Killer Cell Immunoglobulin-like Receptors (KIR), Licensing and Ectosomes in the Regulation of Natural Killer Cell Function

Clinical Implications and Perspectives

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Summary

Natural killer (NK) cells represent the largest proportion of innate effector lymphocytes and account for 10-15% of total peripheral lymphocytes. In contrast to B- and T- cells, NK cell activation does not depend on clonally rearranged and antigen specific surface receptors. Instead, NK cells rely on an array of activating and inhibitory germ-line encoded receptors. When encountering a target cell, activating and inhibitory signals are integrated and a response is formed immediately. Killer cell immunoglobulin-like receptors (KIR) are thereby critical for determination of NK cell activation. KIR receptors can be either activating or inhibitory in nature. Inhibitory KIR receptors not only regulate NK cell function, but are also involved in a developmental process called "licensing". During licensing, NK cells which express an inhibitory KIR capable of recognizing HLA develop increased functional competence. Both, inhibition of mature NK cells and NK cell licensing are based on the interaction of inhibitory KIR with their cognate HLA class I ligands encompassing HLA-C and subsets of HLA-B and HLA-A. NK cell "licensing" is still poorly understood and many questions remain open. So far it remains elusive if the quantity of HLA expression is a variable in NK cell licensing. We took advantage of two recently identified polymorphisms affecting the HLA-C expression on the cellular surface rs9264942 and rs67384697. We genotyped 66 healthy blood donors for these polymorphisms and assessed the quantity of HLA-C expression by FACS using an HLA-C specific antibody. In addition, we assessed the presence of the inhibitory KIR receptors KIR2DL1and KIR2DL3 and their corresponding HLA ligands, being two mutually exclusive groups of HLA-C: HLA-C1 and HLA-C2. In subsequent functional analyses, we observed, in agreement with the concept of licensing, increased functionality of KIR2DL1+ and KIR2DL3+ NK cells in donors expressing the corresponding HLA ligand in a dose dependent manner,. The quantity of HLA-C surface expression, however, did not affect the quality of NK cell licensing.

A unique opportunity to study NK cell licensing is provided during the first months after hematopoietic stem cell transplantation when the NK cell repertoire is rebuilt. Previous studies found that leukemia patients receiving hematopoietic stem cell transplantation (HSCT) from a haploidentical donor, benefit from a survival advantage if KIR ligands are mismatched and NK cell tolerance is irreversibly broken. Recent studies reported controversial findings concerning a survival advantage and NK cell mediated graft versus leukemia effect (GVL) as a consequence of disturbed NK cell licensing early after HSCT, when NK cells are unselectively equipped with functional capacity. Differences in allogeneic HSCT protocols were discussed as possible reason for the opposing results. We investigated reconstitution of NK cell function and NK cell licensing in 56 patients receiving allogeneic (33) or autologous (23) HSCT during the first six months after transplantation. We found that NK cell licensing was maintained after both kinds of transplantation. However licensing effects were less distinct after allogenic compared to autologous HSCT. Additionally, we identified GvHD and pre-transplant ATG administration as variables associated with less prominent licensing in recipients of allogeneic grafts.

Whereas research on inhibitory KIR and NK cell licensing has already influenced treatment modalities of HSCT, much less is known about activating KIR receptors. Ligands to many activating KIR remain unidentified and little is known in which diseases activating KIR may play a role. Recent studies in our lab and elsewhere described associations between KIR genotypes and relative protection from cytomegalovirus (CMV) replication after solid organ transplantation (SOT) and HSCT. We prospectively followed a cohort of 649 patients after SOT, assessed the KIR genotype and recorded common opportunistic viral infections. Subsequent analyses of our data revealed an association of KIR B haplotypes, which encompass many activating KIR, and relative protection from varicella zoster virus (VZV) and a tendency for relative protection from Epstein-Barr virus (EBV) replication. In subsequent analyses we found

that centromeric rather than telomeric activating KIR protect from VZV. In contrast, we detected no association between activating KIR genotype and BK polyomavirus (BKPyV) or Herpes simplex (HSV) replication.

Besides intrinsic factors such as licensing or expression of activating KIR, extrinsic factors determine antitumor and antiviral activity of NK cells. Early after HSCT when NK cell driven GVL is assumed most effective, prophylactic immunosuppression is given and cellular products are administered including erythrocytes (ERY) and platelets (PLT). During storage, blood products release ectosomes. PLT ectosomes can reduce monocyte and dendritic cell function. Furthermore, they favour differentiation of naïve T-cells into T-regulatory cells. However, little is known about their interaction with NK cells. We assessed phenotypical and functional changes on NK cells after co-incubation with PLT ectosomes *in vitro,* and found a reduction of NK cell function and activating surface receptors, mediated through TGF-β on PLT ectosomes.

Introduction

NK cells

Natural killer cells represent the largest portion of innate lymphocytes. Depending on the maturation stage NK cells are located in in bone marrow, secondary lymphoid tissues or in peripheral blood, where they account for 10 to 15% of total lymphocytes.[1,2] In contrast to B- and T-Lymphocytes, NK cells express no clonally rearranged receptors and activation is not triggered upon specific antigen recognition. Instead, NK cells have a wide array of germline encoded activating and inhibitory receptors at their disposal[3]. The NK cell repertoire consists of thousands of different clones. This variety is based on different receptor expression patterns during terminal maturation on the one hand and on clonal distribution of activating and inhibitory KIR receptors on the other hand.[4] The activating NK cell receptors include DNAM1, NKG2C, NKG2D, 2B4, the natural cytotoxicity receptors (NCR) (NKp30, NKp44 and NKp46), CD16, which mediates antibody dependent cytotoxicity (ADCC), and the activating KIR receptors. This array of activating receptors is especially sensitive to detect markers of cellular stress or target cell opsonisation with antibodies. Whereas for most activating NK cell receptors minimally one ligand is known, for several activating KIR receptors the respective ligands remain unknown so far. An overview of activating receptors and corresponding ligands is given in Table 1. The principal inhibitory receptors encompass the heterodimer NKG2A/CD94, ILT2 (LIR1), KLRG1 and the inhibitory KIR receptors.[5,6] Inhibitory NK cell receptors commonly recognize subsets of HLA class I as cognate ligands (Table 1). Apart from engagement of activating receptors, absence or reduction of the normally ubiquitously expressed HLA-class I molecules is required for NK cell activation. The presence of sufficient HLA-class I on target cells strongly counteracts activating signals and constitutes the molecular basis for the “missing self” recognition of NK cells. In consequence, target cells are only susceptible to NK cell lysis if they

express ligands to activating receptors and at the same time HLA class I expression is too low to trigger sufficient signalling through the inhibitory NK cell receptors.[7-9]

