in favour of survival. In addition, gene expression patterns differ between TIML- and cytokine-induced memory-like (CIML)-NK cells with the magnitude of regulated genes being distinctly higher in TIML-NK cells. As such, the tumor-induced conversion of NK cells triggers the emergence of a so far unacknowledged NK cell differentiation stage that might promote GvL effects in the context of adoptive cell transfer.

NKP46-INDUCED DEGRANULATION IN DIFFERENT NK SUBPOPULATIONS: EVALUATION OF ITS SYNERGY WITH CO-ACTIVATING SIGNALS

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Natural killer (NK) cells have the ability to recognize stressed/transformed cells when MHC molecules are downmodulated or absent, finally producing a rapid immune reaction. Target cell recognition by NK cells is a highly dynamic process controlled by the integration of signals from multiple receptors. These receptors can promote adhesion, cytokine secretion, granule polarization and degranulation or they can inhibit NK cell functions. Among the different NK activating receptors, NKp46 is considered one of the most important molecules in tumor recognition. The aim of the present work is to define the synergy of NKp46 stimulation with different co-activating signals (2B4, DNAM-1, CD2) in the degranulation process of different NK cell subsets.

Experiments were performed at different time point after mononuclear cell isolation on resting or IL-2-cultured NK cells from HLA-typed subjects. In order to mimic the engagement of NK cell receptors with target cell ligands we used beads loaded with combinations of functional monoclonal antibodies direct against different NK receptors. To detect NK cell degranulation, we evaluated the surface expression of CD107a protein. NK cells were then stained with different antibodies (anti-CD16, - CD56, -KIRs and -NKG2A) to distinguish CD56dim from CD56bright subsets or licensed from unlicensed NK cells.

Results indicate that the assay has a good sensitivity. In fact, it allows to evaluate the degranulation of the less cytotoxic, resting CD56bright and unlicensed CD56dim NK cells. Degranulation can be induced only after activation with anti-NKp46, while other antibodies (anti-2B4, -DNAM1, -CD2) increased the percentage of degranulation induced by NKp46 or favor the granule polarization (anti-CD18). As expected, the percentages of degranulation were higher in licensed NK cells compared to unlicensed ones. Nevertheless, anti-NKp46 preferentially synergized with anti-2B4 on resting both licensed and unlicensed CD56dim NK cells, as compared to CD56bright NK cells. The variability of the response turned out to be subject dependent suggesting that NK cell reactivity significantly differ from donor to donor. We also noted that the percentage of NK degranulation increased with time of incubation regardless their cytokine stimulation, suggesting that NK manipulation after withdrawal progressively decreased their threshold of activation.

The confocal microscopy data indicate that the polarization process was not a common event. In fact, the polarization of granules occurred only when the NK stimulation was induced in the presence of a specific anti-CD18 (clone MEM-148) monoclonal antibody.

The expression of the C-type lectin-like receptor Nkrp1g and its ligands in the spleen

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In recent years, interaction and crosstalk of NK cells and DCs have been studied extensively. Such interactions shape the innate immune response via DC-mediated NK cell activation and regulate the adaptive immune response through NK-mediated DC maturation and editing.

We previously reported expression of the inhibitory C-type lectin-like receptor (CTLR) Nkrp1g on a subset of intraepithelial lymphocytes (IEL) in the small intestine of mice that is spatially matched by selective expression of the Nkrp1g-ligand Clr-f (Clec2h) on intestinal epithelial cells (IEC) (Leibelt et al., 2015). Here, we report a strain-specific and exclusive expression of Nkrp1g on a minor subset of mouse splenic NK cells that is matched by expression of the Nkrp1g-ligand Clr-g (Clec2i) on activated splenic CD8α+ cDCs.

Immunomodulatory CTLR such as NKG2D are encoded in the Natural Killer Gene Complex (NKC) in both man and mouse. Amongst these are members of the Nkrp1 and Clec2 gene families that have been shown to constitute genetically linked receptor-ligand systems, with their genes intermingled in the NKC. Nkrp1g forms such a receptor-ligand system together with several Clec2 proteins, namely Clr d, Clr f, and Clr-g, respectively. We showed that Nkrp1g is expressed in the spleen exclusively by a small subset of NK cells. Additionally, this expression is strain-dependent and inherent for that particular NK cell population. After activation, Nkrp1g expression is upregulated on the cell surface of such NK cells. With regard to Nkrp1g ligands, we found Clr-d and Clr-f transcripts to be nearly absent from the spleen, whereas Clr-g transcripts were abundantly and rather specifically detected in hematopoietic organs and throughout different splenic leukocyte populations. A newly generated mAb recognizing Clr-g was employed to investigate Clr-g surface expression: a moderate staining of B cells and monocytes was observed when spleens from unchallenged mice were analyzed. However, the splenic Clr-g expression pattern was altered upon poly I:C treatment. While the Clr-g expression of monocytes and B cells became undetectable, CD8α+ cDCs gained a robust Clr-g surface staining. Also Nkrp1g expression levels on NK cells were upregulated upon poly I:C treatment. We also found that the ligation of Nkrp1g by Clr-g dampens NK cell function as assessed by degranulation and cytolysis assays. Currently, we are addressing the functional impact of an interaction between Nkrp1g on activated splenic NK cells and Clr-g on stimulated CD8α+ cDCs as it may occur in the course of an immune activation.

Association of HLA-DR expression in NK cells with their differentiation stages

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Normally, human peripheral blood contains minor subpopulation of NK cells expressing HLA-DR, which increases under stimulation with IL-2 in vitro. In vivo, increased levels of HLA-DR+ NK cells are registered during certain pathological states, such as HIV or CMV infection, multiple sclerosis. The functional significance of this molecule in NK cells is still under debate: there is evidence that NK cells are able to use HLA-DR for antigen presentation, in other works HLA-DR is regarded as a marker of activation or proliferation of NK cells. In this work, we study the relationship between HLA-DR expression and developmental stages of NK cells isolated ex vivo and obtained in an in vitro activation system using IL-2 and K562 cells expressing membrane-bound IL-21 (K562-mbIL21).
Analysis of HLA-DR expression at various stages of differentiation of freshly isolated NK cells showed that the proportion of HLA-DR-positive cells in peripheral blood was much higher in less differentiated CD56bright cells than in relatively more mature CD56dim cells. Besides, the majority of donors displayed a higher level of HLA-DR-positive cells in CD56dimCD57– subset compared to CD56dimCD57+ subset (group 1). However, in some individuals the situation was vice versa: the proportion of HLA-DR-positive NK cells in CD56dimCD57+ subpopulation was significantly higher than in CD56dimCD57– one (group 2). Due to the increased proportion of HLA-DR+CD56dimCD57+ cells in these donors the proportion of HLA-DR+CD56bright cells was reduced. Individuals from group 2 also displayed higher proportion of NKG2C+ cells in HLA-DR-positive subset compared to HLA-DR-negative one. CD56dimCD57+NKG2C+ phenotype suggests that HLA-DR expression may be in certain cases linked to “memory-like” NK cells.
Upon stimulation with IL-2 and K562-mbIL21, but not with soluble IL-21, we observed up to 10-fold increase in HLA-DR-positive NK cell proportion. It happened mostly due to induction of HLA-DR expression de novo, rather than HLA-DR+ NK cells proliferation. Apart from HLA-DR expression increase, most of NK cells changed their phenotype towards CD56brightCD57–NKG2A+ , which is common for “early” NK cells. This data supports the idea of non-linear conversions of NK cells between their developmental stages and functional states. Thus, both in resting state and upon activation, HLA-DR expression was mostly associated with less mature NK cells. However, it can be found in vivo in terminally differentiated NK cells; the origin of this HLA-DR+CD56dimCD57+ subset is yet to be determined.
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UNIQUE DIVERSE NK CELL RECEPTOR REPERTOIRE OF ENDOMETRIAL NK CELLS COMPARED TO PERIPHERAL NK CELLS

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Natural killer (NK) cells are important for successful pregnancy. After implantation, NK cells encounter extravillous trophoblast cells and regulate trophoblast invasion. NK cell activity is amongst others regulated by C-type lectin heterodimer (CD94/NKG2) and killer cell immunoglobulin-like (KIR) receptors. KIR expression on decidual NK cells is affected by the presence of maternal HLA-C and biased towards KIR2D expression. However, little is known about the regulation of NK cell receptor expression on endometrial (eNK) cells prior to pregnancy. In this study, we characterized KIR and NKG2 receptor expression on NK cells obtained from menstrual blood (used as a source for endometrial cells) by 10-color flow cytometry, and compared this to matched peripheral blood NK (pbNK) cells from 19 women. KIR and HLA-C genotypes were determined by PCR-SSOP. We questioned whether the NK cell receptor (NKR) repertoire of eNK cells is different from pbNK cells, already preparing the endometrium for subsequent implantation of an allogeneic fetus. The percentage KIR2DL2/L3/S2, KIR2DL1/S1, KIR3DL1/S1, CD85j, and/or NKG2A positive eNK cells appeared significantly higher compared to pbNK cells. The NKR repertoire of eNK cells was different from pbNK cells, with eNK cells co-expressing multiple NKR. Moreover, although cognate HLA-C ligands can influence KIR expression, KIR expression on eNK cells was independent of HLA-C genotype. Interestingly, the typical NKG2C imprint induced by CMV infection on pbNK cells was not observed on eNK cells from the same individual, suggesting a rapid local turnover of eNK cells and/or a distinct licensing process. The KIR expression pattern of eNK cells collected from the same donor during different menstrual cycles showed very little variation, indicating stable expression. Taken together, our data reveals that in pre-pregnancy endometrium NKR expression profile differs from pbNK cells, which might suggest that these cells are already tuned towards the reception of an allogeneic fetus.

NK cells induce necrosis, apoptosis and mixed forms of target cell death

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One key function of natural killer cells is the elimination of virus-infected or transformed cells. NK cells induce target cell death by at least two different mechanisms, the release of perforin- and granzyme-containing vesicles and the engagement of cell death receptors such as Fas ligand. Perforin added to taget cells at very high concentrations can induce the formation of large pores in their plasma membrane, leading to osmotic swelling and primary necrosis. At lower concentrations, target cells can often repair the inflicted membrane damage, preventing immediate lysis; the duration of membrane damage is however sufficient to allow diffusion of granzymes into the cytosol, leading to caspase activation and subsequent cell death by apoptosis. FasL-mediated cytotoxicity induces caspase activation and apoptosis, as well. We present a technique to follow both cell lysis and caspase activity in single target cells simultaneously in real time. We studied cytotoxicity mediated by primary human NK cells and NK-92 in Fas-expressing and Fas-deficient target cells and observed that NK cells can induce primary necrosis, classical apoptosis characterized by membrane swelling and blebbing, as well as mixed forms of cell death. The extent of membrane disruption and caspase activity can be quantified during the entire process of target cell death, allowing also the discrimination of immediate lysis and secondary necrosis, occurring with delay after apoptotic killing. We observed different subsets of NK cells based on their preferential killing mechanism: some NK cells seem to rely exclusively on necrotic or apoptotic killing whereas another subtype switched from the necrotic to the apoptotic method. This heterogeneity in type and timing of cell death could have interesting implications for immune regulation in vivo; it could also explain the discrepancies observed between different assays of cytotoxicity, using different endpoints and readouts.

