
NK cells in the hepatitis B infected liver: a double-edged sword?

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NK cells constitute a large fraction of lymphocytes within the human liver and have been implicated in diverse roles in liver diseases such as hepatitis B. We have recently defined a large subset of NK cells compartmentalised in the liver that have a distinct transcription factor profile (T-bet^{hi}Eomes^{hi}) from peripheral NK cells (T-bet^{hi}Eomes^{lo}) and are marked by the surface expression of CXCR6 and CD69 (Stegmann et al Sci Rep 2016). In line with the tolerogenic liver environment, these tissue-resident NK cells produce less cytotoxic mediators and pro-inflammatory cytokines than their circulating counterparts. However, in the setting of hepatitis B, the CXCR6⁺ liver-resident subset upregulates TRAIL, which we have previously shown contributes to NK cell killing of hepatocytes (Dunn et al, JEM 2007) and of antiviral T cells (Peppas et al, JEM 2013). The NKG2D pathway is also involved in T cell/NK cell cross-talk in the HBV-infected liver (Huang et al, JI 2017). Our unpublished data reveal that the expression of decoy receptors for TRAIL on hepatic stellate cells allows them to counteract NK cell killing, thereby promoting fibrogenesis. Thus therapeutic harnessing of the antiviral and antifibrogenic potential of NK cells, such as by interferon-based treatment of HBV (Micco et al, J Hep 2013, Gill et al, PLoS Path 2016), may require additional manipulation to optimise beneficial rather than pathogenic effects.

NK cell mediated regulation of anti-viral T cell immunity

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Chronic viral infections display a major health burden worldwide. Viral infections can be eliminated by innate type I interferon production, which can limit viral replication in infected cells. Furthermore, type I interferons can signal to other immune cells such as CD8+ T cells and NK cells during viral infections with the lymphocytic choriomeningitis virus (LCMV). Type I interferon receptor (IFNAR) deficient T cells are dysfunctional during viral infection. Specifically, IFN-I increased expression of NK cell inhibitory receptors. Hence, IFNAR deficient T cell immunity was restored upon NK cell depletion. Accordingly, anti-viral CD8+ T cells were protected against regulatory NK cell functions by type I interferon. Furthermore, NK cell depletion or dysfunction resulted in increased T cell immunity during infection with higher doses of LCMV. Consequently, NK cell depletion resulted into control of an otherwise chronic LCMV infection. Taken together, these data indicate that NK cells may exhibit regulatory effects towards anti-viral T cells in these settings.

NK cell recognition of HIV-1-infected cells

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Adaptive diversification of NK-cell subsets in chronic HBV infection

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Hepatotropic viruses, such as hepatitis B (HBV) and C virus (HCV), shape the human NK-cell repertoire leading to phenotypic and functional alterations. However, little is known about the differentiation pathways of NK cells in chronic viral hepatitis. Recent data revealed that HCMV infection induces epigenetic diversification of NK cells including differentiation in adaptive NK-cell subsets. Phenotypically, these adaptive NK cells are characterized by down-regulation of the epigenetic regulator PLZF. Functionally this has been linked to enhanced antibody-dependent but reduced cytokine-induced effector responses compared to conventional NK cells. Thus, NK-cell subset distribution defines the functional capacity of the present NK-cell repertoire. Since NK-cell functionality is altered in chronic HBV and HCV infection compared to healthy controls (HC), we wondered whether this is also linked to a distinct NK-cell subset distribution and analyzed NK-cell heterogeneity according to the differentiation pathways described in HCMV infection.

We compared phenotypical and functional heterogeneity of circulating NK cells derived from patients chronically infected with HBV (n=49) or HCV (n=45) and HC (n=51) by performing multiparametric flow cytometry. Additionally, methylation patterns of the FCER1G and IFNG promoter regions were analysed via bisulfite conversion. Of note, data were collected with regard to the serological HCMV status of the donors.

Our results revealed an expansion of FcεR1γ- CD56dim NK cells in HCMV-seropositive chronically HBV-infected patients. These FcεR1γ- cells exhibited a transcriptional profile characteristic for adaptive NK cells with down-regulation of PLZF and Helios. According to the expression patterns of FcεR1γ and Helios, CD56dim NK cells could be subdivided into four subsets harboring different characteristics of conventional and adaptive NK cells. Specifically, we observed a differential expression of activating and inhibitory receptors (NKG2A, NKG2C, CD2), natural cytotoxicity receptors (NKP30, NKP46), and signaling proteins (Syk, EAT-2). Functionally, adaptive NK cells exhibited increased IFN-γ production upon CD16 engagement but reduced responsiveness to IL-12/IL-15 and IL-12/IL-18 stimulation. Analyses of the methylation patterns of the FCER1G and IFNG promoter regions showed that the phenotypical and functional properties of these different conventional and adaptive NK-cell subsets in chronically HBV-infected patients are epigenetically regulated.

In sum, HCMV-induced epigenetically regulated adaptive NK cells are expanded in chronically HBV infected patients and show distinct phenotypic and functional characteristics compared to conventional NK cells. Thus, the increased frequency of adaptive NK-cell subsets affects the NK-cell repertoire in chronic HBV infection.