Downregulation of HLA frequently occurs in viral infection or tumor transformation, where loss of HLA is a common strategy to avoid T-cell receptor (TCR) dependent recognition of immunogenic peptides to evade the adaptive immune system.[10-13] Individuals with functional or numerical NK cell deficiency accordingly are at increased risk for viral infections and malignancies.[14] However tumors and viral diseases can establish also in individuals with at first normal NK cell function and numbers, using different strategies as e.g. the creation of an immunosuppressive microenvironment.[15,16]

Once an NK cell encounters a target cell and the activation threshold is reached, it immediately displays its cytotoxic effector functions, such as the release of cytotoxic granules containing perforin and granzymes or the re-allocation of ligands to death receptors such as FasL or TRAIL to the cell surface. In addition, pro-inflammatory cytokines including interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) or granulocyte/macrophage colony stimulation factor (GM-CSF) are released, which set in motion an inflammatory response and attract further immunological effector cells.[17-19] It was this immediate response of a lymphocyte subset against a cellular target that lead to their first description as Natural killer cells 1975 in both mice and humans, since target cells were killed “naturally” and without pre-activation.[20-22]

KIR2DL1	HLA-C2: C*02, C*04, C*05, C*06
KIR2DL2/3	HLA-C1: C*01, C*03, C*07, C*08 Some HLA-C2: C*0501, C*0202, C*0401 Some HLA-B: B*4601, B*7301
KIR2DL4	HLA-G
KIR2DL5A/B	unknown
KIR3DL1	HLA-B expressing Bw4 epitope Some HLA-A: A*23, A*24, A*32
KIR3DL2	HLA-A: A*03, A*11
KIR3DL3	unknown
KIR2DS1	HLA-C2: C*02, C*04, C*05, C*06
KIR2DS2	HLA-A*11, HLA-C1?
KIR2DS3	unknown
KIR2DS4	HLA-C: C*0501, C*1601, C*0202
KIR2DS5	unknown
KIR3DS1	unknown
KLRG1	E/N/P-Cadherin
ILT2(LIR1)	Multiple HLA class I
NKG2A	HLA-E
NKG2C	HLA-E
NKG2D	MICA/B ULBP1-6
NKp30	B7H6, HCMV-pp65
Nkp44	Viral hemagglutinin
NKp46	Viral hemagglutinin
CD16	Fc portion of IgG
DNAM1	CD112, CD155
2B4	CD48

Tbl.1 NK cell receptors and ligands. Depicted is a list of the most important NK cell receptors with their corresponding ligands. Adapted from Ref no. 5 and 52.

KIR – Receptors

The human KIR receptors are a family of membrane spanning glycoproteins, which are critical determinants not only of NK cell activation and tolerance, but also of development and education. KIR receptors are expressed on mature NK cells and some T-cell subsets.[23,24] The human KIR genes are located along with other genes of the immunoglobulin super family on chromosome 19q13.4.[25] The KIR gene family encompasses 15 highly homologous genes, which have evolved through duplication from one common ancestor KIR gene with 3 immunoglobulin-like domains KIR3DX.[26] In total, 13 different KIR genes are expressed and two pseudogenes have been described: 2DL1, 2DL2/3, 2DL4, 2DL5A, 2DL5B, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1/S1, 3DL2, 3DL3, 2DP1 and 3DP1. KIR2DP1 and 3DP1 represent the two pseudogenes, which do not code for proteins.[27-29] Since the duplication of the KIR2DL5 KIR gene locus approximately 1.7 million years ago, a centromeric (KIR2DL5B) and a telomeric (KIR2DL5A) locus code for two different KIR2DL5 alleles.[30]

All expressed KIR receptors consist of two or three extracellular Immunoglobulin-like domains, a stem region, a transmembrane section and a cytoplasmic tail. The number of extracellular immunoglobulin-like domains (2D versus 3D) and the length of cytoplasmic tails (S for short and L for long) are thereby the fundament to KIR nomenclature. The last digit in the KIR nomenclature (1 to 5) indicates the order of historical description. [31,32]

KIR receptors can either transduce inhibitory or activating signals. All inhibitory KIR receptors share the common feature of a long cytoplasmic tail. The long cytoplasmic tails carry two immunoreceptor tyrosine based inhibitory motifs (ITIM), which provide inhibitory signal transduction.[33-35] Activating KIR receptors on the other hand possess short cytoplasmic tails. In contrast to inhibitory KIR, activating KIR signalling depends on immunoreceptor tyrosine based activation motifs (ITAM). However, the short

cytoplasmic tails of activating KIR do not comprise an ITAM. Instead, activating KIRs associate with DAP12 containing an ITAM.[36] KIR2DL4 represents the only exception of activating KIRs with a unique long cytoplasmic tail, which can transduce inhibitory and activating signals.