Characterization of NK cell populations and clones stimulated with IL-2 and modified K562 feeder cells

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Stimulation of NK cells with IL-2 and genetically modified K562 cell expressing membrane-bound IL-21 (K562-mbIL21) induces their intensive proliferation and may be used for NK cell clone generation. Characterization of the clones obtained from different NK cell subsets was performed in this work. Changes in phenotype of the clones were compared with the changes in cultures of whole NK cell populations activated by IL-2 and K562-mbIL21. Human NK cells were obtained by magnetic separation. Cloning was performed by single cell sorting to wells of 96-well plates contained irradiated K562-mbIL21 cells and IL-2. The stimulation conditions necessary for the induction of NK cell proliferation, affected the phenotype of the clones and whole NK cell cultures. Decrease in CD57+ cell proportion, together with CD56 upregulation, was usually observed in whole NK cell populations stimulated with IL-2 and K562-mbIL21. Several collections of clones were generated from CD56bright, CD56dimCD57– and CD56dimCD57+ NK cell subsets. The greatest numbers of cells (up to 15-25×106) were detected in clones derived from CD56dimCD57– NK cells; the same clones had highest lifetime (14 weeks and more). CD57+ NK cells were characterized by low frequency and lifetime of clones generated using IL-2 and K562-mbIL21 stimulation. All obtained clones expressed activating receptor NKG2D and cell adhesion molecule CD56, though the expression levels varied in clones derived from different individuals and/or different subpopulations of NK cells. Most of the clones were characterized by relatively low expression of CD16. Heterogeneity of the clones was established by the presence or absence of expression of KIR receptors, NKG2A, NKG2C, natural cytotoxicity receptor NKp46, CD57. The highly proliferated clones varied largely in CD57 expression (from 0 to 100% cells in a clone). Interestingly, even NK cell clones derived from CD57+ subset contained a proportion of CD57– cells, suggesting a reversibility of CD57 expression in some conditions.

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Analysis of pigment cell-specific NK cell memory

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Background: NK cells can acquire functional properties consistent with immunological memory, a function that had traditionally been assigned to T- and B cells. Recently, the pigment cell-dependent contact sensitizer monobenzone was shown to induce a memory NK cell response specifically directed at pigmented cells (melanocytes). Here, we aim to characterize these highly specific NK cells phenotypically and functionally and investigate their relation to other ILC subsets.
Methods: Mice were sensitized with monobenzone by repeated cutaneous application and lymphocytes were isolated from the liver. FACS analysis of lineage negative (CD3-, CD19-, Ter119-) cells was conducted with regard to the surface markers NK1.1, CD49b and further NK differentiation- and maturation markers. Expansion of distinct cell subsets in response to monobenzone was also traced in vivo using Ki67 and BrdU.
Results: We discovered that several phenotypically distinct hepatic NK- and ILC populations respond in the course of monobenzone sensitization. Using flow cytometry cell sorting we show that these populations exhibit distinct transcription factor expression-profiles.
Conclusion: The different cell populations identified may represent various developmental stages toward memory NK cell formation. The comprehensive characterization of this maturation process may provide novel insight into memory NK cell biology and could provide momentum to the use of these pigment cell-specific memory NK cells for melanoma immunotherapy. By single NK cell sequencing we plan to define which receptors, and possibly ligands, are involved.

Natural killer cell activation in liver cancer patients with enhanced NKG2D expression, interferon gamma and perforin production after ex vivo expansion

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Primary liver cancer is the second leading cause of cancer death globally, and the incidence is steadily increasing in Western countries. The majority of patients are diagnosed at an advanced stage with limited treatment options and 5-year survival rate less than 5%. More efficacious therapeutics are warranted. Immunotherapy including natural killer (NK) cells infusion has become clinically validated for cancer treatment.

The current study aims to characterize NK cells isolated from liver cancer patients, expand and activate ex vivo, and examine their anti-tumor properties. In the pilot study, ex vivo NK cell expansion protocols induced an average of 738-fold expansion and purity 92.6% after 21-day of culture in defined medium without feeder cells. Expanded NK cells, when compared with baseline, exhibited enhanced expression of NKG2D and CD69, increased production of perforin and IFN gamma, and augmented cytotoxic activity against liver cancer cells.

The patient and tumor characteristics have been further examined to understand the effect on NK features. The expanded NK cells were continued to culture in medium supplemented with healthy serum or autologous patient serum respectively. NK cells exposed to autologous serum decreased in cell number, reduced expression of NK stimulatory receptors, diminished production of perforin and IFN gamma when compared to the NK counter parts exposed to healthy serum. There could be inhibitors in patient serum that hindered NK. We have previously demonstrated that NK cell number and activity were significantly lower and negatively correlated with granulin-epithelin precursor (GEP) expression levels in liver cancer patients [Cheung et al. Oncoimmunology 2015]. Notably, GEP is a hepatic cancer stem cell marker [Cheung et al. Oncotarget 2016; Wong et al. Mol Cancer Ther 2014] able to evade immune recognition and modulating the NK cytotoxicity through enhanced shedding of MICA [Cheung et al. Cancer Immunol Res 2015]. In the current study, we demonstrated corroborating data that liver cancer patients with high serum GEP/MICA showed reduced NK abundance. Furthermore, these NK cells after isolation from patients and cultured ex vivo, in the absence of autologous serum / away from inhibitors, showed higher expansion ability.

In summary, ex vivo expansion and activation of peripheral NK cells isolated from liver cancer patients were demonstrated. Further investigation include functional efficacy and tumor homing efficiency of the expanded NK populations. The present study demonstrated the feasibility on NK infusion to boost immunity for liver cancer treatment.

Generation of tumor-specific NK cells by differentiation of CAR-gene transduced hematopoietic progenitors

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Natural killer (NK) cells hold promise for adoptive cancer immunotherapy. Like T cells, the antitumor activity of NK cells can be enhanced by expression of chimeric antigen receptors (CARs) that facilitate selective recognition and killing of malignant cells. CARs consist of an extracellular single-chain antibody fragment (scFv) for recognition of a cell surface antigen, linked to an intracellular signaling moiety such as CD3ζ or CD3ζ fused to a costimulatory protein domain. The engagement of CARs on NK cells triggers antigen-specific lysis of target cells, hence bypassing the need for the activation of endogenous cytotoxicity receptors.

For adoptive immunotherapy, NK cells are usually isolated from peripheral blood, and expanded ex vivo with cytokines before infusion into patients. Experimentally, NK cells have also been generated from hematopoietic stem cells (HSCs) by ex vivo differentiation following different protocols. CAR NK cells may be generated from CAR gene transduced HSCs following a similar approach. To explore this strategy, we established a protocol for ex vivo expansion and subsequent differentiation of CD34+ HSCs into NK cells. Mobilized CD34+ cells isolated from peripheral blood of healthy donors and cultured ex vivo in a specific cytokine mix gave rise to CD56+ NK cells in high proportions. To prevent CAR expression in hematopoietic lineages other than NK cells that may arise during the differentiation process, in parallel we constructed a lentiviral vector encoding an ErbB2-specific CAR under the control of an NK-specific NCR1 promoter. In a pilot experiment, CD34+ cells transduced with this vector and cultured ex vivo differentiated into ErbB2 CAR-expressing NK cells, albeit at low frequency. Importantly, the ex vivo generated CAR NK cells were functionally active, displaying enhanced cytotoxicity against ErbB2-expressing tumor cells. Ongoing work aims at optimizing the transduction protocol to improve yield of CAR NK cells, and thereby allow further functional studies.

Targeted NK cells display potent activity against glioblastoma and induce protective antitumor immunity

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Significant progress has been made over the last decade towards realizing the potential of natural killer (NK) cells for cancer immunotherapy. In addition to donor-derived primary NK cells, also continuously expanding cytotoxic cell lines such as NK-92 are being considered for adoptive cancer immunotherapy. High cytotoxicity of NK-92 has previously been shown against malignant cells of hematologic origin in preclinical studies, and general safety of infusion of NK-92 cells has been established in phase I clinical trials. To enhance their therapeutic utility, here we genetically modified NK-92 cells to express a chimeric antigen receptor (CAR), consisting of an ErbB2-specific scFv antibody fragment fused via a linker to a composite CD28-CD3 zeta signaling domain. GMP-compliant protocols for vector production, lentiviral transduction and expansion of a genetically modified NK-92 single cell clone (NK-92/5.28.z) were established. Functional analysis of NK-92/5.28.z cells revealed high and stable CAR expression, selective cytotoxicity against ErbB2-expressing but otherwise NK-resistant tumor cells of different origins in vitro. Ongoing work focuses on the development of these cells for adoptive immunotherapy of ErbB2-positive glioblastoma. We evaluated the activity of NK-92/5.28.z cells against a panel of glioblastoma cell lines and primary glioblastoma cultures and demonstrated selective in vitro cell killing that was dependent on ErbB2 expression by the target cells. Potent in vivo antitumor activity of NK-92/5.28.z was observed in orthotopic glioblastoma xenograft models in NSG mice, leading to a marked extension of symptom-free survival upon repeated stereotactic injection of CAR NK cells into the tumor area. In immunocompetent mice, local therapy with NK-92/5.28.z cells resulted in cures of transplanted syngeneic GBM in the majority of animals, induction of endogenous antitumor immunity and long-term protection against tumor rechallenge at distant sites. Our results suggest adoptive transfer of ErbB2-specific NK-92/5.28.z cells as a promising new immunotherapy approach for glioblastoma. A phase I clinical trial investigating NK-92/5.28.z cells as a treatment for recurrent ErbB2-positive glioblastoma is in preparation.

Harnessing NK cells to target chronic lymphocytic leukemia: design and efficacy of novel trispecific immunoligands

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Chronic lymphocytic leukemia (CLL) is the most prominent B cell malignancy among adults in the western world and is characterized by a clonal expansion of B cells. There is emerging evidence that natural killer (NK) cells play a pivotal role in the immunosurveillance of CLL. Currently available mAbs are partially efficient in CLL and new agents that are more specific and effective are needed.

Our approach is to design bi- and tri-specific recombinant proteins (immunoligands) which simultaneously bind to tumor cells and NK cells resulting in NK cell-mediated killing of the former while bypassing their immune-escape mechanisms. One such trispecific immunoligand (ULBP2-aCD19-aCD19) aims to target CD19 antigen on CLL cells while a second arm activates NK cells through ULBP2, a ligand for the activating receptor NKG2D. In vitro data with ULBP2-aCD19-aCD19 revealed an enhanced NK cell-dependent lysis of CLL cell line and primary CLL cells in allogenic and autologous settings. This effect could be successfully replicated in an immunedeficient mouse model (NSG) as a part of in vivoanalysis.

On the other hand, novel trispecific immunoligands are being analysed for their ability to enhance the efficacy of NK cell activation. This design includes a single chain (scFv) recognizing CD19 and two arms engaging different NK cell receptors CD16 (via anti-CD16 scFv) and NKG2D (via ULBP2). Based on the evidence that activating receptors on NK cells can synergize to enhance NK cell effector functions, we hypothesize that such trispecific immunoligands will be more potent immunotherapeutic agents.

Irradiation increases the expression of ICAM-1 on tumor cells, resulting in enhanced sensitivity to NK cell-mediated cytotoxicity

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NK cells adhere to target cells through the interaction between NK cell receptors, lymphocyte function-associated antigen 1 (LFA-1), and their ligands, endothelial intercellular adhesion molecule-1 (ICAM-1). However, the contribution of enhanced expression of ICAM-1 by irradiation for NK cell cytotoxicity is unclear. The purpose of our investigation was to evaluate the effect of expression of ICAM-1 in cancer cells by irradiation to cytotoxicity of NK cells. Expression level of ICAM-1 on the target cell surface before and after irradiation of 5 human cancer cell lines (HL-60, SK-BR-3, HCT-116, U937, and U251) were analyzed using flow cytometry. Cytotoxicity of NK cells was analyzed by WST-8 assay. Expanded NK cells from day 14 with an NK purity over 90% were used for cytotoxicity assay. Blocking assay was performed using monoclonal antibody of ICAM-1 in HL-60 cells and/or LFA-1 in NK cells. The expression of LFA-1 was increased on expanded NK cell. The expression of ICAM-1 was induced by irradiation after 24 hours in various cell lines including HL-60, SK-BR-3, HCT-116, and U937, although the level of expression was various depending on cell lines. In a cell line of HL-60, ICAM-1 was expressed more than 3 times compared to non-irradiated cancer cells at 24 hours after irradiation (relative MFI: 8.0 vs. 29.9). There was no expression of ICAM-1 before and after irradiation in U251 cells. Enhanced expression of ICAM-1 after irradiation was followed by increased susceptibilities to NK cell-mediated cytotoxicity. Especially in HL-60, ICAM-1 was most abundantly increased after irradiation and cytotoxicity of NK cells was also increased the most at E:T ratio, 0.5:1 (83.3% vs. 43%, $p<0.001$), 1:1 (97.5% vs. 62.5%, $p<0.001$), and 2:1 (110.3% vs. 83.5%, $p=0.005$), respectively. There was no significant difference with or without irradiation in U251 cells at E:T ratio, 0.5:1, 1:1, and 2:1, respectively. These results suggest that radiation therapy would improve the antitumor effect of NK cells thorugh LFA-1 and ICAM-1 interaction.