NKG2Cpos NK cells associated with CMV infection inhibit expansion of activated virus-specific CD8 T cells

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Background and aims: Infection with the human Cytomegalovirus (CMV) imprints both the T cell and the NK cell compartment often resulting in expansion of CMV-specific CD8+ T cells and NK cells expressing NKG2C, an activating NK cell receptor binding to HLA-E. In the elderly, infection with CMV has been associated with impaired immunity. Here, we analyzed the impact of CMV-associated changes in the NK cell compartment on antiviral CD8 T cells.

Results: To analyze the impact of NK cell activation on virus-specific T cells, PBMCs of CMV seronegative and seropositive donors were cultivated in the absence or presence of irradiated NK cell target cells (K562). Virus-specific CD8 T cells targeting HLA-A*02-restricted epitopes of CMV or influenza A virus (IAV) were identified with dextramers. In CMV seropositive donors, antigen-specific expansion of CMV- or IAV-specific memory CD8 T cells was inhibited in the presence of K562 cells. In contrast, expansion of IAV-specific CD8 T cells was not inhibited in CMV seronegative donors. As previously described, CMV was associated with high levels of the activating receptor NKG2C on NK cells. We therefore hypothesized that expression of the NKG2C-ligand HLA-E on CD8 T cells may play a role in negative regulation. Indeed, expression of HLA-E increased with CD8 T cell differentiation to effector memory cells and was upregulated upon unspecific T cell activation and upon stimulation of CMV-specific or IAV-specific CD8 T cells with the cognate antigen in vitro. Importantly, this upregulation of HLA-E on activated virus-specific CD8 T cells was decreased in the presence of K562 cells in CMV seropositive donors but not in CMV seronegative donors, suggesting that negative regulation of CD8 T cells by activated NK cells is promoted in CMV infected donors. To address if cytolytic effector functions of NKG2C+ NK cells against HLA-Ehigh cells play a role in negative regulation of CD8 T cells, an HLA-Ehigh K562 target cell line was used. Upon activation with HLA-Ehigh target cells expression of the degranulation marker CD107a was significantly higher on NK cells from CMV seropositive donors compared to CMV seronegative donors and was associated with high NKG2C levels.

Conclusion: The CMV-associated expansion of NKG2Cpos NK cells promotes negative regulation of virus-specific CD8 T cells presumably via the interaction between NKG2C and HLA-E on activated CD8 T cells. This enhanced negative regulatory effect on T cells may contribute to the immune senescent state observed in elderly CMV seropositive individuals.

Regulation of antiviral T-cell response via modulation of NK cell function

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CD8+ T cells are central effectors of the adaptive immune response that serve the elimination of virus-infected cells. Fast-replicating virus strains or high viral burden are often associated with defective CD8+ T cell function, a condition that favors the establishment of chronic infection. Recently, natural killer (NK) cells have been shown to control CD8+ T cell responses at an early stage of infection, thereby contributing to virus persistence. While perforin plays a role in this process, it is not known whether other apoptotic mediators may also contribute to this NK-cell dependent regulation of the CD8+ T cell response.

Using a model of virus-induced hepatitis, we found a role for Tnfsf10/Trail in modulating the immune response and the subsequent immunopathology. Trail-deficient mice showed a higher virus-specific CD8+ T cell response than wild-type animals, resulting in better pathogen clearance and reduced liver pathology. This was strictly dependent on NK cells, as indicated by depletion studies. Further investigation disclosed a Trail-mediated mechanism modulating the function of NK cells in a cell-intrinsic and apoptosis-independent manner.

Taken together, our data reveal an unpredicted function of a well-known pro-apoptotic molecule in determining NK cell function independently of apoptosis signaling. They also show the relevance of this novel regulatory mechanism for the immunomodulation of T cell-mediated liver disease.

Generation of NK cell memory

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Natural Killer cells have the capacity to undergo clonal expansion, contraction, and the generation of a self-renewing population of NK cells with enhanced immune functions upon reencountering the same pathogen or stimulus. We have demonstrated mouse NK cells expressing the CMV-specific Ly49H or alloantigen-specific Ly49D receptor can acquire memory and have identified key receptors and molecules that enable this process. In this presentation, we will give an overview of NK cell memory and highlight new advances in this field.

The Secretary Lysosome as a Programmable Signalling Hub?

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Decoding the HLA immunogenetics underlying NK cell education reveals NK cell subsets with enhanced anti-viral and anti-tumor functions

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Polymorphic human leukocyte antigen (HLA) class I educates NK cells through interactions with killer cell immunoglobulin-like receptors (KIRs) and by supplying peptides that bind HLA-E to form ligands for CD94/NKG2A receptors on NK cells. HLA-B dimorphism in the leader peptide modulates this latter function: –21methionine (–21M) delivers functional peptides, but –21threonine (–21T) does not. Genetic analysis of human populations worldwide showed that haplotypes with –21M HLA-B rarely encoded the KIR ligands Bw4+HLA-B and C2+HLA-C. Thus, there are two fundamental forms of HLA haplotype: one preferentially supplying CD94/NKG2A ligands and the other preferentially supplying KIR ligands. This –21 HLA-B dimorphism divides the human population into three groups: M/M, M/T, and T/T.