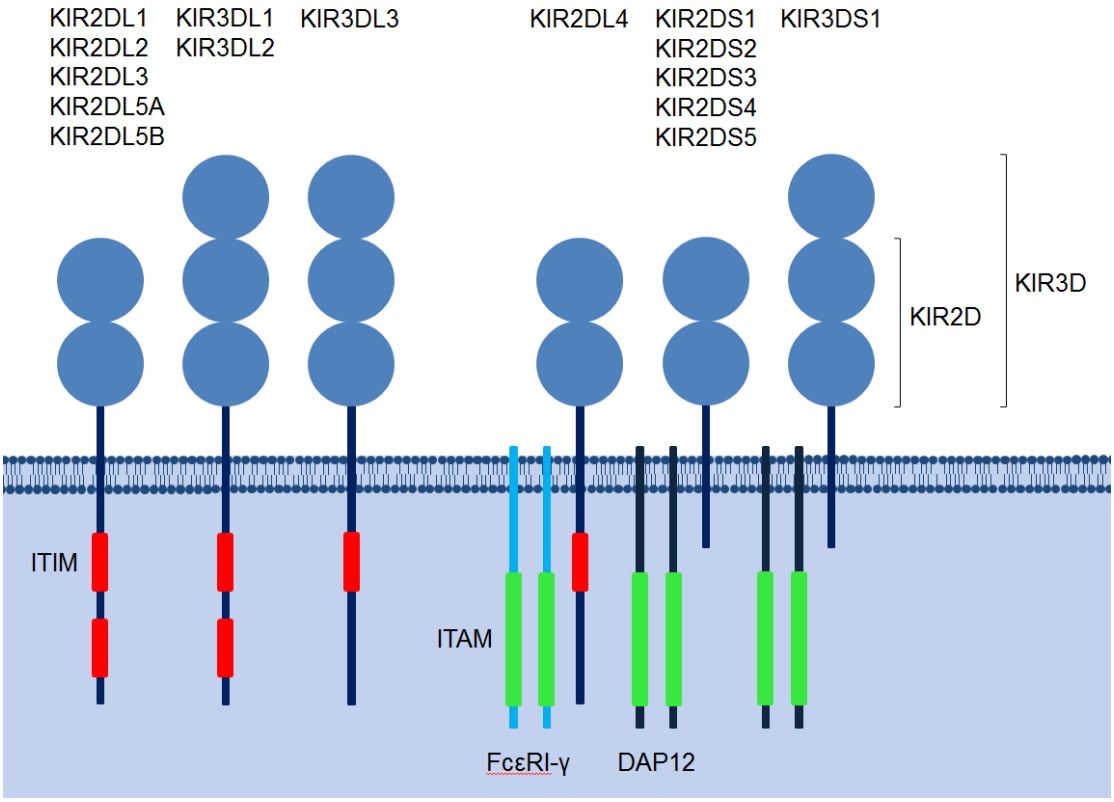

Fig. 1 Structure of killer cell immunoglobulin like receptors (KIR). KIR receptors have either two or three extracellular immunoglobulin-like domains (2D versus 3D) and a short of long cytoplasmic tail (S for short and L for long) which is reflected in the nomenclature. The last digit in the KIR nomenclature (1 to 5) indicates the order of historical description. Inhibitory KIR carry an ITIM for inhibitory signalling on their long cytoplasmic tail and activating KIR associate with DAP12 carrying an ITAM for activating signalling. KIR2DL4 is an exception and carries only one ITIM and can also associate with FcεRI-γ for activating signalling.

inhibitory signals are mediated via one single ITIM on its long cytoplasmic tail (Fig1).[37] Proximal signalling of activating KIR receptors further involves the recruitment of tyrosine kinases Syk/ZAP-70 to the ITAM containing DAP12. Further downstream signalling resembles activating T-cell receptor signalling in large parts.[36,38]

KIR receptors occupy a special position among NK cell receptors. Unlike other inhibitory and activating NK cell receptors, the KIR receptor repertoire is highly variable between individuals and clonally distributed among mature NK cells.[39] Most other NK cell receptors are expressed on the majority of NK cells and vary mainly between different maturation steps (e.g. CD57) or between activated and resting NK cells (e.g. NKp44).[40,41] Therefore, the influence of KIR receptors on NK cells is privileged for inter- and intra-individual investigations.

Individual KIR gene clusters are commonly subdivided into haplotypes A and B, based on different KIR gene content. The basic structure of both haplotypes is defined by a canonical set of four KIR genes. Whereas KIR3DL3 and KIR3DL2 define the centromeric and telomeric end of the KIR gene loci, KIR3DP1 and KIR2DL4 are located in between and separate the KIR gene loci into a centromeric and a telomeric group. KIR A haplotypes have a fixed KIR gene content and encompass in addition to the four framework genes also KIR2DP1, KIR2DL1, KIR2DL3, KIR3DL1, KIR2DS4, with KIR2DS4 as the single activating KIR. On the other hand KIR B haplotypes distinguish themselves through harbouring a varying number of additional, mostly activating KIRs. The KIR B haplotype is defined by the presence of one or more of the following KIR genes: KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5 and KIR3DS1. A KIR B haplotype may contain maximally 5 additional activating KIR genes. Based on varying combinations and numbers of expressed KIR receptors, dozens of different B-haplotypes have been found so far.[27,42-47] Further variation originates from telomeric and centromeric recombination of KIR A and B haplotypes: While KIR receptors within the telomeric and centromeric part are in high linkage disequilibrium, combinations of centromeric KIR A haplotype with telomeric KIR B haplotype and centromeric KIR B with telomeric KIR A haplotype occur more frequently.[48]

Activating KIR& antiviral immunity

Only few ligands to activating KIRs have so far been detected and for many activating KIR receptors no ligands have yet been found. The identified ligands currently encompass exclusively HLA class I molecules: KIR2DS1 recognizes HLA-C2 alleles as corresponding ligands and KIR2DS4 specifically binds to subsets of HLA-C1, HLA-C2 and

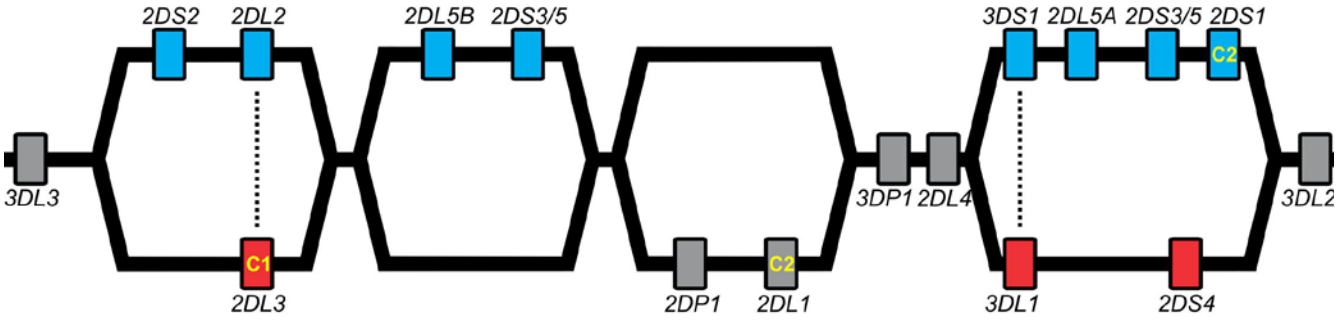

Fig. 2 Organisation of the human KIR gene locus. The branches show KIR gene content of KIR A and KIR B haplotypes, whereas KIR genes typical for KIR A haplotypes are coloured red and for KIR B haplotypes blue. Frame work genes are in grey. From (47) © 2012 The Royal Society