Umbilical cord blood stem cell derived NK cells as universal treatment for metastatic colorectal cancer using EGFR independent killing mechanisms

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Background: Therapeutic monoclonal antibodies (mAbs) against the epidermal growth factor receptor (EGFR) act by inhibiting EGFR downstream signaling and by eliciting an NK cell-mediated anti-tumor response. The IgG1 mAb cetuximab has been used for treatment of RASwt metastatic colorectal cancer (mCRC) patients, showing limited efficacy. Methods: In the present study, we address the potential of adoptive NK cell therapy to overcome these limitations investigating two allogeneic NK cell products, i.e. activated peripheral blood NK cells (A-PBNK) and umbilical cord blood stem cell derived NK cells (UCB-NK). Results: While cetuximab monotherapy was not effective against EGFR- RASwt, EGFR+ RASmut and EGFR+ BRAFmut cells, A-PBNK were able to initiate lysis of EGFR+ colon cancer cells irrespective of RAS or BRAF status. Cytotoxic effects of A-PBNK (but not UCB-NK) were further potentiated significantly by coating EGFR+ colon cancer cells with cetuximab. Of note, a significantly higher cytotoxicity was induced by UCB-NK as compared to A-PBNK in EGFR-RASwt (67 ± 7% versus 42 ± 8%), EGFR+ RASmut (37 ± 6% versus 20 ± 2%) and EGFR+ BRAFmut (43 ± 7% versus 23 ± 3%) colon cancer cells and equaled the cytotoxic efficacy of the combination of A-PBNK and cetuximab. The anti-tumor efficacy of UCB-NK cells against cetuximab resistant human EGFR+ RASmut colon cancer cells was further confirmed in an in vivo preclinical mouse model where UCB-NK were effective in controlling the growth of EGFR+ RASmut colon cancer; moreover, an increased median survival was observed for treatment with UCB-NK + cetuximab (62 days) as compared to UCB-NK only (48 days). Conclusion: As UCB-NK have been proven safe in a recently conducted phase I clinical trial in acute myeloid leukemia, a fast translation into clinical proof of concept for mCRC could be considered.

NK cell therapy in combination with anti CXCR4 antibody for the treatment of paediatric sarcomas.

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BACKGROUND:
Metastatic sarcoma in children and adolescents has a 5-year survival rate of less than 20% and current therapies, consistent in radical surgery and neo-adjuvant chemotherapy, remain ineffective. Consequently, new therapeutic venues are required.
Chemockine receptor CXCR4 over expression in sarcomas mediates directed metastasis to those organs where its ligand, CXCL12, is expressed. CXCR4-CXCL12 interaction activates cell signalling cascades promoting cell survival, proliferation, adhesion and/or migration of CXCR4+ cells leading to an enhanced progression and metastatic capacity of CXCR4 expressing sarcomas. A newly developed antibody, ulocuplumab, is able to block CXCR4 signalling.
In addintion, our group has described that Natural Killer (NK) cells have high cytotoxic capacity against CXCR4+ tumor initiating cells through NKG2D receptor. We have tested the synergistic effect ofNK cell therapy in combination with anti CXCR4 antibody immunotherapy inhibiting sarcoma metastasis.
METHODS:
Expression of CXCR4 by different sarcoma celllines (U2OS, MG-63, 143B, A673, A4573, RH30) was analyzed by flowcytometry and qRT-PCR.
NK-92mi cell line invitrocytotoxicity against sarcoma cells was evaluated by flow cytometry using CFSE and 7AAD to trace death target cells.
Ulocuplumab and NK92mi cell line mediated inhibition of CXCR4+ sarcoma cellsmigration towards a gradient ofhuman recombinant CXCL12 chemockine was tested using Transwell plates.
Lentiviral particles expressing GFP andluciferase were used to transduce sarcoma cell lines.GFP+ Luc+ sarcoma cells were inoculated intravenously and orthotopicaly in immunodeficient NSGmice (NOD.Cg-Prkdcscid Il2rg tm1WjlSzJLos) to generate an invivomodel of metastatic sarcoma to test the effectiveness of anti CXCR4 and NK cell combined therapy.
RESULTS:
We have observed an heterogeneous CXCR4 expressionin different sarcoma cell lines (osteosarcoma, Ewing sarcoma, rhabdomyosarcoma), finding the highest CXCR4 expression in alveolar rhabdomyosarcoma RH30 cell line.
NK-92mi cell line cytotoxicity against sarcoma cell lines is also heterogeneous and not enhanced by ulocuplumab antibody.
Both ulocuplumab and NK-92mi inhibit CXCR4+sarcoma cell lines migration toward CXCL12chemockine in invitroassays.
Luc+ RH30 cells metastasize in our immonodeficient mouse model into those organs where CXCL12 is expressed.
CONCLUSIONS:
Anti CXR4 antibody combined with NK cell therapy is a promising new immunotherapy for metastatic CXR4 expressing sarcomas in paediatric patients. Its synergistic effect should be further evaluated ininvivostudies prior to its implementation in future clinical trials.

CBP/p300 acetyltransferases regulate the expression of NKG2D ligands on tumor cells.

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Tumor surveillance of natural killer (NK) cells is mediated by the cytotoxicity receptor natural-killer group 2 member D (NKG2D). Ligands for NKG2D are generally not expressed on healthy cells, but induced on the surface of malignant cells. To date, NKG2D ligand (NKG2D-L) induction was mainly described to depend on the activation of the DNA damage response, although the molecular mechanisms that regulate NKG2D-L expression remain largely unknown. Here, we show that the acetyltransferases CBP (CREB-binding protein) and p300 play a crucial role in the regulation of NKG2D-L on tumor cells. Loss of CBP/p300 decreased the basal cell surface expression of human ligands and reduced the upregulation of MICA/B and ULBP2 in response to histone deacetylase inhibitors or DNA damage. Furthermore, CBP/P300 deficiency abrogated the sensitivity of stressed cells to NK cell-mediated killing. CBP/p300 were also identified as major regulators of mouse NKG2D ligand RAE-1 in vitro and in vivo using the Eμ-Myc lymphoma model. Mechanistically, we observed an enhanced activation of the CBP/p300 binding transcription factor CREB (cAMP response element-binding protein) correlating to the NKG2D-L upregulation. Moreover, increased binding of CREB and CBP/p300 to NKG2D-L promoters and elevated histone acetylation were detectable. This study provides strong evidence for a major role of CBP and p300 in orchestrating NKG2D-L induction and consequently immunosurveillance of tumors in mice and humans. These findings might help to develop novel immunotherapeutic approaches against cancer.

Engineering antigen-specific Natural Killer cells via TCR gene transfer: A novel source for adoptive immunotherapy.

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Recent decades have witnessed extraordinary improvements in the use of cellular immunotherapy against malignancies. A commonly used approach is the isolation and expansion of tumor-associated antigen (TAA)-specific cytotoxic T lymphocytes (CTLs) for adoptive transfer. The technology of T cell receptor (TCR) gene therapy has improved this approach by supplying large populations of antigen-specific T cells generated by delivery of genes for a TCR in order to redirect CTLs towards selected epitopes of tumor antigens.

The assembly and surface expression of the TCR introduced by gene delivery is a complex process, requiring pairing of the introduced TCR-α and TCR-β chains to form a heterodimer that then associates with the four CD3 chains. The TCR-α and TCR-β chains introduced by gene delivery have a risk of pairing with the complementary TCR-β or TCR-α chains that are endogenously expressed by the T cell. This phenomenon called mispairing, has the potential to produce TCRs of unpredictable specificity that may cause a lethal GvHD-like syndrome in vivo. Despite the promise of TCR gene therapy, the mispairing problem constitutes a bottleneck in the development of effective and safe therapies.

To date, several NK cell based anti-cancer products have been taken to clinical trials with promising results. However, to manufacture efficient NK cell products, it is essential to develop strategies to increase safety, efficiency and specificity. In this study, we use NK cells for TCR gene therapy, aiming to reprogram them to selectively target tumor antigens in complex with major histocompatibility complex (MHC).

Introduction of functional TCR complex to NK cells enhances their efficiency to identify and kill tumor cells while circumventing the potential risk of TCR mispairing. Our results demonstrate that the ectopic expression of CD3δ, CD3γ, CD3ε and TCR α/β heterodimer but not CD3ζ is necessary for establishment of a functional TCR complex on the surface of NK-92 and YTS cells. These cells efficiently detect an antigenic epitope presented by MHC-I and robustly kill targets expressing the antigen.

Our strategy does not only have the potential to open up a whole new chapter in the field of cancer immunotherapy but also provides a final and definitive solution for the mispairing problem observed in TCR gene therapy. Further studies to better characterize the pros and cons of this approach compared to using T cells and direct comparison of in vivo efficiencies are warranted.

RETROVIRAL TRANSFER INTO IL-2/FEEDER-CELL-ACTIVATED HUMAN NK CELLS

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Natural killer (NK) cells have capabilities for rapid and efficient recognizing and killing tumor cells that makes them a promising agent for cancer immunotherapy. Still, genetic reprogramming of NK cells may improve their anti-tumor efficacy. Here we report the efficient retroviral vector transduction of primary human NK cells stimulated with IL-2 and modified K562 cells expressing membrane-bound IL-21. The retroviral vectors including RD114 providing a better penetration into lymphoid cells were applied for gene transfer into human cells. Transfection was performed using calcium phosphate-based method. 24-well plates pretreated with RetroNectin solution were used for transduction procedure. To validate the model we performed originally transduction of human T-cell line Jurkat. Cell fractions expressing reporter protein were 60% and 80% for green fluorescent protein (GFP) and nerve growth factor receptor (NGFR) , respectively. NK cells were isolated from peripheral mononuclear cells of healthy volunteers by magnetic separation. The activation of NK cells with the combination of IL-2 and feeder cells expressing membrane-bound IL-21 allowed us to overcome the NK cell resistance for the gene introduction. Upon the stimulation for 7 days these NK cells expressed low level of CD57, and high levels of HLA-DR and activating receptors NKG2D, NKp30 and CD16. The NK cell transduction efficacy reached 50% in individual experiments. Highly differentiated CD57+ NK cells were shown to be resistant to the retroviral transduction. These data may help for selecting and preparing NK cells assigned for genetic manipulations based on retroviral transfer.