Mass cytometry and assays of immune function demonstrated that M/M and M/T individuals have CD94/NKG2A⁺ NK cells that are better educated, phenotypically more diverse, and mount more potent ADCC and missing-self responses. Conversely, NK cells from T/T individuals are represented by a dominant population of highly sensitized KIR⁺ NK cells correlating with the KIR ligands present in the individual. In T/T individuals, the educated KIR⁺ NK cell subsets are complemented by larger numbers of less educated CD94:NKG2A⁺ NK cells that are more effective at anti-viral immune responses through production of IFN- γ and MIP-1 β .

Reappraisal of genome-wide association studies (GWAS) of HIV-1 infection in 9,771 infected, treatment-naïve subjects suggest that expression of HLA-E is likely the most critical factor distinguishing elite controllers from chronic progressors. Linear mixed effects models demonstrate elegant synergy between -21 HLA-B dimorphism and allele-dependent HLA-A expression in regulating levels of HLA-E on the cell-surface, mechanistically consistent with CD94/NKG2A-mediated inhibition impairing clearance of HIV-infected targets. These mechanistic insights into CD94/NKG2A involvement of NK cells in HIV afforded by use of genetic data provide strong argument for attempts at clinical manipulation through blockade of CD94/NKG2A and HLA-E interactions in the management of viral infection and hematologic cancers.

Antigen specificity of NKG2C+ adaptive NK cells

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Human Cytomegalovirus (HCMV) infection shapes the repertoire of human Natural Killer (NK) cells. HCMV infection is associated with the clonal-like expansion and persistence of an adaptive subset of NK cells expressing the activating receptor NKG2C and undergoing global epigenetic remodeling, similar to memory CD4+ Th1 and CD8+ cytotoxic T lymphocytes. 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 antigen specificity is a hallmark of adaptive NK cells and that recognition of viral ligands from different HCMV strains together with pro-inflammatory signals controls the expansion of NKG2C+ NK cells in HCMV-infected individuals and promotes their phenotypic shift from conventional to adaptive cells.

Tissue-resident NK cells in the human lung

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Recent data indicate that a subset of NK cells resides in tissues such as the liver and lung. In both compartments, NK cells comprise the major innate lymphocyte subset and may therefore play an important role in pathologies associated with these tissues. However, whereas tissue-resident hepatic NK cells have been characterized in several studies, phenotypic and functional traits of tissue-resident NK cells in human lung are largely unknown.

Here, we have analyzed NK cells in the human lung and demonstrate that the majority of lung NK cells are comprised of terminally differentiated, hypofunctional CD56dimCD16+ cells. Furthermore, on average 20% of NK cells in the lung expressed CD69, indicating tissue-residency. A subset of the CD69+CD16- NK cells co-expressed CD49a and CD103. Tissue-resident NK cells in the lung expressed high levels of NKG2A and Eomes but lacked CD57. Additionally, in some donors CD49a expression was associated with characteristics of clonal-like expansions of NK cells including co-expression of KIR and NKG2C. In some but not all donors with NK cell expansions in the lung, we could identify peripheral blood NK cell expansions, classified by a CD56dimCD16+NKG2A-CD57+KIR+NKG2C+ phenotype.

Together, these findings suggest that NK cells with distinct phenotypes can be selectively expanded in different compartments such as the lung and peripheral blood.

Hobit expression defines a subset of tissue-resident CD56bright NK cells in the human liver

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Background & aims: Immune responses show a high degree of tissue specificity, and this specificity is generated in part by factors influencing tissue egress and retention of immune cells. Hobit was recently shown to act in concert with Blimp-1 to regulate tissue-residency in mice, if it acts in a similar capacity in humans remains so far unknown. Our aim was to assess the expression and contribution of Hobit to tissue-residency of Natural Killer (NK) cells in the human liver.

Methods: We have investigated the NK cell compartment in matched liver and blood samples from patients undergoing liver transplantation by multi-color flow cytometry.

Results: The human liver is enriched for CD56bright NK cells showing an increased frequency ($p=0.03$) and expression ($p=0.0006$) of the transcription factor Hobit. Hobitpos CD56bright NK cells in the liver exhibited high levels of CD49a, CXCR6 and CD69 ($p=0.02$ for all 3 groups), markers previously linked to tissue residency of human NK cells. Finally the Hobitpos CD56bright NK cells in the liver expressed a unique set of transcription factors with higher levels of T-bet and Blimp-1 ($p=0.02$, respectively) when compared to Hobitneg CD56bright NK cells.

Conclusions: Taken together we show that the transcription factor Hobit identifies a subset of NK cells in human livers that express a distinct set of adhesion molecules and chemokine receptors consistent with tissue residency. Suggesting that Hobit is involved in regulating tissue-residency of human intrahepatic CD56bright NK cells and that Hobit-expression is essential for a subset of liver-resident NK cells in inflamed livers.

mTOR acts as a molecular rheostat controlling Natural Killer cell education

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Natural Killer cell education is the process by which NK cells acquire reactivity through activating NK cell receptors (NKaR). This process is mediated by inhibitory NK cell receptors (NKiR) upon chronic engagement by MHC-I molecules. The molecular details governing NK cell education remain however mostly unknown. Using a screen for post-transcriptional modifications in educated NK cells, we found that basal activity of the mTOR/Akt pathway was proportional to the number of educating NKiR expressed and strictly dependent on signaling via SHP1. mTOR was essential for NK cell education and the level of mTOR activity was tightly correlated with NK cell ability to respond through NKaR. At the molecular level, mTOR was an amplifier of NKaR signaling, and boosted both the calcium response and LFA1 integrin activation. Finally, restoration of uneducated NK cell reactivity via IL-15 stimulation requires an intact mTOR pathway. Altogether, our results demonstrate that mTOR acts as a molecular rheostat of NK cell reactivity controlled by NK cell receptors during education, and clarify how IL-15 stimulation overcomes NK cell education.