HLA-A (Tbl.1). [49-52] Recently HLA-A11 has been identified as ligand to KIR2DS2.[53] The physiological importance of these interactions remains to be investigated. In addition, the HLA molecules identified as activating KIR-ligands may not be exclusive, as previous research suggests non-HLA ligands for KIR2DS4, though these remain to be identified.[54] Well characterized ligands for KIR2DS3, KIR2DS5 and KIR3DS1 are so far elusive. KIR2DL4, as the only KIR receptor with a long intracellular tail capable of activating and inhibitory signal transduction, is discussed in the section "inhibitory KIR receptors".[37]

Incidence and outcome of several malignant diseases, reproductive failure and viral infections were found to be associated with the activating KIR gene content of patients' genomes. Activating KIR genes reduce the susceptibility to pre-B-cell leukemia, the most common form of leukemia in western pediatric patients.[55] In addition, different

allogeneic HSCT scenarios showed reduced leukemia relapse in case the stem cell donor genome contains KIR-B haplotype associated activating KIR genes.[56-58] Moreover, maternal activating KIR receptors (KIR-B haplotype) provide protection from preeclampsia.[59] A growing number of studies recently started to enlighten the protective capacity of activating KIR against different viral infections. Patients infected with human immuno deficiency virus (HIV) benefit from a slower progression to AIDS if they carry the KIR3DS1 gene and the HLA-B Bw480IIe allele.[60] Patients with primary immunodeficiencies affecting NK cells suffer from recurrent herpes virus infections.[14] Infection/reactivation of CMV is a common side effect of immunosuppression following allogeneic HSCT and SOT.[61,62] A correlation between the presence of activating KIR receptors in SOT transplant recipients or the stem cell grafts of HSCT and the occurrence of CMV replication has been extensively investigated. Two studies demonstrated an inverse correlation between CMV infection/reactivation after SOT and the number of activating KIR.[63,64] Subsequent analyses have mapped the the responsible activating KIR receptors to the telomeric part of the KIR haplotype.[65] In addition, a greater number of activating KIR genes in the stem cell graft after HSCT was shown to reduce CMV infection/reactivation rates.[66,67] Whereas NK cells are known to be involved in anti-Epstein Barr Virus (EBV) immunity, protective effects of KIR genes are poorly investigated. Similar to EBV, NK cells are suspected to be important in anti-Varicella Zoster virus immunity but no correlation between VZV and the KIR genes has been done yet. However, cellular assays, suggested a binding of KIR2DS1 to the HLA-C subset C2, if target cells present EBV peptides.[68,69] The clinical course of a Herpes simplex virus (HSV) infection was previously shown to be influenced by the receptor-ligand pair KIR2DL2 and HLA-C1, whereas activating KIR displayed no influence on course of the disease. In kidney transplant patients the frequency of KIR3DS1 was lower in patients with BK virus infection.[70] A further viral infection influenced by the KIR genome is the hepatitis C virus (HCV). A recent study compared HCV positive and negative people and

several adverse effects concerning the development of HCV related complications were found associated with both inhibitory and activating KIR.[71]

Inhibitory KIR & licensing

The main functions of inhibitory KIR receptors comprise recognition of "self" and assurance of NK cell tolerance towards healthy tissue through the recognition of HLA class I.[9,72,73]

The ligands to inhibitory KIR receptors encompass different subsets of HLA class I molecules. KIR2DL1 and KIR2DL2/3 recognize two mutually exclusive groups of HLA-C alleles as their ligands, HLA-C2 and HLA-C1 respectively. HLA-C molecules can be subdivided in two groups according to the amino acids at position 77 and 80. Group 1 HLA-C share the amino acids serine 77 and asparagine 80, and group 2 asparagine 77 and lysine 80.[42,74] While traditionally the HLA-C ligands to KIR2DL1 and KIR2DL2/3 were considered as strictly confined by the different amino acids at position 77 and 80, recent research has shown that also HLA-C2 molecules can be ligands to KIR2DL2/3.[75] KIR3DL1 receptors recognize HLA-B alleles with the Bw4 epitope. Similar to HLA-C, two mutually exclusive groups of HLA-B are discriminated, one expressing the Bw4 epitope and the other Bw6. HLA-Bw6 are not known being ligand to KIR receptors. KIR3DL1 receptors recognize additionally a small number of HLA-A alleles as their ligand, including HLA-A: A*23, A*24, A*32.[76-78] For KIR3DL2 so far only two HLA–A alleles (A*03 and A*11) have been found as corresponding ligands and binding seems furthermore peptide dependent.[79] KIR2DL4 and KIR3DL3 are the only KIR receptors which have only one ITIM. Furthermore KIR2DL4 is the single inhibitory KIR receptor, which can also associate with FcεRI-γ for activating signalling, due to a positive charged residue in its membrane spanning region.[37] KIR2DL4 is not clonally distributed, but the single KIR receptor expressed in all NK cells and predominantly in endosomes.[27,80,81]

Previous work found that KIR2DL4 binds HLA G and heparin and heparanated proteoglycans.[81-83] KIR3DL3 is a poorly investigated KIR, which is hardly expressed on NK cells and no ligands have been identified.[84] The ligands to KIR2DL5A/B genes have not been identified yet, but previous evidence suggests that inhibitory signalling can be transduced.[85,86]

Inhibitory KIR receptors are not only crucial to save healthy tissue from NK cell mediated damage, but they are also important during the educational process referred to as "licensing", when functional competence of NK cells is determined. After completion of the licensing process, NK cells expressing an inhibitory KIR for which the corresponding HLA is present in the respective person are equipped with more functional competence, compared to NK cells which express only KIR for which the corresponding HLA-ligand is absent.[87-90] In a more premature state of NK cell development before NK cell function is fine-tuned during licensing, but when cytotoxic effector functions have already developed, NK cell tolerance is assured by NKG2A. NKG2A recognizes HLA-E on target cells, which present the leader peptides of classical HLA class I molecules. NK cells expressing NKG2A are functionally comparable to fully licensed NK cells expressing only inhibitory KIR and no NKG2A.[91,92]