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Influence of high fat feeding on NK cells in mice

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Introduction
Obesity is a widespread disease accompanied by secondary complications like cardiovascular disorders, diabetes and cancer. Previous studies demonstrated that essential functions of natural killer (NK) cells such as targeting tumor cells are disturbed in obese individuals. To investigate the influence of different feeding regimes on NK cells, this study aimed to analyze NK cell related parameters in mice fed a normal or high fat diet under a restrictive or ad libitum feeding regime.
Methods
To induce obesity, male C57BL/6 mice received either a normal fat diet (NFD, 4 % fat) or a high fat diet (HFD, 34 % fat). Mice were fed the respective diet either ad libitum or received 90 % of the daily food intake of the corresponding ad libitum group (restrictive feeding regime). Food intake was documented daily and body weight was determined every week. Eighteen weeks after start of feeding, mice were sacrificed and blood as well as visceral fat mass was collected. Different blood immune cells were analyzed by flow cytometry and specific plasma cytokines were determined by multiplex immunoassay.
Results
Ad libitum as well as restrictive feeding of mice with the HFD showed significantly higher body weight, visceral fat mass and blood leukocyte number compared to the NFD group. Additionally, number of blood leukocytes showed a positive correlation with body weight and fat mass.
Plasma levels of IL-2 and IL-6 were increased in mice fed the HFD ad libitum compared to the NFD ad libitum group, whereas mice received the diets restrictive showed no differences in these cytokine plasma concentrations.
Independent of the regime, feeding the HFD compared to NFD, led to increased absolute numbers of blood NK cells, while the percentage of NK cells was decreased. In addition, the frequency of NK cells bearing the activation receptor NKG2D tends to be lower in HFD fed animals compared to the NFD group, pointing to an inhibited functionality of these cells.
Conclusion
The present study on different feeding regimes and diets shows that ad libitum feeding of a high fat diet leads to an increase of IL-2 and IL-6 plasma levels, which was prevented by restrictive feeding. Feeding a high fat diet also increased blood leukocytes, among them NK cells, whereas percentage of NKG2D+ NK cells was reduced. Therefore this study gives new insights in the influence of high fat feeding on NK cell characteristics.

A Feeder-Cell-Free Two-Phase NK Cell Expansion Protocol for Adoptive Immunotherapy of Rhabdomyosarcoma

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Despite intensive research metastatic or advanced stage rhabdomyosarcoma have bad prognosis as they are aggressively spreading and prone to relapse.
New therapeutic concepts in tumor therapy aim to modulate the patient's immune system to increase its aggressiveness or targeted effect towards the tumor cells. Besides direct application of cytokines or antibodies, adoptive immune cell therapy is a promising approach. The present study focusses on natural killer (NK) cells that have been shown to be cytotoxic against transformed and dysregulated cells. NK cells recognize their targets without prior sensitization and get activated by an imbalance of signals from inhibitory or activating receptors. Moreover, NK cells can be stimulated by cytokines among which the common-gamma-chain cytokines seem reasonably powerful. Our project concentrates on the most recently identified member of this family, IL-21, exploiting it for a new feeder-cell-free NK cell expansion protocol. In combination with other cytokines IL-21 enhances the maturation and cytotoxicity of NK cells, but hinders NK cell expansion. To minimize the latter, but strengthen its positive effect, we established a two-phase expansion protocol. IL-15 is used for the early expansion phase, followed by a short IL-21 exposition phase. Thereby, the cytotoxic activity of the NK cells can be increased, specifically shown by lysis of rhabdomyosarcoma cells. Functional analyses further revealed an enhanced degranulation and elevated secretion of pro-inflammatory cytokines, such as IFN-γ and TNF-α. Additionally, we show the effect of adoptive NK cell transfer in a preclinical study, using bioluminescence imaging in a luciferase transduced rhabdomyosarcoma xenograft model.
In summary, the two-phased feeder-cell-free ex vivo expansion protocol combines efficient expansion and high cytolytic functionality of NK cells for treatment of rhabdomyosarcoma in an autologous setting.

Primary CAR NK cells demonstrated increased cytotoxicity against AML cells based on enhanced cell-cell interactions as revealed by time-lapse microscopy

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BACKGROUND AND AIMS: Natural killer (NK) cells are a subset of lymphoid effector cells within the innate immune response and are important for antitumor surveillance. However, cancer cells are able to undermine this immunosurveillance by development of tumor immune escape mechanisms (TIEMs). To overcome these immune-evasive mechanisms and to restore specific antitumor surveillance, primary NK-cells were gene modified to express chimeric antigen receptors (CARs) against CD123, a marker widely expressed on multiple relapsed acute myeloid leukemias (AML).

METHODS: After efficient transduction with a newly constructed alpharetroviral vector containing anti-CD123 CAR and EGFP, primary human CAR NK-cells were sorted and expanded for 14 d in vitro in NK MACS medium containing IL-2 (1000 U/ml), which was replenished every 2-3 d. Cytotoxicity assays of CAR NK-cells versus the resistant CD123+ AML cell line KG1alpha (ratio 5:1, incubation for 5 h or 20 h) were accomplished and release of several cytokines was quantified. To monitor retargeted migrations and interactions between effector (NK) and target cells (KG1alpha), both cell types were stained with CFSE (NK) or eFluor450 (KG1alpha) and spread at a ratio of approximately 5:1 on a slide coated with a monolayer of human lung microvascular endothelial cells (HLMVEC), which served as a control for side effects. Immunofluorescence time-lapsed imaging was performed up to 8 h with a fluorescence microscope (IX81, Olympus) and effector to target cell interactions were statistically evaluated with ScanR software (Olympus).

RESULTS: Gene-modified and EGFP-sorted anti-CD123 CAR NK-cells demonstrated an increased cytotoxicity against CD123+ leukemic cells. Cytotoxicity was inhibited by CD123 blocking antibodies or recombinant CD123 peptides. In addition, CAR NK-cells exhibited enhanced directed interactions and migration against CD123+ blasts compared to controls, with only minor side effects against HLMVEC, suggesting specific elimination of AML cells by CAR NK-cells. In addition, increased levels of IL-10, Interferon-gamma, and Granzyme B were detected after 20 h incubation of CAR NK-cells with AML blasts. Imaging experiments revealed higher specific interactions between anti-CD123 CAR NK-cells and CD123+ AML cells compared to controls (non-transduced or EGFP-expressing NK-cells) as well as to specifically blocked primary CAR NK-cells.

CONCLUSIONS: These results clearly demonstrate specificity and functionality of the chimeric receptor molecule on primary human NK cells and provide an important base line for additional experiments to develop new clinical protocols using CAR NK-cells for both improved specificity and cytotoxicity against AML.

Prognostic role of KIR-B haplotype and HLA-C in 7-years follows up after HSCT in a cohort of AML patients from a community of high consanguinity

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NK cells activity is largely tuned by a balance of activating and inhibitory signals that are transmitted via respective receptors, including killer immunoglobulin- like receptors (KIRs). The impact of NK cells towards GVL effects after HSCT has been established in many reports. However, this effect is sometimes combined with adverse immune responses leading to Graft-versus-host-disease (GvHD). The link between KIR/HLA interaction and GvHD has not been well investigated. Herein, we studied the impact of KIR/HLA interaction in the HSCT outcome after longitudinal follow up of HLA-matched cohort with high consanguinity. 174 HSCT including 82 AML donor/patient pairs were screened. DNA was collected from peripheral blood and KIR and HLA was genotyped for donors and recipients respectively. A total of 47% patients encountered (GvHD). Our analysis revealed that, in all HSCT and AML an increased incidence of aGvHD was associated with matching of KIR2DL1_C2 (P=0.02), KIR2DS2_C1 and KIR2DS_C2 (P=0.04) especially in AML donor-patient pairs. More cGvHD was also observed in AML patients who were matched for KIR2DL1_C2 (P=0.02), KIR2DS1-C2 (P=0.003) and have both KIR2DL2/2DS2 genes(P=0.03). In conclusion, genes of KIR B-haplotype especially 2DL2, 2DS1 and 2DS2 in the donors and HLA-C2 in the recipients were associated with cGvHD and good prognosis outcome after HSCT.

Killer cell immunoglobulin-like receptor 3DL1 polymorphism defines distinct hierarchies of HLA class I recognition

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The Killer Immunoglobulin receptor (KIR) family are Natural Killer (NK) cell surface receptors that recognise HLA on target cells and control NK activation. KIR3DL1 is a polymorphic inhibitory receptor recognising HLA containing the Bw4 motif that encompasses positions 77-83 on the α1 helix of the HLA. KIR3DL1 and its interaction with ligand are implicated in altered disease course in chronic viral diseases such as HIV-1 as well as in graft versus host disease (GVHD) and leukemic relapse following human stem-cell transplantation (HSCT). However, determination of how HLA class-I (HLA-I) and killer cell immunoglobulin-like receptor 3DL1 (KIR3DL1) polymorphism impact disease outcomes remains unclear. In this study tetramers for multiple KIR3DL1 allotypes including *001, *005 and *015 were screened for reactivity against a panel of 100 common HLA-I molecules. This revealed distinct hierarchies of specificity for each KIR3DL1 allotype, with KIR3DL1*005 recognising the widest array of HLA-I ligands. These differences were further reflected in functional studies using NK clones expressing these specific KIR3DL1 allotypes. Unexpectedly, the Ile/Thr80 dimorphism in the Bw4-motif did not categorically define strong/weak KIR3DL1 recognition and the extent of the influence of this dimorphism differed between allotypes. Although several of the KIR3DL1*001, *005, and *015 polymorphisms are remote from the KIR3DL1–HLA-I interface, structures of these three KIR3DL1–HLA-I complexes showed that the broader HLA-I specificity of KIR3DL1*005 correlated with an altered KIR3DL1*005 inter-domain positioning and increased mobility within its ligand-binding site. Collectively, we provide a framework for understanding the impact of KIR3DL1 polymorphism on the recognition of HLA-I.

Relevant Publications:
Killer cell immunoglobulin-like receptor 3DL1 polymorphism defines distinct hierarchies of HLA class I recognition.
Saunders PM*, Pymm P*, Pietra G, Hughes VA, Hitchen C, O'Connor GM, Loiacono F, Widjaja J, Price DA, Falco M, Mingari MC, Moretta L, McVicar DW, Rossjohn J, Brooks AG, Vivian JP
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Role of educating inhibitory receptors in the killing of renal cell carcinoma cells induced by DC vaccines

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In an attempt to improve DC vaccination against cancer the role of NK cells in renal cell carcinoma (RCC) killing was evaluated. Alternative FastDC stimulated with TLR ligand- and IFNg-containing cocktails induced a stronger NK cell activation than gold standard DC, which resulted not only in enhanced IFNg secretion and degranulation in response to classical MHC class I negative target cells, but also in degranulation to HLA-ABC positive RCC cell lines. Determination of the degranulation of KIR- or NKG2A-educated versus uneducated NK cells highlighted that alternative DC were able to induce enhanced activation of uneducated cells. Comparison of NKG2A and KIR education revealed an impaired activity of NKG2A-educated versus KIR-educated cells in killing RCC53 cells, which was associated with the consitutive HLA-E expression but neither of HLA-Bw4 nor of HLA-C1/C2 molecules on RCC53 cells. Treatment of RCC53 cells with IFNg resulted in an upregulated expression of HLA-Bw4 and -C2 molecules, whereas HLA-E expression levels remained unaffected. As a consequence, a lower degranulation was induced in NK cells with a particular loss of degranulation by KIR-educated NK cells. While the enhanced activity of uneducated NK cells requires further evaluation of possible negative consequences on non-tumorigenic control cells, these data postulate an enhance tumor cell killing by DC vaccination mediated by the activation of educated as well as uneducated NK cells.