Modulation of NK cell antitumor reactivity by platelets

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**Novel platform for studying infiltration, migration and cytotoxicity of human
Natural Killer cells in solid tumors**

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Natural Killer (NK) cells represent a vital component of the immune response against tumor and are interesting candidates for cancer immunotherapy, since they do not require prior sensitization, do not induce graft-versus-host disease and are not HLA-restricted. However, NK cell-based immunotherapies to treat solid tumors have been largely unsuccessful. Here we applied a microchip platform using ultrasonic standing waves (USWs) to induce 3D tumor cell cultures for studying NK cell-solid tumor interaction. The system produces one hundred parallel microtumors ($\approx 0,2$ mm size) isolated in individual wells. Here we made microtumors from thyroid carcinoma and renal carcinoma cells that were characterized in terms of viability and NK ligands expression. A significant modulation of MICA/B, PVR and ICAM-1 expression was observed in 3D cultures compared to 2D cultures, possibly caused by differences in the tumor microenvironment. From 3D co-cultures of NK cells and tumor cell we determined NK cell viability, tumor cell death and expression of NK receptors involved in development and tumor recognition. The platform is versatile allowing formation of 3D microtumors of different cell types and supports characterization of NK cell-tumor interaction by, e.g. live cell imaging, light-sheet microscopy and flow-cytometry. The method could also help to develop strategies for personalized anti-tumor treatment.

Liver resident Eomeshi Tbetlo natural killer cells undergo apoptosis in metastatic tumour microenvironment.

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Natural Killer (NK) cells are innate lymphocytes crucial for anti-viral and tumour immunity. NK cells are enriched in human liver, accounting for 30-50% of intra-hepatic lymphocytes. We recently characterised a population of human tissue resident NK cells characterised by an Eomeshi Tbetlo phenotype. Here we investigate changes in hepatic NK cell populations in metastatic liver cancer and primary hepatocellular carcinoma. The healthy liver is significantly more vulnerable to metastatic disease than primary cancer and we propose that liver resident NK cells are more susceptible to changes in the microenvironment of metastatic tumour cell growth.

Liver biopsies were obtained from patients undergoing surgical resection for hepatic colorectal metastasis (n=10) and hepatocellular carcinoma (n=4). In addition mononuclear cells were isolated from liver perfusate (n=33), biopsies (n=12) and matched blood samples (n=33) taken during liver transplantation.

Nearly a third of hepatic lymphoid cells from donor biopsies were NK cells (30.7±4.1%). CD56bright NK cells are enriched in liver tissue (55.4±5.8%) compared to PB (9.7±1.1%). Hepatic CD56bright NK cells were Eomeshi Tbetlo (89.5±1.8%), a phenotype virtually absent from peripheral blood. These NK cells express CXCR6 and CD69, markers of tissue residency, which are absent from hepatic CD56dim and blood NK cells. Interestingly, NK cells are depleted from metastatic colorectal tumours (10.9±2.3%) but not the surrounding healthy tissue (26.2±5.8%, p=0.0019), with the largest depletion in Eomeshi Tbetlo CD56bright NK cells (31.8±2.8%, p=0.001). This pattern of depletion was not seen in primary hepatocellular carcinoma, where a similar proportion of Eomeshi Tbetlo CD56bright NK cells was observed in the tumour (39.9±4.6%) and surrounding healthy tissue (45.2±3.2%). Conditioned media generated from metastatic tumour bearing liver tissue induces significant apoptosis of Eomeshi Tbetlo NK cells (43.7±4.7%) compared to hepatic T cells (23.6±3.7%) and other NK cell populations (8.1±2.4%, n=4, p=0.0046).

We have previously described a unique hepatic population of Eomeshi Tbetlo CD56bright NK cells which are phenotypically distinct from peripheral blood and show enhanced cytotoxicity in healthy human liver. These highly cytotoxic cells are reduced in metastatic liver tumours but not primary hepatocellular carcinoma. These cells appear highly sensitive to changes in the microenvironment caused by metastatic tumour growth. Elucidating the mechanism of this depletion may provide novel therapeutic targets for metastatic liver cancer.