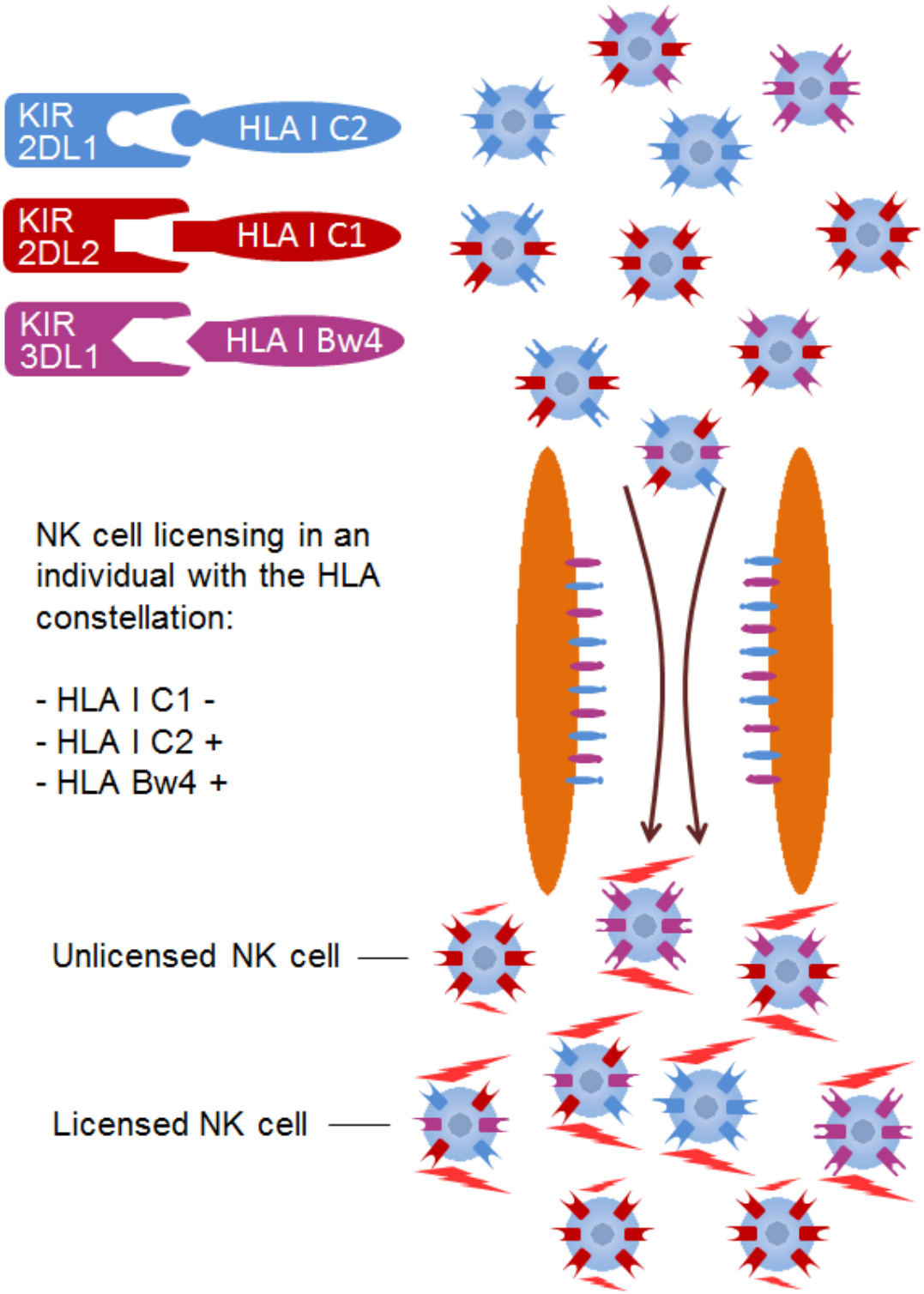

Fig. 3 NK cell licensing. In an educational process called licensing only those NK cells are equipped with maximal functional competence, which are able to recognize HLA through their inhibitory KIR receptor.

Hematopoietic Stem cell transplantation, NK cell licensing and GVL

When allogeneic HSCT was established for leukemia treatment more than four decades ago, transfer of hematopoietic stem cells initially served to rescue the hematopoietic system after high dose chemotherapy or total body irradiation (TBI). Only subsequently it was unravelled that donor derived lymphocytes exert GVL and thereby significantly contribute to improved outcome of high dose chemotherapy or TBI combined with hematopoietic stem cell rescue. Donor derived T-lymphocytes were first identified to exert GVL. However, T-cell mediated GVL comes with the risk of GVHD and must be controlled by immunosuppressive prophylaxis.[93,94] NK cells were only later found to exert antitumor immunity but without the risk of causing GVHD. NK cell mediated GVL was initially described in a haploidentical HSCT scenario. NK cells are licensed according to donor HLA and consequently, NK cell tolerance is permanently broken in NK cells expressing only an inhibitory KIR for which the corresponding HLA ligand is absent in the patient. Such a constellation favours NK cell driven GVL, whereas no graft derived NK cell mediated GVHD has been observed yet.[95-99]

After HLA matched allogeneic HSCT, KIR ligands are by definition identical between donor and recipient. Recent investigations demonstrated a lower relapse risk also after HLA matched HSCT, if one or more KIR ligands are absent in the donor/recipient pair.[100,101] A possible explanation for this observation is offered by a study which found a temporary broken NK cell tolerance during reestablishment of the KIR repertoire in the first months after HSCT. Resulting from the non-proper licensing process, all NK cells were equipped with enhanced functional competence. In parallel, inhibitory signalling through KIR/HLA interaction was not affected.[102] However, both functional data of reconstituting NK cell function and survival data after allogeneic HLA-matched HSCT are equivocal regarding the benefit of missing KIR ligands after allogeneic matched HSCT. Several studies failed to confirm a survival advantage of patients lacking KIR ligands after allogeneic HLA matched