Applying CRISPR/Cas9-based genetic screens to study interaction of NK cells with tumor cells

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NK cells distinguish transformed from healthy cells with the help of various receptor-ligand interactions and are regulated by a balance of inhibitory and activating signals to trigger anti-tumor immunity but maintain self-tolerance. However, the spectrum of factors regulating NK cell cytotoxicity, including the multitude of ligands and regulatory mechanisms of their expression on tumor cells are far from being completely resolved. Here, we applied unbiased genome-wide loss-of-function screens using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system to study factors involved in promoting NK cell sensitivity of the erythroleukemic tumor cell line K562. We identified B7H6, the ligand for the activating NK cell receptor NKp30, as the dominating factor involved in promoting NK cell cytotoxicity towards K562s. Furthermore, as underlying mechanisms of its selective expression on tumor cells are poorly understood, we applied CRISPR interference (CRISPRi)-based screens to identify regulatory elements for B7H6 expression in K562s, using a single-guide RNA library tiling the genomic region around the B7H6 locus and sorting cells that lost B7H6 surface expression by flow cytometry. We discovered a potential role of the promoter of the neighboring gene NUCB2 in regulating B7H6 expression. This cis-regulatory interaction has to be further validated and might give insights in the selective expression pattern of B7H6. Lastly, to identify novel ligands and ways of regulating ligand expression on tumor cell lines for two selected activating NK cell receptors (KIR3DS1 and NKp44), we performed genome-wide CRISPR screens and sorted cells that lost binding to soluble receptor constructs. Thereby, we could identify heparin sulfate proteoglycans (HSPGs) as novel ligands for the KIR3DS1 receptor, whose expression is associated with outcome of different diseases including cancer. Since HSPGs have previously been described as co-ligands for other NK cell receptors, including NKp44, our results indicate a global role of HSPGs in regulation target cell recognition and NK cell function that has to be further investigated. Additionally, we detected a potential novel role of the calcium pump SPCA1 encoded by ATP2C1 in regulation of NKp44 ligand expression. Overall, our results demonstrate that CRISPR-based genetic screens are useful tools to study NK cell - tumor cell interactions, paving the way for further investigations contributing to a better understanding of the complexity of different signals acting together to promote or prevent NK cell recognition of unhealthy and transformed cells.

Comparison of human innate lymphoid cells in peripheral blood and synovial fluid

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Introduction: Group 1 to 3 innate lymphoid cells (ILCs) represent very small populations in peripheral blood (PB) and synovial fluid (SF) in humans. Due to their potential to produce cytokines such as IL-17 and IL-22 ILCs might contribute to the pathogenesis of rheumatoid arthritis (RA).
Objective: To compare human ILCs obtained from peripheral blood of healthy controls (HC) and RA patients as well as synovial fluid of RA patients.
Methods: We determined numbers of ILCs in PB of healthy controls (HC) and RA patients as well as SF by flow cytometry.
Results: When comparing the ILC subsets of different subject groups and compartments, respectively, we found that the profiles of the ILC subsets from PB of healthy controls and RA patients were quite similar. In contrast, in SF ILC2/ILC1 ratio was significantly inverted due to decreased numbers of ILC2 and a concomitant increase of ILC1. Furthermore, in SF of 12 out of 13 RA patients we identified CD56-NCR+ILC1, a population which has not been described so far.
Conclusion: ILC subset composition in HC and RA patients is comparable. In contrast, ILC subsets obtained from SF differ significantly from PB-ILC. Intraindividual comparison indicates a specific recruitment of group 1 ILC to the site of inflammation.

Role of peroxiredoxin 1 in functioning of NK cells

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Peroxiredoxin 1 (PRDX1) is a is a member of thiol-dependent antioxidant enzymes family responsible for protecting cells against peroxides toxicity. Initially, PRDX1 was identified as a Natural Killer Cell Enhancing Factor A (NKEF-A) that improves NK cells cytotoxicity when added exogenously. Interestingly, Prdx1-/- mice have been shown to have deep impairment in NK cell number and function. However, the role of PRDX1 protein in NK cells has not been profoundly investigated. Therefore, our ongoing studies are focused on understanding the significance of PRDX1 protein in NK cells' antitumor activity.
We observed that PRDX is one of the most up-regulated antioxidant enzymes in activated NK cells. We found that PRDX1 is upregulated in IL-2 and IL-15 activated NK cells at the protein level. Moreover, RNA sequencing data performed in different NK cell subsets (CD56 bright, NKG2A-KIR-, NKG2A+KIR-, NKG2A+KIR+, NKG2A-KIR+, NKG2C+iKIR+, NKG2A-aKIR+) from 10 healthy donors reveal that PRDX1 expression follows NK cell maturation and is up-regulated in more differentiated NK cell subsets. Furthermore, inhibition of endogenous PRDX1 with adenanthin resulted in impairment of NK cell cytotoxicity, degranulation and cytokine production. Moreover, upon PRDX1 inhibition several activating receptors: (NKp30, NKp44, NKG2D, DNAM-1) and activation markers (CD25, CD69) were significantly down-regulated . To further investigate the role of PRDX1 in functioning of NK cells we created two experimental models with knockout and overexpression of PRDX1 in NK cells. PRDX1 knockout has been done in NK92 cell line using CRISPR/Cas9 genome editing. We found that NK92 cells with PRDX1 knockout are more sensitive to H2O2. Recently, we generated model of human primary NK cells with elevated level of PRDX1 using mRNA electroporation.
In the future we are planning to further investigate whether PRDX1 is a potential candidate to be explored as a factor increasing the therapeutic potential of NK cell-based therapies.

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The role of transcription factor Runx2 in human NK cell and ILC development and function

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Natural killer (NK) cells and other innate lymphoid cells (ILC) are major players of the innate immune system. Their main function is to kill cancerous cells and pathogens including viruses, bacteria and parasites directly via cytotoxicity or indirectly by secreting cytokines that boost other cells of the innate and adaptive immune system. Although mouse models have provided much insight into crucial mechanisms and factors involved in NK cell and ILC development, the amount of knowledge concerning these complex processes in human remains largely unknown. Research has demonstrated that the expression of the transcription factor Runx2 is affected in early T-cell progenitor acute lymphoid leukaemia, which is characterised by a block in NK/T-cell development. Here, we induced Runx2 knockdown in umbilical cord blood derived hematopoietic stem cells using shRNA. This resulted in a distorted NK cell and ILC3 development in vitro, suggesting that Runx2 is essential for the generation of mature NK cells and ILC3s. We continued to investigate this effect by inhibiting Runx2 expression in specific NK cell precursors using shRNA, which confirmed that Runx2 also plays an important role at later NK cell developmental stages.

Is therapeutic modulation of NK cell development feasible?

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5-Aza-cytidine (5-AzaC) is a cytostatic drug that is able to induce depletion of maintenance DNA methyltransferase 1 (Dnmt1). Previous work from other groups showed direct cytotoxic effects upon 5-AzaC treatment of leukemic cells but treatment of mature NK cells led to increased inhibitory KIR expression and impaired granzyme B and perforin release resulting in suppressed NK cell cytotoxicity. In contrast to that our recently published work provides evidence that low dose 5-AzaC enhances the graft-versus-leukemia (GvL) effect. We applied a humanized xenotransplantation model in NOD SCID IL2Rγc-/- (huNSG) mice that predominantly harbors immature KIR negative NK cells to study the effect of 5-AzaC on developing NK cells. Along with an increased GvL effect we observed a higher number of bone marrow-residing immature and mature NK cells. It is currently unclear whether these NK cell subpopulations directly mediated anti-leukemic activity, whether bystander cells were involved, and how this increase in anti-leukemic activity is regulated on the molecular level. Considering that 5-AzaC induces the reversal of DNA hypermethylation and as a result the selective silencing of multiple genes involved in cell differentiation, we hypothesize that 5-AzaC promotes NK cell differentiation or functionality via modulation of relevant transcription factors. Thus, we are currently studying NK cell development and its epigenetic modulation in the presence of 5-AzaC (a) in vitro during stem cell-NK cell differentiation and (b) in vivo in reconstituting C57BL/6J mice. With these models we are mimicking modulation of the GvL effect after hematopoietic stem cell transplantation where progenitor cells liberally proliferate. As a result, modulation of NK cell development and functionality by 5-AzaC could be applied in the early post-transplantation period of patients with leukemia to enhance GvL effects.

Involvement of NFAT Transcription Factors in NK Cell Reactivity

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NK cells are lymphoid components of innate immunity and play an important role in tumor immunosurveillance. One of the major transcriptional regulators in lymphoid cells is NFAT (Nuclear Factor of Activated T Cells), as highlighted by its important role in T and B cell development and function. With regard to NK cells, available data indicate that NFAT is dispensable for development. However, several lines of evidence including the observation that the immunosuppressive drugs cyclosporin A and tacrolimus, which mediate their effects through inhibition of calcineurin and consecutively NFAT, influence NK reactivity implicate a role of this family of transcription factors in NK cell reactivity and function. Here we employed different genetic mouse models on the C57BL/6 background to directly study the functional role of NFAT in NK cells.

We found that except for NFAT3 mRNA and protein of all family members (NFAT 1, 2, 4 and 5) was expressed in resting NK cells of wild type (WT) mice with NFAT1, 2 and 4 being most abundantly detectable. When we employed NK cells with knockout (KO) of NFAT 1, 2, and 4 in comparative in vitro analyses, we surprisingly found that lack of NFAT resulted in enhanced NK cell activation, degranulation and release of immunomodulatory cytokines like IFN-γ after co-culture with YAC-1 target cells as well as increased production of granzyme B and perforin after cytokine activation. The inhibitory effect of NFAT on NK cell effector function was further confirmed in vivo by employing WT and germ line NFAT KO animals in the syngeneic B16 melanoma model, which revealed a significantly reduced metastatic burden in NFAT KO mice. Depletion of NK cells in this model system in turn resulted in increased metastasis, however, with WT animals displaying significantly higher metastatic burden compared to NFAT KO mice. As this pointed to the fact that NFAT influences metastasis via both NK-dependent and independent mechanisms, we further generated mice with a NK cell-specific (conditional Ncr1-Cre dependent) NFAT2 KO. When these animals were employed again in analyses of B16 lung metastasis, comparative analyses with WT animals confirmed the inhibitory effect of NFAT on NK tumor immunosurveillance.

Taken together, these results provide the first direct evidence for the functional involvement of NFAT in NK cell antitumor reactivity and, in contrast to T and B cells, identify NFAT as a negative regulator of NK cell function.

Perforinopathy: a spectrum of immune disease caused by defective perforin delivery or function

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The pore-forming protein perforin (PRF) is an essential effector molecule of cytotoxic T-lymphocytes (CTL) and natural killer (NK) cells (Law, 2010). It is stored in secretory granules and released into the immune synapse upon the recognition of a target cell by CTLs or NK cells. There, PRF forms large transmembrane pores (Lopez, 2013) and allows the entry of pro-apoptotic serine proteases, granzymes, which kill the virus infected or cancerous target. The remarkable efficiency of perforin is determined by a unique mechanism of pore formation (Leung, 2017). The loss of PRF function in humans results in the catastrophic collapse of immune homeostasis, manifested as a lethal immune dysregulation, Familial Haemophagocytic Lymphohistiocytosis (FHL) (Voskoboinik, 2015). We discovered that this was due to the failure of non-functional CTL/NK cells to detach from the target leading to cytokine hyper-secretion (Jenkins, 2015).

Animal studies have indicated a critical role of PRF in immune surveillance of cancer, but no evidence has yet linked defective perforin cytotoxicity with cancer susceptibility in humans. Although some patients with bi-allelic PRF1 mutations present with FHL in early infancy, a significant cohort of individuals develop atypical forms of the disease and/or other primary pathologies in adolescence or adulthood (Voskoboinik, 2015). Almost 50% of such patients, who lived to at least 10 years of age without FHL, developed a haematological malignancy. Remarkably, we found that mutant PRF misfolding was one of the major causes of impaired CTL/NK function leading to immune-mediated diseases (Chia, 2009). Furthermore, we recently identified a family in which a wide range of inflammatory and oncological diseases have occurred over three generations and was associated with PRF misfolding mutations.

Compiling our data across all of the PRF variants tested, there was a striking direct relationship between the age and severity of onset of disease, and the degree of PRF mifolding and dysfunction. Consistent with these patterns, the least misfolded PRF mutants were invariably associated with atypical late-onset FHL, cancer or aggressive viral infections, while a complete loss of function was always associated with FHL in infants.

These studies provide the most compelling evidence for a role for PRF and cytotoxic lymphocytes in human cancer immune surveillance, establish the novel paradigms for PRF misfolding as a novel immune-mediated disease and strongly suggest PRF mutations as a cause of hereditary cancer predisposition.