CD16A-specific tumor targeting of NK cells by tetravalent AFM13 enhances NK cell cytotoxicity and proliferation even in the presence of serum IgG

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AFM13 is an NK-cell engaging CD30/CD16A bispecific tetravalent TandAb antibody currently in clinical development as mono- and combination therapy in Hodgkin lymphoma (HL) and other CD30+ malignancies. In the current study, we present evidence that the anti-CD16A domain of AFM13 is highly specific for CD16A and not prone to adverse competition by high levels of serum IgG, possibly due to its epitope localization. Therefore, tumor targeting is restricted to CD16A+ immune cells like NK-cells and can be achieved at lower dosing compared to conventional IgG-based engagers. Importantly, we show that CD16A engagement of primary human NK-cells by AFM13 amplifies IL-2 and IL-15-mediated NK-cell proliferation, which is associated with an activated NK-cell phenotype such as the upregulation of CD25, CD69, CD132, and CD137. In addition, AFM13 induces strong NK-cell cytotoxicity towards CD30+ tumor cells, which is more pronounced than cytotoxicity observed for an anti-CD30 IgG1 antibody. Interestingly, cytokine stimulation of NK-cells is able to recover CD16-downregulation and the selective NK-cell dysfunction observed after CD16 engagement and signaling. These data will be instrumental in designing future application regimens for CD16-based immune cell engagers. In summary, these data demonstrate that our proprietary anti-CD16A domain is a versatile binding platform suitable for combination with novel tumor antigen-specific antibody domains.

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Regulation of ILC functions

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Innate lymphoid cells and the control of organ homeostasis

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Innate lymphoid cells (ILC) are a recently discovered branch of the lymphoid lineage that belongs to the innate immune system. ILC are tissue-resident cells that are particularly numerous in the intestinal lamina propria. While ILC perform traditional functions associated with immune cells (such as defense against microbial infections), it is an emerging new paradigm, that ILC are involved in processes not normally linked to the immune system. For example, ILC play important roles in maintaining tissue homeostasis and are important regulators of metabolic processes. We have been interrogating two interconnected signaling networks that are involved in unconventional functions of ILC. ILC express the aryl hydrocarbon receptor (AhR), a transcription factor, activity of which is controlled by environmental (e.g., nutrient-derived) metabolites and toxins that directly initiate transcription of a battery of genes some of which metabolize toxins to non-toxic intermediates. In recent years, the importance of AhR signaling in immune cells has been receiving attention. For example, mice genetically lacking the AhR in group 3 innate lymphoid cells (ILC3) showed a profound defect in the maintenance of ILC3. In addition, AhR signaling in ILC3 is required for the expression of interleukin (IL)-22, an unusual cytokine that exclusively acts on non-hematopoietic cells (e.g., epithelial cells, stroma cells) and has been linked to host resistance to certain types of bacterial and viral infections. Curiously, ILC3 are positioned in proximity to the crypts of Lieberkuehn in the small intestine and they produce high levels of IL-22 during steady-state (i.e., in the absence of infections or inflammation). The effects of IL-22 on epithelial cells have not been thoroughly explored. ILC3-derived IL-22 has been shown to induce the production of anti-microbial proteins by the intestinal epithelium, protecting the mucosal layer against pathogenic infections. In addition, IL-22 has been shown to protect crypt-resident stem cells against damage in a mouse model of graft-versus-host disease, though the IL-22-regulated targets in stem cells remain unidentified. Considering the high conservation of the AhR and its importance for IL-22 production, we investigated the function of the AhR-IL-22 axis in response to genotoxic stress in intestinal stem cells caused by carcinogens such as azoxymethane (AOM). Indeed, mice deficient in IL-22 or lacking AhR in ILC3 showed an increased rate of AOM-induced colorectal cancer. Interestingly, IL-22 controlled molecular circuitry that protects the intestinal stem cell niche from genotoxic stress. The implications of these findings for the development of human cancer will be discussed.

CD25-dependent IL-2 signals contribute to physiologic responses of NK cells and ILC1

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NK cells and recently discovered innate lymphoid cells (ILC) execute critical immunological functions, including the killing of infected cells, the production of proinflammatory cytokines as well as the secretion of tissue-trophic factors. These activities contribute to multicellular immune responses that require tight control and coordination in order to optimize immunity, and to prevent unopposed tissue damage. IL-2 is a cytokine that can drive the proliferation, differentiation and activation of different lymphocytes. Competition for IL-2 is considered a key mechanism for regulation of immune responses. Others and we have previously shown that Foxp3+ regulatory T cells and CD4+ T helper cells can contribute to the regulation of Eomes+ NK cells and Eomes- CD127+ ILC-1 at least in part through regulating the availability of IL-2. Several groups have demonstrated that both NK cells and ILC1 can express CD25, the high-affinity receptor chain for IL-2, which is generally believed to be required for cells to be able to access IL-2 in vivo. To test the cell-intrinsic physiologic relevance of CD25-dependent IL-2 signaling for NK cells and ILC1 we have established novel mouse models enabling the specific deletion of CD25 in these cells. We will discuss the results of our current studies in experimental models of viral infection, tumors and inflammatory diseases, suggesting a physiologic role of CD25-dependent sensing of IL-2 for type 1 innate lymphoid cells.

Lack of Nkp80 expression define a liver-resident CD49a(+) NK cell subset with ILC1-like features

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Background:

The human liver contains a unique CD49a+ NK cell subset that resembles murine liver-resident NK cells. At the moment it is not fully understood if these cells have conventional NK cell or ILC-(innate lymphoid cells) type 1-like features. For this reason we have analyzed liver CD49a(+) NK cells in more detail and compare them with similar CD49a(+) cells from the gastrointestinal (GI) tissues.