HSCT and a recent study investigating NK cell function after HSCT found no evidence for a temporarily abnormal licensing process.[98,103] However, the effect of graft characteristics and transplant associated complications such as cellular composition of the graft, GVHD, its prophylaxis and treatment, and CMV infection on NK cell licensing remain poorly or not investigated.[103] A transplantation setting very close to the fully matched transplantation is autologous HSCT, where retrospective cohort studies provide evidence for a survival advantage of pediatric patients lacking a KIR ligand and no survival advantage in adults. At the same time many factors which were previously discussed as being responsible for different findings between studies are absent.[104,105]

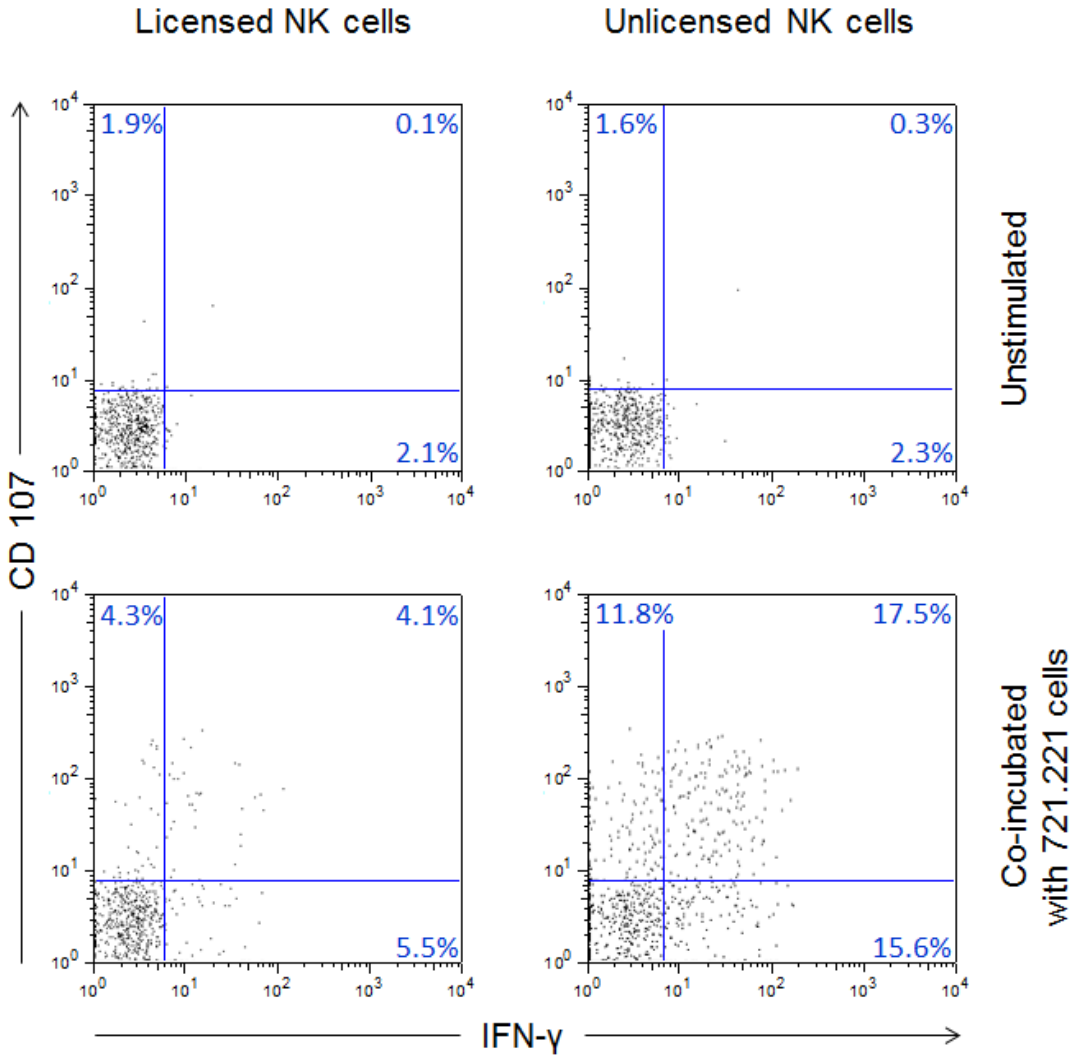

Fig. 4 NK cell function of licensed and unlicensed NK cells Full PBMC were co-incubated with the HLA deficient target cells 721.221 and degranulation and cytokine production was measured. In subsequent FACS analysis it was gated on single KIR+NKG2A- licensed (KIR-ligand present in the donor) and unlicensed (KIR-ligand absent in the donor) NK cells. 2000 events are shown per FACS plot.

Ectosomes & NK cell fuction

Among the most frequent recipients of PLT transfusions are hemato-oncological patients, especially after hematopoietic stem cell transplantation (HSCT).[106-109] Transplant recipients are per se an increased risk of infection and PLT transfusions may additionally contribute to the susceptibility to infections, as PLT infusions have been previously identified as risk factor for infectious diseases.[110-114] In parallel PLTs can be involved in cancer growth and metastasis. Since decades increased PLT counts are known to correlate with worse outcome in ovarian and other cancer patients. Only recently mechanisms were discovered, how PLTs support tumor cells to evade immunological consequences.[115-117]

Ectosomes released by PLTs exert powerful immunosuppressive effects on different cell types of the immune system. PLT-Ecto are subcellular micro vesicles, which are continuously shed from the surface membrane as a physiological process of PLT aging. They express beside PLT markers such as CD61, high levels of immunomodulatory molecules including phosphatidylserine (PS) and TGF-β1. During PLT storage in the blood bank, Ecto accumulate and are in large amounts concomitantly infused to patients with each PLT transfusion. Previous work has revealed that PLT-Ecto have immuno-suppressive impact on different cell types of immune system: PLT-Ecto cause a reduction of pro-inflammatory cytokine release by macrophages, whereas in parallel inducing a release of TGF-β1. In addition, PLT-Ecto disturbed phagocytic activity and cytokine release of DCs as well as monocyte-derived DC differentiation[118]. Recent work found, that PLT-Ecto can support differentiation of naïve CD4+ T cells towards functional regulatory T cells.[119]