Identifying lincRNAs associated with key transcription factors of NK cell development employing RNAseq

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Long noncoding RNAs (lncRNA) have recently been identified to play essential roles in positively or negatively regulating the expression of protein-coding genes. Notably, pairs of regulated gene and lncRNA gene often map closely together. We performed RNA seq analysis of 90 samples from flow cytometry-sorted cells (NK=67, T=15, CD34=4, B=2, monocytes=2) derived from blood, bone marrow, liver, and tonsil as well as NK cell in vitro differentiation assays to analyze the transcriptome of various functional NK cell subsets (CD56dim/bright, licensed/non-licensed, CMV memory and other memory), and developmental stages. Differential gene expression analyses led to the identification of several lncRNA genes that were either specifically expressed in NK cells (as compared to B, T, and monocytes) or were found at defined stages of NK cell differentiation. The expression of RP11-222K16.2, RP11-291B21.2, and LINC00299 lncRNAs turned out to be correlated with those of EOMES, NKG2C, and ID2, respectively. Remarkably, the lncRNA genes are located close to (2-300 kb) their respective correlated protein-coding genes. A further interesting pair is given by AL450992.2 and RORC, which are 10 kb apart and show a highly correlated expression in ILC3 cells isolated from tonsil samples as well as in ILC3 that were generated from cord blood-derived hematopoietic progenitor cells.

No function has so far been described for the identified lncRNAs, except for a single report of LINC00299, which is disrupted in a patient with neurodevelopmental disabilities. Notably, also dysregulated expression of ID2 was previously reported to result in abnormal brain development. Currently we are performing single-cell RNA seq of 160 NK cells derived from peripheral blood to confirm co-expression of lncRNAs and their correlated genes at single cell level. Moreover, we are modulating lncRNA expression in hematopoietic progenitor cells to unravel their role during NK cell differentiation in vitro.

Reprogramming of mature T8 cells into cells with NK cell-like phenotype from BCL11b-k.o. mice

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Natural killer cells are key effectors in tumor cell surveillance. However, the clinical application to combat cancer is limited so far. New strategies for a better availability of NK-cells for therapeutic interventions are needed. Starting from genetic analysis of T-cell malignancies a crucial relevance of the BCL11b gene for the T-cell fate was identified. Subsequent studies showed that genetic loss of this gene at any level of the T-cell development induced reprogramming of these cells into ITNK cells with strong tumor killing potential. To better understand underlying processes of this reprogramming in T-cells we used an inducible genetic Bcl11b knock out mouse model. Sequential gene expression analysis and flow cytometry characterization after knock down was performed to get insight in the reprogramming. Materials and methods: RNA-Sequencing was performed by next-generation sequencing on a SOLiD 5500xl Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). RNA was extracted usingTrizolR. rRNA was removed from samples using a RiboZero Kit (Epicentre, Madison, WI, USA. RNA was fragmented by chemical hydrolysis, phosphorylated and purified. Adaptors were ligated to the RNA fragments prior to reverse transcription. cDNA was used for preparation of a sequencing library (Bouter et al. 2014) and 75 nucleotides were sequenced in the forward direction and 35 nucleotides in the reverse direction. Sequence reads were mapped to the mouse genome reference sequence mm10 (HYPERLINK "ftp://hgdownload.cse.ucsc.edu/goldenPath/mm10/" \t "_blank" ftp://hgdownload.cse.ucsc.edu/goldenPath/mm10/).
Results: T8 cells from lymph nodes of Cre and Wt control mices were prepared by negative magnetic cell sorting and expanded for 7 d in the presence of IL2 and a.-CD3 / CD28 beads (Miltenyi), followed by the knock down of BCL11b by the addition of OHT for 2 d.
In all, control and BCL11b-k.o., cultures a strong shift into memory cell phenotypes was seen. But only in BCL11b-k.o. cultures a strong de novo expression of surface and cytoplasmic NK cell markers (NKp46, NK1.1, granzyme B) on up to 70 % of all vital cells, associated with strong changes in gene expression, size and shape were seen starting around d 3-8 after OHT removal. Further work is done to proof functional capacities of the resulting NK-like cells and to expand them in cell culture.
Our results demonstrate that even in the case of terminal differentiated memory T8 cells availability of BCL11b is necessary to maintain T cell identity.

Accumulation of Dense-core Granules Determines Functional Potential in Educated NK cells

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The development of functional competence during natural killer (NK) cell education depends on interactions between inhibitory receptors and host MHC class I molecules. However, the mechanism that connects inhibitory signaling to the downstream functional potential of NK cells remains elusive. We found that education through self-MHC specific inhibitory killer cell immunoglobulin-like receptors (KIR) is linked to the accumulation of large, granzyme B-dense cytotoxic granules. This unique morphological phenotype persists independently of transcriptional programs that regulate metabolism, granule biogenesis and expression of effector molecules. Disruption of the granular compartment using lysosomotropic agents reduced both target-specific degranulation and cytokine production by self-specific NK cells, associated with loss of Ca2+ flux and distal signaling. Conversely, chemically induced fusion of lysosome-related organelles led to a lower threshold for activation, resulting in gain of function. Thus, integration of receptor signaling during NK cell education translates into qualitative differences in the granular compartment, which in itself acts as a signaling hub that determines functional potential in NK cells. The granular matrix thus represents a form of molecular memory that allows priming and effector functions to be temporally separated, enabling a state of functional persistence characteristic of NK cell education.

Characterization of immature NK cells inside the inflamed CNS by the co-expression of CXCR3 and the activatory receptor DNAM-1

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We have recently shown that the CX3CR1 receptor contributes to the migration of protective CD11b+ mature NK cells into the central nervous system (CNS) in the mouse model of multiple sclerosis, the experimental autoimmune encephalomyelitis (EAE). The CX3CR1-deficient mice presented an increased proportion of immature CD27+ NK cells in the CNS during EAE. However, it is still unclear which chemokine receptor mediated the migration of these immature NK cells that are also present in the healthy and inflamed CNS of WT mice. We have performed an extensive analysis of the chemokine receptors expressed on immature NK cells and showed that inside the CNS, immature NK cells express both CXCR3 and the activatory receptor DNAM-1. Interestingly, DNAM-1-high expressing NK cells could be segregated into CXCR3+ and CXCR3- cells. DNAM-1highCXCR3+ NK cells have an entire pre-mature and immature phenotype defined by CD27-CD11b- and CD27+CD11b-, respectively, while the loss of CXCR3 expression on the DNAM-1-high subset coincides with a progressive NK cell maturation identified by the expression of CD11b. Thus, CXCR3 serves to discriminate two subtypes of immature DNAM-1-high NK cells, while lack of CXCR3 and low expression of DNAM defined the mature NK cell fraction.
During EAE, DNAM-1highCXCR3+ NK cells are reduced in the CNS at disease peak while DNAM-1highCXCR3- NK cells increase, compared to the naïve mice. However, in the chronic stage of EAE, the DNAM-1highCXCR3+ population is restored to proportions found in naïve mice. Using the Ki67 marker of cell cycle we identified that the most proliferating population of NK cells in the inflamed CNS is the DNAM-1highCXCR3-. Ongoing experiments will elucidate whether the ligation of CXCR3 with its ligands actively produces a phenotypical and functional change on NK cells as it has been shown for T cells. Additionally, we will define the implication of these NK cell populations in the course of EAE disease.

Killer-Specific Secretory Protein of 37 kDa in Natural Killer Cells

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Natural killer cell differentiation is linked to series of transcriptional and epigenetic changes associated with a shift from cytokine- to receptor-driven functional responses.
Transcriptional profiling of discrete subsets of NK cells revealed that the killer-specific secretory protein of 37 kDa (KSP37) increased gradually with NK cell differentiation and was one of the most abundant transcripts in terminally differentiated adaptive NK cells. KSP37 is a secretory protein with a yet poorly defined function that was identified by Ogawa et al. in 2001 1 and known to be expressed in CD4+, CD8+ and in γδ T-cells, in addition to CD16+ NK cells. Confocal microscopy revealed that Ksp-37 was pre-formed in resting NK cells and stored in vesicular structures that were distinct from granzyme B and perforin-containing secretory lysosomes. Furthermore, KSP37 was more polarized in mature cells, suggesting that packaging of KSP37 into vesicles is tightly regulated in mature cells.

To study the release of these KSP37 containing vesicles, NK cells were subjected to increasing concentrations of IL-15, resulting in depletion of KSP37 stores in a dose-dependent manner. This effect was visible after only four hours of stimulation; however, continuous stimulation did not deplete KSP37 stores completely. This is supported by transcriptional data showing an increased transcriptional rate at 24 hours following stimulation with IL-15, which then declines with time, suggesting that the transcriptional regulation of KSP37 is partly influenced by the IL-15 signaling pathway, but not necessarily by STAT5, which acts as a second messenger early on in this cascade. We also used Brefeldin A, an agent that specifically block the transport between the ER and Golgi, which resulted in accumulation of KSP37 in NK cells, and supporting a consecutive secretion pattern. Utilizing high-dimensional flow cytometry revealed an accumulation of KSP37 in mature unstimulated NK cells, this phenotype was also present after IL-15 stimulations, indicating that differentiated NK cells are capable of retaining more KSP37 than immature cells.

Thus, Ksp-37 is an abundant pre-formed effector molecule with a subset specific distribution that is static both under the resting and activated state of NK cells. We hypothesize that KSP37 release from NK cells is part of an until now unknown helper-function involving recognition of tumor cells via the NKG2D pathway.

Quantitative analysis of NK cell reactivity using latex beads

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Natural Killer (NK) cell responses are regulated by a variety of 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 chose to functionalize latex beads that have approximately the same size as lymphocytes with specific antibodies directed against distinct activating NK cell receptors. This enabled us to investigate NK cell reactivity in a defined, clean and controllable system.
The latex beads were covalently coated with antibodies against various activating NK cell receptors or control antibodies via a chemical crosslinker. The antibodies were used in different concentrations and the exact amount of antibodies bound to one bead was calculated with the aid of a flow cytometry based quantification kit.
NK cells were stimulated with beads that were coated with different amounts of antibodies and the effector functions induced by this were analyzed. We quantified degranulation and used fitting curves to describe the dose dependence in detail. This enabled us to compare the different receptors in their sensitivity and efficacy to activate NK cells. The ITAM coupled receptors appeared to be most potent with CD16, NKp30 and NKp46 inducing the strongest maximal degranulation and CD16 and NKp44 needing the lowest stimulation to reach half-maximal degranulation. The only receptor with significant differences to the other receptors tested was 2B4, showing only a weak stimulation. In addition to degranulation we also detected the production of IFN-γ and MIP-1β. 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.
Our data demonstrate that activating receptors differ in their effectiveness to activate NK cells. Coating our latex beads with defined amounts of recombinant ligands for these receptors will enable us to also address differences in receptor/ligand interactions in the future.

Diversification of both KIR and NKG2 NK cell receptors in macaques

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Killer immunoglobulin-like receptors (KIR) as well as their MHC class I ligands display enormous genetic diversity and polymorphism in macaques. Recently, we have shown that also the NKG2C genes are expanded in rhesus macaques. Bioinformatic analysis of two overlapping BAC clone sequences revealed the presence of three NKG2C genes in addition to single-copy NKG2A and NKG2F genes. According to phylogenetic tree analysis and amino acid comparisons no NKG2E-corresponding gene is present. The three NKG2C proteins are 90-95% identical. Comparison with human NKG2C suggests that all three rhesus macaque NKG2C proteins may interact with Mamu-E, the ortholog of HLA-E.
So far we have no evidence for genomic polymorphism with respect to presence or absence of NKG2C genes. However, analysis of NKG2C cDNA sequences derived from 8 individuals revealed polymorphisms in the three NKG2C genes. Besides some variations in the ligand-binding C-type lectin-like domain, polymorphisms are found in regions encoding the stalk and transmembrane. Interestingly, the stalk region of mouse activating and inhibitory Ly49 molecules is known to bind to mouse cytomegalovirus immunoevasin m157. Thus, we hypothesize that exposure to rhesus CMV (and possibly further herpesvirus family members) has driven expansions and diversification of NKG2C genes in macaques.