Material and Methods:

Human tissue-infiltrating lymphocytes were isolated from 15 liver-perfusates, 5 -resections, 17 -explants, and 28 biopsies from normal gastrointestinal tissues. Lymphocytes were phenotypically characterized by multicolor flowcytometry and tested for cytokine production following PMA/ ionomycin stimulation or K562 cell co-culture experiments.

Results:

Analyzing liver infiltrating CD49a(+) CD94(+) CD56(+)CD3(-)NK cells, we found 2 distinct subsets defined by expression of the NK cell activating receptor Nkp80. Unlike CD49a(+)Nkp80(+) cells, the CD49a(+)Nkp80(-) subset is negative for the transcription factor EOMES and express the cytolytic effector molecule perforin at low levels. Consistent with these findings, cytolytic degranulation (CD107a expression) is hardly detected in CD49a(+)Nkp80(-) cells following PMA stimulation or co-culture with K562 cells. However, the capacity to produce IFN γ do not vary between these cells. Surprisingly, frequency of liver-resident CD49a(+)Nkp80(-) cells from healthy donors is very low (0.15% of lymphocytes) and is not significantly different to CD49a(+)Nkp80(+) (0.17%).

CD49a(+)Nkp80(-) CD94(+) NK cells could also be detected in the gastrointestinal tract. Of note, frequencies of duodenal CD49a(+)Nkp80(-) NK cells (1.08% of lymphocytes) are significantly higher in comparison to liver perfusates (0.15%) or liver-resections (0.16%), whereby frequencies in esophagus (0.6%), stomach (0.8%), ileum (1.3%) and colon (0.4%) are not significantly different to liver-resident cells. Markers for tissue resident lymphocytes like CD69 (38%) and CD103 (39%) are partly expressed on liver-resident CD49a(+)Nkp80(-) NK cells, whereas these markers are abundant on the intestinal CD49a(+)Nkp80(-) NK cells. More importantly, the expression pattern of intestinal CD49a(+)Nkp80(-) NK cells is similar to the recently described CD103(+) intraepithelial ILC1 subset.

Conclusion:

Liver CD49a(+)Nkp80(-) NK cells share some features of non-conventional NK cells and are maybe a counterpart of mucosal CD103(+) intraepithelial ILC1.

Functions of innate lymphoid cells

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ILCs are a new type of lymphocyte. ILCs include natural killer (NK) cells and three other main subsets: ILC1, ILC2 and ILC3. Since their discovery, ILCs have been shown to contribute to wound healing and defense against infection, however, much of the role of ILCs remains to be elucidated, particularly given the diversity of these cells, which adds to the complexity of their analysis. Intestinal T cells and group 3 innate lymphoid cells (ILC3 cells) control the composition of the microbiota and gut immune responses. NCR+ ILC3 cells were redundant for the control of mouse colonic infection with *Citrobacter rodentium* in the presence of T cells. However, NCR+ ILC3 cells were essential for cecal homeostasis. Our data show that interplay between intestinal ILC3 cells and adaptive lymphocytes results in robust complementary failsafe mechanisms that ensure gut homeostasis. We also attempted to dissect the contribution of ILCs to immunity in natural conditions in humans. We investigated the presence of ILCs in a cohort of patients with severe combined immunodeficiency (SCID). All ILC subsets were absent in SCID patients carrying mutations of IL2RG or JAK3. T-cell reconstitution was observed in SCID patients that had undergone allogeneic hematopoietic stem cell transplantation (HSCT) in the absence of myeloablation, but all patients remained ILC-deficient in this setting. Remarkably, the observed ILC deficiencies were not associated with any particular susceptibility to disease, over periods of 7 to 39 years after HSCT. We thus report here the first cases of selective ILC deficiency in humans, and show that ILCs may be dispensable in natural conditions, if T cells are present and B-cell function is preserved (intrinsically or by Ig substitution). Together with earlier findings for mice, these results provide evidence for redundancy of the immune function of ILCs in the presence of a functional adaptive immune system.

MCM10 IS REQUIRED FOR HUMAN NK CELL DEVELOPMENT AND HOMEOSTASIS

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Mature human NK cells are characterized as CD56bright and CD56dim, with each subset possessing unique phenotypic and functional properties. While the ontogeny of each subset is unclear, they likely represent discrete stages of differentiation, with the CD56bright subset thought to be the direct precursor of the CD56dim subset. The importance of NK cell function in human health is demonstrated by isolated NK cell deficiency (NKD) that arises from single gene defects. NKD leads to severe viral infection and malignancy as a result of absent NK cell subsets or aberrant function. As such, requirements for NK cell development can be informed by monogenic disorders that lead to impaired maturation as reflected by loss of specific NK cell subsets.

Using whole-exome sequencing, we have identified deleterious compound heterozygous mutations in minichromosome maintenance complex 10 (MCM10) in a boy with fatal susceptibility to cytomegalovirus. While T and B cell function were normal, the patient had impaired NK cell development leading to decreased NK cell number and over-representation of the CD56bright subset. As with other MCM complex members, MCM10 is required for eukaryotic cell cycle progression and DNA damage repair. Knockdown of MCM10 in an NK cell line led to decreased proliferation and cell cycle arrest, chromosome segregation defects, and increased induction of DNA damage repair pathways. To specifically address the role of MCM10 in NK cell development, we performed CRISP-Cas9 gene editing of MCM10 in healthy donor CD34+ hematopoietic stem cells. In vitro NK cell differentiation led to impaired terminal maturation of MCM10-deficient NK cells when compared to controls, as was previously observed in our patient with MCM10 deficiency.