Erythrocytes (ERY) are, similar to PLTs, frequently infused to both hematooncological patients and patients requiring surgery. Previously it has been shown that the storage time of ERY concentrates correlates with increased infection rates.[120] In analogy to PLT,

ERY continuously shed Ecto during storage. However they express a different set of molecules on their surface. While ERY-Ecto also express PS, no TGF-β1 is found on their surface. Phosphatidylserine on ERY-Ecto was previously identified responsible for ERY-Ecto induced inhibition of macrophages.[121,122]

Ectosomes are also released from various immune cells within the blood stream and an increasingly investigated form of cellular communication under physiological and pathophysiological circumstances. Immunomodulatory influence of Ecto released from polymorphonuclear (PMN) cells has previously been found for different cell types.[123,124] Recently it has been demonstrated, that PMN-Ecto can change the cytokine profile of NK cells and reduce macrophage function, whereas the influence of PLT and ERY ectosomes on NK cells remains non-investigated.[125]

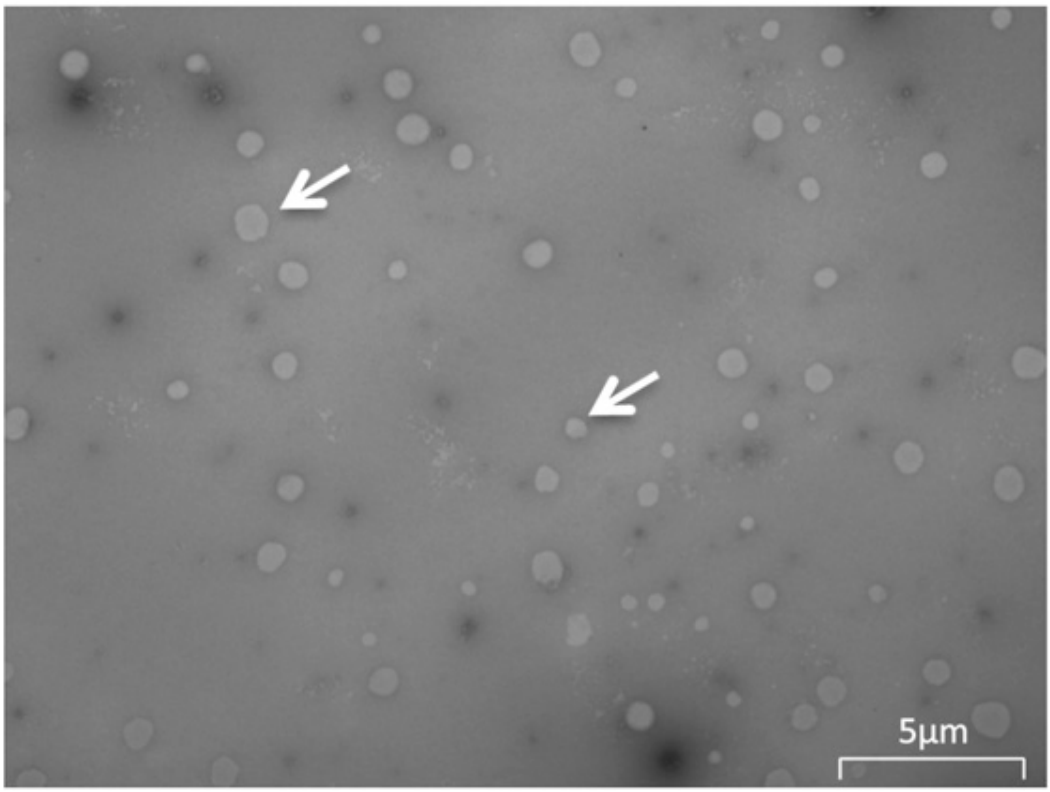

Fig. 5 PLT ectosomes Depicted are PLT ectosomes collected from PLT supernatant of stored platelets. From (118) Copyright © 2011 by The American Association of Immunologists, Inc

Aims

Compared to the lymphocytes of the adaptive immune system, NK cell regulation is still poorly understood. The dual function of inhibitory KIR, the equipment of functional capacity during the educational process "licensing" and inhibition of mature NK cells, is known for more than a decade. How NK cell licensing operates as well as potential influence factors remains poorly understood. Further, for several activating receptors both cognate ligands and physiological significance remain elusive.

If the quantity of HLA surface expression is a variable in the licensing process is unknown. The previously detected polymorphisms rs9264942 and rs67384697 were shown to interfere with HIV progression and to affect the surface expression of HLA-C. In the first project we investigated if the quantity of HLA-C surface expression correlates with the licensing status.

In the second project we investigated functional reconstitution of NK cells after HSCT. Previous studies presented controversial findings regarding the existence of a temporal disturbance of NK cell licensing associated with a survival advantage of transplant recipients lacking inhibitory KIR ligands. Here we aimed to elaborate if HSCT per se affects NK cell licensing or if allogeneic HSCT related factors are involved and compared licensing after both types of transplantation. Subsequently, we aimed to determine variables that could be involved by comparing NK cell licensing in subgroups of patients receiving allogeneic HSCT.

Solid organ transplantation is with the exception of syngenic transplantation always accompanied by immunosuppressive therapy and provides a unique setting to study opportunistic viral infections. In the third project we aimed to investigate the influence of the KIR genotype on the incidence of frequently occurring viral infections other than CMV, where a protective influence of activating KIR receptors had been described

previously. In particular, we asked if replication of VZV, EBV, HSV or BKPyV correlates with the presence of KIR B haplotype and if protective KIR are found among telomere or centromeric genes.

After HSCT, NK cells are the first lymphocytes to repopulate peripheral blood and it was shown that they can reduce viral infection rates and the probability of disease relapse.[126,127] The early post-transplant phase is also the time when frequently PLT and ERY are infused to counteract low PLT and ERY numbers. Whether PLT- or ERY-Ecto interfere with NK cell function is not known, but may be relevant in particular for patients, whose therapy includes HSCT. PMN-Ecto can change the cytokine panel release of NK cells. If cytotoxic effector functions are affected remains unclear. We aimed to evaluate the potential immune-regulatory impact of PLT-, ERY- and PMN-Ecto on NK cells.