Comparative analysis of IL-21 and autologous feeder cell-driven ex vivo expansion after separation of primary NK cells using a fully-automated platform

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BACKGROUND AND AIMS: The infusion of ex-vivo expanded Natural killer (NK) cells as potential antitumor effector cells appears to be suitable for effector cell-based immunotherapies in high-risk cancer patients. However, the GMP-conform manufacturing of clinical-grade NK cells at sufficient high numbers represent a great challenge. Therefore, we improve and optimized previous expansion protocols for those effector cells through the use of newly developed culture medium, IL-21 and autologous feeder cells.
METHODS: Separation of primary human NK cells (CD3-CD56+) was carried out with the CliniMACS Prodigy® (Prodigy) in a 2-step process starting with 0.1–1.24 × 10e10 leukocytes collected by lymphapheresis or buffy coats. Enriched NK cells were adjusted to starting cell concentrations within 2–3 x 10e6 cells/ml and cultured in comparative expansion experiments for 14 days with interleukin-2 (IL-2, 1000IU/ml) in different GMP-compliant media (X-VIVO™10, CellGro®, TexMACS™ and NK MACS®). After medium optimization, we investigated beneficial effects for functionality and phenotype at the beginning of cell expansion with irradiated (25Gy) autologous feeder cells in a ratio of 20:1 (feeder:NK) in presence and absence of IL-21 (100ng/ml).
RESULTS: The Prodigy manufacturing process revealed high target cell viabilities (median: 95%, range: 83–99%), adequate NK cell recovery (median: 51%, range: 41–100%) and purity of 93% (range: 78–98%) in regard to CD3-CD56+ target cells. The process in its early phase of development led to a median T-cell depletion of log 3.1 (range: 1.6–3.9) after CD3 depletion and log 3.3 (range: 1.7–4.4) after the whole process including CD3 depletion and CD56 enrichment step. Followed manually performed media experiments with NK MACS® resulted in significant higher NK cell expansion rates and an approximately equal distribution of CD56dimCD16pos and CD56brightCD16neg NK subsets on day 14. Moreover, manual effector cell expansion in NK MACS® containing IL-2 and, at the initiation of the culture, irradiated autologous feeder cells in combination with IL-21 induced a 78-fold (range: 67–88) NK cell proliferation. Compared to freshly isolated NK cells, 14 days-expanded NK cells revealed significant higher levels of NKp30, NKp44, NKG2D, TRAIL, FasL, CD69 and showed comparable cell viabilities and killing/degranulation activities against tumour and leukemic cell lines in vitro.
CONCLUSIONS: After fully-automated NK cell separation process on Prodigy, we generated a new NK cell expansion protocol that resulted in high numbers of NK cells with potent antitumor activity. Next steps are the integration of the manual expansion procedure in the fully-integrated platform for a standardised GMP-conform overall process in this closed system.

Standardized and flexible eight colour flow cytometry panels harmonized between different laboratories to study human NK cell phenotype and function

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Background: Advancements in multi-colour fluorescence activated cell sorting (FACS) panel warrant harmonized procedures to obtain comparable data between various laboratories. The intensifying clinical exploration of Natural Killer (NK) cell-based immunotherapy demands standardized and harmonized NK cell FACS panels and acquisition protocols. Methods: Eight-colour FACS panels were designed to study human NK cell phenotype and function within peripheral blood mononuclear cells (PBMC). The panels were designed around fixed backbone markers and channels, covering antigens for non-NK lineage exclusion (CD3, TCRγδ, CD19, CD14, SYTOX® Blue) and NK cell selection (CD45, CD56, CD16), complemented with variable drop-in markers/channels to study NK-cell phenotype (NKG2A, NKG2C, NKG2D and KIR2D) or NK cell function and activation (CD25, NKp44 and CD107a). Results: Harmonized FACS set-up and data analysis for three different flow cytometers at three different sites has been established, leading to highly comparable and reproducible data sets using the same PBMC reference samples (n=6). Further studies of NK cells in fresh or cryopreserved PBMC samples (n=12) confirmed that freezing and thawing of PBMC samples did not significantly affect NK phenotype or function. Conclusion: Our data demonstrate that cryopreserved PBMC samples analyzed by standardized FACS panels and harmonized analysis protocols will generate highly reliable data sets for multi-center clinical trials under validated conditions.

Improving kinetics expansion of human natural killer cells used for adoptive immunotherapy

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INTRODUCTION: Expansion ex vivo of natural killer cells is a source of producing a large number of these cells for immunotherapy. NK cell expansion protocols are required to optimize clinical scale based immunotherapy. The procedure is based on an existing expansion process: 14 days co-culture with k562-mb15-41BBL cell line kindly provided by Professor Dr. D Campana (NUH Hospital, Singapore).34 kinetics expansion processes were evaluated using four different growth culture media: RPMI-1640 medium (Lonza, Belgium); SCGM medium(Cellgenix, Germany); NkMACS medium and TexMACS GMP medium (Miltenyi Biotec, Germany)
METHODS:
Peripheral blood mononuclear cells (PBMC) were obtained from 9 healthy adult donors. Lymphocyte subpopulations were determined by flow cytometry with the following panel of conjugated antibodies: CD45-FITC;CD19/20-PE; 7AAD-PerCpcy5;CD3-PECy7; CD56-APC;CD16-APCCy7. K562-mb15-41BBL was used to expand NK subpopulation after 3 weeks of co-culture. All media were supplemented with 10% human AB serum and IL-2 10 IU/mL first week and 100 IU/mL thereafter. % of NK cells were monitored every week. The cytotoxicity was evaluated by europium-TDA release assays. Degranulation was analyzed by means of flow cytometry using expression of CD107A. Cytokine production was measured at different expansion time by CBA assay
RESULTS: After analysing the process of expansion (21 days) in the different culture media, the ratios of increase in the NK subset were the following (Range 10,4-86 fold day 7;161-404 fold day 14;180-526 fold day 21; n=12) (+- SD): RPMI-1640 medium yielded 170-fold increase; SCGM medium a 292-fold increase; NkMACS medium a 395-fold increase; TexMACS GMP medium a 409-fold increase. All the processes resulted in a highly pure NK cell product (CD56+CD3- >80%). The effector functions of NK cell products on stimulation with k562 target cells revealed no differences in the production of pro inflammatory cytokines (TNFa, IL10, IL6, IL-1β, IL8), similar levels of degranulation and cytolytic activity. Three procedures were performed in the CliniMacs Prodigy (automated expansion system) using the TexMACS (best choice in GMP grade) medium with results similar to those obtained in manual expansion processes.
CONCLUSIONS: Our results demonstrate that NkMACs /TexMACs media are the most effective options for increasing the cell expansion process. All NK cell products had similar phenotypic and functional profiles; hence, this culture media can be used to improve clinical expansion procedure in both a fully automated system (CliniMacs Prodigy) and in manual expansion procedures under Good Manufacturing Practice.

FULLY AUTOMATED TWO STEP SEPARATION PROCEDURE FOR CLINICAL PURIFICATION OF NK CELLS USING A SINGLE INSTRUMENT

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Donor derived Natural Killer (NK) cells are promising for the treatment of cancer patients. Studies reveled that allogeneic NK cells mediate graft versus leukemia (GvL) effects while they don't increasing graft versus host disease (GvHD), in contrast to allogeneic T cells. Prior to infusion, NK cells products are often purified and T cells are removed from the graft. Therefore, depletion of CD3+ cells and enrichment for CD56+ cells Is performed sequentially in two semi-automated procedures, requiring multiple manual hands on steps. To optimize the procedure and to enable fully automated clinical purification of NK cells, we developed a combined CD3 depletion and CD56 enrichment within a single automated process.
Based on magnetic cell separation (MACS) and the CliniMACS® Prodigy platform, the entire process for NK cell purification was procured fully automated, including sample preparation, all washing steps as well as labeling and separation of the cells. Sampling can be done aseptically at different process steps for counting & flow cytometry, if required for in-process control. The process was performed in a closed system consisting of only one single use tubing set. Leukapheresis products from healthy donors were used as starting material and the developed process resulted in efficient depletion of unwanted CD3+/CD56- T cells (4.0-4.3 Log) and B cells (2.3-3.2 Log). The final cell products consisted of >98% CD3-/CD56+ cells with a low frequency of remaining T (<0.07%) and CD3+/CD56+ NK-like T cells (<0.6%) but up to 18% CD3-/CD56+/CD14+ monocytes. In relation to the starting numbers, 26-74% of the NK cells (CD3-/CD56+/CD14-) were recovered in the cell product after processing.
In summary, we present a novel process for clinical NK cell purification in a fully automated way. The high level of automation enables standardized, consistent and operator independent processing of NK cells for therapeutic applications. Most importantly, the process allows efficient depletion of T cells for adoptive transfer of the cell product in allogeneic settings.

A disturbed IL-12 signaling in CD56bright NK cells contributes to an impaired immune response to Staphylococcus aureus in severely injured patients

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Severely injured patients are at high risk for nosocomial infections due to the development of immunosuppression of unknown origin. Despite advances in modern intensive care medicine such opportunistic infections frequently result in life-threatening sepsis. The rise in antibiotic resistance worldwide demands novel therapeutic concepts that restore the immune defense in injured patients. CD56bright natural Killer (NK) cells play an important role in the immune defense against diverse bacterial infections through the release of Interferon (IFN) gamma that activates phagocytes for the eradication of the microbes. In the present study, we analyzed the ability of human CD56bright NK cells from injured and healthy subjects to respond to Staphylococcus aureus infection. Blood mononuclear cells (PBMC) from severely injured patients were isolated at different time points (1 d to 3 weeks) after injury and were stimulated with inactivated S. aureus bacteria. PBMC from healthy volunteers served as control. CD56bright NK cells from patients were suppressed in IFN-gamma synthesis throughout the observation period and could not be rescued by the addition of recombinant IL-12. The suppressed IFN-gamma synthesis correlated with a reduced expression of the IL-12 receptor (IL-12R) beta2 chain and downstream with decreased Signal Transducer and Activator of Transcription (STAT) 4 phosphorylation. Exposure of the patients' PBMC to S. aureus in the absence of autologous serum increased the production of IFN-gamma and the expression of the IL-12Rbeta2 chain in/on NK cells. In turn, the transfer of the patients' sera on PBMC of healthy volunteers caused a reduced S. aureus-induced IFN-gamma production, IL-12Rbeta2 expression, and STAT4 phosphorylation as found for the patients' NK cells. The suppressive effect of the patients' serum on NK cells from healthy subjects was at least partly mediated by TGF-betaRI signaling. However, TGF-beta and activin as known TGF-betaRI ligands were not responsible for the inhibitory activity of the patients' sera. In summary, severe injury is associated with inhibition of CD56bright NK cell function that is characterized by a disturbed IL-12 signaling and might contribute to the enhanced risk for nosocomial infections.

Cellular immunotherapy with multiple infusions of ex vivo expanded haploidentical natural killer cells after autologous transplantation for patients with plasma cell myeloma

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Plasma cell myeloma (PCM) is currently treated with chemotherapy and autologous stem cell transplantation (ASCT), but relapse rates remain high. Adoptive transfer of mature haploidentical natural killer (NK) cells is a promising approach to provide PCM patients with immunocompetent effector cells with anti-myeloma function after transplantation. Here we report on the current clinical Phase I/II trial of multiple preemptive infusions of good manufacturing practice (GMP) expanded NK cells to PCM patients (clinicaltrials.gov NCT01040026).