In summary, our results define MCM10 deficiency as a novel NKD and demonstrate a critical role for MCM10 in human NK cell homeostasis and maturation. The requirement for this conserved cell cycle complex member specifically in NK cell terminal maturation and acquisition of function, as has also been demonstrated in the case of previously reported MCM4 deficiency, demonstrates a new paradigm in human NK cell development.

How killer cells are protected from their own weaponry: A sweet end to the perforin tail

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Natural killer cell and cytotoxic T lymphocyte cytotoxicity is critically reliant upon the pore forming protein, perforin. Prior to delivery to target cells by exocytosis, perforin is stored in acidic secretory granules where it remains functionally inert. However, how cytotoxic lymphocytes remain protected from their own perforin prior to its export to secretory granules, particularly in the Ca²⁺-rich endoplasmic reticulum, remains unknown. Here we show that N-linked glycosylation of the perforin C-terminus within the endoplasmic reticulum inhibits oligomerisation of perforin monomers and thus protects the host cell from premature pore formation. Subsequent removal of this glycan occurs through proteolytic processing of the C-terminus within secretory granules and is imperative for perforin activation prior to secretion. Despite evolutionary conservation of the C-terminus, we found that processing is carried out by multiple proteases, which we attribute to the unstructured and exposed nature of the region. In sum, our studies reveal a post-translational mechanism essential for the regulation of perforin function and, in turn, the survival of perforin expressing cytotoxic lymphocytes.

The regulation of Natural Killer cell detachment as a key factor for serial killing and effector function

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Natural Killer (NK) cells are highly effective in recognizing diseased cells and they can effectively eliminate several such cells in a process called serial killing. It is well studied how NK cells are activated and how they form conjugates with target cells, but the process of detachment, which is essential for serial killing, remains mostly unclear. Here we analyze the regulation of NK cell detachment.

We use fresh, IL2-activated or cultivated human NK cells and analyze the formation of conjugates and detachment over time by flow cytometry-based approach.

Our data show that cultured human NK cells detach after an average time of 40-50 minutes from K562 tumor cells. The detachment depends on early signaling via Src- and Syk-family kinases and ATP but not on downstream signaling via MAP kinases or PKC-theta. Detachment was different between fresh, IL2 activated and cultured NK cells while the formation of conjugates was almost unchanged. Interestingly, blocking NK cell cytotoxicity by depleting mature Perforin inhibited detachment. Preventing the cell death on target cell site also reduced the detachment of NK cells and increased the production of the cytokines IFN- γ and TNF-alpha. Induction of NK cell independent target cell death leads to the opposite effect. We can show, that a loss of ligands for activating NK cell receptors and the majority of adhesion molecules on the target cell membrane after cell death is responsible for the NK cell detachment. Additionally the uptake of apoptotic bodies by NK cells seems to accelerate the detachment.

Therefore, detachment of NK cells is a regulated process, which depends on the activation status of the NK cell, ongoing receptor-proximal signaling events and changes in the protein profile after death of the target cell. Additionally, delayed detachment and increased conjugation time has a clear influence on the type of NK cell effector function.

Development of the mucosal NK cell compartment in infants

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Background

Innate lymphocyte cells 1 (ILC1) play a critical role in the protection against viral infections in infants, as adaptive T cell and antibody responses are not fully developed early in life. In addition CD127⁻ ILC1s, referred hereafter as NK cells, are also increasingly recognized for their role in tissue modeling and tissue growth. However, most of our understanding of the infant immune system is based on studies in blood. Here we investigate the establishment of early NK-cell populations in the human gastrointestinal mucosa.

Methods

NK cells from human infant (median 9.6 months) and adult (median 37 years) intestines (N=14) were isolated from the gastro-intestinal epithelial layer and the lamina propria. NK-cell populations were characterized using 18-parameter flow cytometry.

Results

NK cells were defined as lin⁻CD127⁻CD7⁺CD56⁺ cells, both in the epithelial layer and the lamina propria. The gating strategy was confirmed using antibodies directed against NKp46, which was highly expressed on this NK cell population. The frequency of tissue-resident NK cells (CD103⁺) was similar in infants compared to adults, and corresponded with other tissue residency markers including CD69 and CD49a. Both CD103⁺ and CD103⁻ NK cells consisted of approximately a third of granzyme B⁺ NK cells in infants and adults. Perforin expression was largely restricted to mucosal CD103⁻ NK cells compared to CD103⁺ NK cells in infants but not in adults. Lastly, we assessed KIR expression on NK cells. KIR⁺ NK cells were present in similar frequencies in adult and infant epithelium, however, in the lamina propria, KIR⁺ NK cells were significantly enriched in infants compared to adult tissue.

Conclusion

Tissue-resident NK cells are present early in life, produce granzyme B, perforin and express KIRs. These features allow NK cells to perform the locally required responses in the infant mucosa. However, KIR⁺ NK cells were enriched in the lamina propria in infants, suggesting a potential role of these mucosal NK cells early in life in tissue development and host defense.

Expanded NK cells for cancer therapy

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CAR expressing NK cells for cancer treatment

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In contrast to donor T cells, natural killer (NK) cells are known to mediate anti-cancer effects without the risk of inducing graft-versus-host disease (GvHD), which makes them a valuable source for haploidentical or even third-party-donor immunotherapy as we and others showed in various clinical studies. However limitations appeared due to tumour immune escape mechanism, which led to the overall goal to improve NK-cell specificity and cytotoxicity against cancer. Similar to the auspicious efforts, which have been made with chimeric antigen receptor (CAR) expressing T cells, NK cells can be modified by retroviral or lentiviral vector transduction to express single-chain antibody fragments (scFv) for cancer retargeting. This will be reviewed in the context of mainly pre-clinical data published on retargeting NK cells so far, with an outline of possible advantages using these short-lived donor effector cells. Our results on both gene-modified NK92 cells and transduced primary human NK cells redirected against leukemic and tumor cells will be presented, respectively. Finally, first international efforts to harmonize the manufacturing and approval process of CAR effector cells will be summarized.

The tumorigenicity of pluripotent stem cells transplanted into the heart is reduced by the activity of natural killer cells

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Transplantation of stem cells represents an upcoming therapy for many degenerative diseases. For clinical use, transplantation of pluripotent stem cell-derived cells should lead to integration of functional grafts without immune rejection or teratoma formation. Our previous studies showed that the risk of teratoma formation is highly influenced by the immune system of the recipients after subcutaneous transplantations. In this study, we investigated the effects of NK cells upon transplantation of pluripotent stem cells into the heart. Moreover, we determined whether an immunosuppressive treatment with cyclosporine A (CsA) affects the NK cell activity and the teratoma risk.

So-called multipotent adult germline stem cells (maGSCs), which are an alternative pluripotent cell type as demonstrated by teratoma assays and other means, were transplanted into the heart of T, B, and natural killer (NK) cell-deficient RAG2-/- γ c-/- mice, into RAG2-/- mice, which still have NK cells, and into C57BL/6 wild type mice. The recipients were either treated or not with the immunosuppressive drug cyclosporine A (CsA) at a dose of 10 mg/kg/day. The NK cell activity was determined directly ex vivo by 51Cr release assays.

A higher teratoma formation rate was observed in RAG2-/- γ c-/- mice than in RAG2-/- mice. Notably, in both strains the teratoma formation rate was significantly reduced by CsA. Thus, CsA had a profound effect on teratoma formation independent of its immunosuppressive effects. The transplantation into the hearts of RAG2-/- mice led to an activation of NK cells, which reached the maximum 14 days after transplantation and was not affected by CsA. The in vivo activated NK cells efficiently killed YAC-1 and also maGSC target cells. This NK cell activation was confirmed in wild type mice whether treated with CsA or not. Sham operations in wild type mice indicated that the inflammatory response to open heart surgery rather than the transplantation of maGSCs activated the NK cell system.

An activation of NK cells during the transplantation of stem cell-derived in vitro differentiated grafts might be clinically beneficial by reducing the risk of teratoma formation by residual pluripotent cells.

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IL-2 receptor modulation restores impaired NK-mediated regulation of T-cell activity in multiple sclerosis

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Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS), resulting from a breakdown in peripheral immune tolerance. Although a beneficial role of NK-cell immune regulatory function has been proposed, it still needs to be elucidated whether it is impaired as part of the disease. Immunohistochemistry revealed occurrence of granzyme K expressing NK cells in active MS lesions in close proximity to T cells. In accordance to an enrichment of the CD56bright NK-cell subset in the cerebrospinal fluid, CD56bright NK cells exhibited a higher migratory capacity in an in vitro model of the human blood-brain barrier in comparison to their CD56dim counterparts. Investigating MS patients treated with Natalizumab revealed that transmigration of CD56bright NK cells depends on the $\alpha 4\beta 1$ integrin VLA-4. While the migratory capacity of NK-cell subsets derived from MS patients did not differ from the one of healthy individuals, NK cells of MS patients exhibited a reduced cytolytic activity in response to autologous antigen-activated CD4 T cells. Impaired NK-cell immune-regulatory function was mainly due to T-cell evasion caused by impaired DNAM-1/CD155 interactions. On one hand, NK cells derived from MS patients expressed lower levels of the activating receptor DNAM-1, a genetic alteration consistently found in MS association studies. This most likely enhances the threshold for NK cell activation in MS. On the other hand, CD4 T cells of MS patients showed a diminished up-regulation of the DNAM-1 ligand CD155 upon antigen-activation. Daclizumab is a humanized monoclonal antibody directed toward the IL-2 receptor alpha chain of the high affinity IL-2 receptor (IL-2R) that has been recently approved for treatment of relapsing-remitting MS. Therapeutic modulation of the IL-2R with daclizumab, did not only enhance the cytolytic activity of NK cells, but also restored the defective NK-mediated immune-regulation by increasing the proportion of CD155 expressing CD4 T cells. Thus, rendering them more sensitive to NK-mediated lysis. (Gross et al., PNAS 2016).