Ten PCM patients were recruited (seven males, three females, median age: 59y). All patients received four cycles of VTD chemotherapy (reaching CR: 4x, VGPR: 5x and PR: 1x) before high dose therapy with melphalan 200mg/m2 and ASCT. Patients' stem cells were mobilized and cryopreserved after the third VTD cycle.

NK cells from haploidentical family donors were purified from unstimulated leukapheresis by a 2 steps method consisting of T cell depletion and NK cell selection using CliniMACS. Highly pure NK cells were expanded ex vivo for 19 days with autologous irradiated feeder cells in GMP-medium containing interleukin-2 and -15. NK cell numbers increased 54-fold (range: 38- to 76-fold). In three NK cell productions T cell contents were above the limit of 1x10e5 cells/kg BW and were successfully reduced by a 2° CD3-depletion. NK cell products were cryopreserved in escalating doses before administration.

The PCM patients received from 65 to 460x10e8 expanded NK cells (median: 3.8x10e8 cells/kg BW, range: 0.9 – 5.7x10e8 cells/kg BW) as 3 – 8 infusions (median 6 DLIs). The NK-DLIs were administered between day 2 and 21 after ASCT as escalating dose from 1.3x10e6 to 1.0x10e8 cells/kg body weight (BW). The NK-DLIs were well tolerated without any acute adverse events. No signs of acute or chronic graft-versus-host disease were observed in any of the patients, and engraftment after ASCT occurred between days 13–24 (median: 16 days). Infused donor NK cells were monitored by short-tandem repeats PCR and detected in peripheral blood of PCM patients 1 and 20 hours post infusion, indicating NK cell survival in recipients in the absence of IL-2 support in vivo.

These results demonstrate the feasibility of large-scale GMP expansion and safety and tolerability of multiple high-dose infusions of human NK cells as immunotherapy after stem cell transplantation for PCM.

The first experience of application of natural killer cells in the therapy of acute myeloid leukemia in children in Belarus

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The subject of the study was the analysis of preliminary results of haploidentical NK cells immunotherapy in the first line of de novo AML treatment.
5 patients (aged form 2 to 13) of the intermediate risk group with de novo AML were treated according to the original AML-MM-2014 protocol. According to this protocol the patients of the intermediate risk group were randomized to the arm with 5 course of chemotherapy and the arm with 4 course of chemotherapy followed by haploidentical NK cells immunotherapy. The conditioning before NK cells infusion included cyclophosphamide (60 mg/kg, -7 day) and fludarabine (25 mg/m2, from -6 to -2 days).
NK cells from haploidentical donors were selected from the product of apheresis after two steps of isolation (CD3 depletion, CD56 selection) by Clinimacs. NK cells for 3 patients were activated in vitro (18-72 h) and for 2 patients – in vivo with IL-2 ("Roncoleukinum"). Purity of selected NK cells was 83,1±7,6 %, the mean dose of infused NK cells was 17,1±4,8 x106/kg.
Adverse effects of NK immunotherapy were estimated according to CTCAE scale (version 4.0). Leucopenia and neutropenia (grade 4) were the main side events. The mean duration of neutropenia (<500 neutrophils in mkl) was 23 days (from 14 to 56 days).
Patients 1 developed secondary leukemia (ALL) after 2,5 month after the end of the treatment and died from the disease progression. Patients 2-5 are alive in remission from 3 to 27 months.
In conclusion, natural killer cells immunotherapy for the children with AML is feasible, safe and tolerable.

Donor NK cell alloreactivity influences on immune recovery after HSCT in children

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In the study we compared the parameters of immunological recovery after hematopoietic stem cell transplantations (HSCT) in children depending on donor NK cell alloreactivity.
Recovery of B-, NK-, T-cells and its subpopulations during the first year after HSCT was studied. The data of 69 matched (31 related, 38 unrelated) HSCT was analyzed. Bone marrow was the source of stem cells in all cases. Donor NK cell alloreactivity was evaluated according to the receptor-ligand mismatch model (the donor has an inhibitory receptor for which the cognate ligand is absent in the recipient). Alloreactivity of donor NK cells was found in 46 transplantations.
At +30 day after HSCT we did not find any significant differences in the recovery of lymphocyte subpopulations between the groups of patients with alloreactivity or without one. Significantly decreased absolute number of NK cells was detected at +60 and +100 days after transplantations in the group of patients with alloreactivity compared with the group of patients without one. At +180 and +365 days in the group of patients with alloreactivity was found the significantly higher level of CD3+CD4+ T-helper cells and its subpopulations. Thus, in the group of patients with alloreactivity the absolute level of T-helper cells was 0,29 (0,2-0,3) x109/l at +180 day and 0,68 (0,56-0,8) x109/l at +365 day when in the group without alloreactivity it was 0,18 (0,1-0,2) x109/l and 0,4 (0,2-0,58) x109/l, correspondently. At +365 day the levels of the naïve T-helper (CD4+CD45RA+) cells and the recent thymic emigrants (CD4+CD45RA+CD31+) were significantly lower in the group of patients without alloreactivity.
In conclusion, the patients who received transplantations without donor NK alloreactivity showed the higher level of NK cells at the earlier time points after HSCT and the slower speed of recovery of T-helper cells.

Influence of irradiated PBMCs in natural killer (NK) cell ex vivo proliferation and preferential expansion of HLA-DR-expressing NK cells

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The feasibility of clinical NK cell studies has demonstrated promising potential to treat cancer, increasing the development of methods for the generation of high NK cell numbers for clinical applications. Expansion methods based on the use of peripheral blood mononuclear cells (PBMCs) as feeder cells stimulate significantly ex vivo NK cell proliferation. Nevertheless, few is known about the controlling mechanisms behind.
We investigated the influence of irradiated PBMCs to boost NK cell proliferation in the presence of OKT3 and IL-2. Physical contact between feeder cells and NK cells seemed to be indispensable to enhance NK cell proliferation ex vivo, not only due to occurrence of receptor-ligand interactions, but also due to soluble factors produced in co-culture which sustain proliferation. We observed that among these feeder cell-NK cell interactions, both T cells and monocytes played a key role in supporting NK cell proliferation, meanwhile presence of B cells was dispensable.
Moreover, a whole genome microarray analysis of highly proliferating and non-proliferating NK cells revealed important phenotypic changes on 5-days cultured NK cells observed with and without feeder cells. Reduction of Siglec-7 and 9 expression characterizes NK cells in active proliferation as opposed to non-proliferating and resting NK cells (day 0), independently of the presence of feeder cells. Interestingly, proliferating NK cells cultured with feeder cells contained increased frequencies of cells expressing RANKL, B7H3 and HLA class II molecules, particularly HLA-DR, as compared to resting NK cells or expanded with IL-2 only.
Our results highlight the importance of the crosstalk between T cells, monocytes and NK cells in feeder cell-based expansion protocols as well as support a preferential expansion of HLA-DR-expressing NK cells ex vivo.

Subset-dependent stress response in human NK cells following IL-15 starvation

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Adoptive transfer of allogeneic NK cells hold great promise for cancer immunotherapy. There are several alternative protocols to expand NK cells in vitro, most of which are based on stimulation with cytokines, feeder cells or combination of both. Although IL-15 is essential for NK cell homeostasis in vivo it is commonly used at supra-physiological levels to induce NK cell proliferation in vitro. Therefore, adoptive transfer of IL-15 addicted NK cells is associated with cellular stress due to sudden cytokine withdrawal. Here, we established an in vitro culture model with high- or low-dose IL-15 concentrations to achieve either a robust NK cell proliferation or a more homeostatic culture condition. NK cells pretreated with a high-dose IL-15 concentration demonstrated a massive loss in cell numbers upon IL-15 withdrawal, whereas pretreatment with low-dose IL-15 resulted in stable NK cell numbers over 48h of IL-15 starvation. We observed a strong up-regulation of active Caspase-3 and down-regulation of anti-apoptotic BCL-2 family proteins, especially in strongly dividing cells (≥2 cell divisions). Moreover, NK cells expressing NKG2A or a single educating KIR were more susceptible to IL-15 withdrawal than their respective counterparts.

It has recently been shown in mice that autophagy is playing an important role during NK cell development and the formation of NK cell memory upon MCMV infection. Autophagy is a lysosomal degrading system and is typically activated upon cellular stress (e.g. nutrition and growth factor starvation or oxidative stress). During IL-15 starvation we observed a time-dependent increase of autophagosomes, which was further increased by chloroquine treatment indicating that IL-15 withdrawal promote autophagy flux in starved NK cells. The effect was significantly stronger in NK cells pretreated with high-dose IL15 as well as in strongly dividing cells (≥2 cell divisions).

Thus, our data show that the stress response in NK cells following IL-15 withdrawal depends on both NK cell differentiation and education and involves dynamic changes in the autophagy and anti-apoptotic machineries determined by the IL-15 dose and degree of cell division. Further insights into the regulation of autophagy during NK cell starvation may pave the way for new means to prime NK cells for adoptive transfer following in vitro expansion.

Natural Killer Cells Associated Profiles in Cancer and Pregnancy as Sensors of Clinical Outcome.

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Natural Killer (NK) cells are innate lymphocytes with the ability to eliminate target cells, secrete cytokines and contribute to tissues remodeling. NK cells functions are being regulated by an axis of activating and inhibitory receptors that recognize various ligands on target cells. Hence, alterations in NK cells receptors (NKR) or in their ligands profiles result in different NK cells function. Moreover, disease related microenvironment factors can influence NKR and ligands profiles. Therefore, profiling of NKR and their ligands can be used as a tool for disease diagnostics and prognosis. Profiles examination of NKR and their ligands in Acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), various solid tumors, and in pregnancy disorders pointed to a condition related profiles. In ALL, NKG2D higher mRNA expression and NK cells mediated function results in central nervous system (CNS) ALL. Moreover, profiling of NKG2D ligands (ULBP1, ULBP2, ULBP3) in patients BM samples (n=68), significantly differentiate between CNS negative, CNS positive and CNS relapse cases. In AML, a new role of NKp44 was discovered after profiling NKp44 splice variants from blood samples of newly diagnosed AML patients (n=36, TCGA data). Solitary expression of NKp44(ITIM+) variant (NKp44-1) was associated with poor survival. Moreover, dominant expression of NKp44-1 was seen in 80% of various solid tumors cases (TCGA and qPCR data). Anatomically, NKp44 expression is seen in human Decidua. Decidua tissues from first trimester spontaneous abortion lack dominant expression of NKp44-1 in 80% of the cases (n=21) and was associated with cytokines regulating the NCR2 gene. Taken together, this data shows new aspects associated with NKG2D and NKp44 in cancer and pregnancy clinical outcome.

Hepatitis C Virus-induced Natural Killer cell proliferation involves monocytes and the OX40/OX40L axis

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Chronic infection with the hepatitis C virus (HCV) occurs in around 3% of the world population and is a major cause of liver disease, including liver cirrhosis and hepatocellular carcinoma.
Natural Killer (NK) cells are important effector cells in HCV infection. However, the mechanisms through which NK cells are activated and proliferate in response to HCV remain largely elusive.

Using the HCV replicon cell culture model, we show that after co-culture of replicon-containing human hepatic cells with peripheral blood mononuclear cells (PBMCs), an NK cell subset increases expression of the high-affinity IL-2 receptor chain CD25, proliferates and produces IFN-□. This NK cell activation was dependent on IL-2, cell-cell contact and monocytes. We found the TNF-receptor superfamily member OX40 was increased on the activated NK cell subset and blocking of OX40L, which was induced on monocytes co-cultured with replicon-containing cells and on hepatocytes, abrogated HCV-induced proliferation of NK cells. This effect was independent of the hepatic cell line and the HCV strain used.

In conclusion, our data uncover a mechanism of HCV-mediated NK cell activation and proliferation requiring cell-cell contact with monocytes and HCV-containing cells and a previously unappreciated involvement of the OX40/OX40L. These results reveal a novel mode of cross-talk between innate immune cells during viral infection.