List of Publications & Manuscripts

This PhD thesis bases upon two published manuscripts and two submitted manuscripts.

H. N. Charoudeh, **L. Schmied**, A. Gonzalez, K. Czaja, K. Schmitter, Laura Infanti, A. Buser and M. Stern. *Quantity of HLA-C Surface Expression and Licensing of KIR2DL+ Natural Killer Cells. Immunogenetics. Oktober 2012 Jul 7.*

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Project 1

Quantity of HLA-C Surface Expression and Licensing of KIR2DL+ Natural Killer Cells

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Contribution to the study:

Design and performance of specific experiments, analysis and interpretation of the data, writing parts of the manuscript.

Abstract

Natural killer (NK) cells require interaction of inhibitory surface receptors with human leukocyte antigen (HLA) ligands during development to acquire functional competence in a process termed "licensing." The quantity of HLA required for this process is unknown. Two polymorphisms affecting HLA-C surface expression (rs9264942 and rs67384697) have recently been identified, and shown to influence progression of HIV infection. We typed a cohort of healthy donors for the two HLA-C-related polymorphisms, KIR2DL1 and KIR2DL3, and their respective HLA-C ligands and analyzed how HLA ligands influenced licensing status of killer cell immunoglobulin-like receptor (KIR)+ NK cells in terms of degranulation and cytokine production in response to HLA-deficient target cells. The presence of respective HLA class I ligands increased the function of KIR2DL1+ and KIR2DL3+ NK cells in a dose dependent manner. In contrast, neither of the HLA-C-related polymorphisms nor the quantity of cell surface HLA-C had any significant effect on NK cell function. Interestingly, HLA-Cw7—an HLA-C allele with low surface expression—licensed KIR2DL3+ NK cells more strongly than any other KIR2DL3 ligand. The quantity of cell surface HLA-C does not appear to influence licensing of NK cells, and the HLA-C related polymorphisms presumably influence HIV progression through factors unrelated to NK cell education.

Introduction

Natural killer (NK) cells are a subset of lymphocytes involved in the defense against viral infection and malignant transformation. Upon target recognition, NK cells synthesize and secrete cytokines and lyse target cells without the need for pre-activation (Lanier 2005). NK cell function is regulated by the integration of signals derived from activating and inhibitory receptors. Among inhibitory receptors, killer cell immunoglobulin-like receptors (KIR)−which bind to class I human leukocyte antigens (HLA)−play a pivotal role in discriminating normal from pathologic tissue (Biassoni et al. 2003). The inhibitory signal derived from the KIR/HLA interaction renders NK cells tolerant towards autologous tissue, whereas cells lacking HLA class I may trigger an NK cell response resulting in target cell lysis. Recent evidence has pointed out that this function is acquired during NK cell development in a process termed "licensing": only NK cells expressing inhibitory receptors for self-HLA obtain the functional competence to respond to the lack of HLA ligands on a target cell (Anfossi et al. 2006; Kim et al. 2008; Yokoyama and Kim 2006). In contrast, NK cells whose inhibitory receptors did not interact with their cognate ligands during development remain unlicensed (Brodin and Hoglund 2008). A previous study analysing the interaction of the KIR3DL1 receptor with its ligand−HLA with the Bw4 motif−showed that NK cells from donors carrying two Bw4 alleles showed higher levels of licensing compared to NK cells from donors carrying no or only one Bw4 allele (Kim et al. 2008). As a further potential variable in the equation, HLA antigens are expressed on the cell surface at varying quantities. A single nucleotide polymorphism (SNP; rs9264942) located 35 kb upstream of the HLA-C gene−termed HLA-C −35—was recently identified to associate with the abundance of cell surface HLA-C (Thomas et al. 2009). HLA-C −35 has been demonstrated to be a major factor governing viral load and progression of human immunodeficiency virus (HIV) infection. "Set point" viral load in untreated patients was lower, and progression of HIV was

delayed in patients carrying C alleles−associated with higher amounts of cell surface HLA-C−compared to carriers of T alleles (Fellay et al. 2007; Pereyra et al. 2010). Following this, a novel single nucleotide deletion polymorphism in the 3′ untranslated region (UTR) of HLA-C (rs67384697) has been shown to influence binding of the microRNA hsa-miR-148, and thereby associated with HLAC surface expression (Kulkarni et al. 2011). Similar to HLAC−35, this single nucleotide polymorphism was shown to associate with HIV set point viral loads. As NK cells participate in the control of HIV replication (Alter et al. 2011), we hypothesized that the association of HLA-C −35 and the 3′ UTR HLA-C deletion SNP with HIV progression might be due to a role for the quantity of HLA-C in the NK cell licensing process. To address the question experimentally, we assessed in a large cohort of healthy donors both HLA-C-related polymorphisms and HLA-C surface expression on lymphocytes. We correlated these data to the functional competence of NK cells in terms of cytokine production and degranulation after exposure to class I negative tumor cells. NK cell function was compared in NK cell subsets with or without expression of the inhibitory KIR receptors KIR2DL1 and KIR2DL3, which recognize mutually exclusive subsets of HLA-C ligands termed C1 and C2 (Winter et al. 1998). The HLA C1 group includes HLA Cw antigens carrying asparagine at position 80 (e.g., HLA Cw1, Cw3, Cw7, and Cw8), whereas the HLA C2 group includes HLA Cw antigens carrying lysine at position 80 (e.g., HLA Cw2, Cw4, Cw5, and Cw6; Winter and Long 1997).

Patients and methods

Antibodies and flow cytometry

The following mAbs were used: anti-CD3 Pacific blue (OKT3, eBioscience), anti-CD56 PE-Cy7 (HCD56, Biolegend), anti-CD107a PE (clone H4A3, BD), anti-IFN-γ PE (clone B27, BD), anti-KIR2DL1 FITC (clone 143211, R&D), anti-KIR2DL3 APC (clone 180701, R&D), and the pan-HLA-C antibody DT9 (kindly provided by Mary Carrington, National Institute of Health, Bethesda, USA) which was used in conjunction with goat anti-mouse IgG2b PE (Southern Biotech). To evaluate HLA-C surface expression, we used QuantumTM R-PE MESF Kit (Polysciences, Inc., Warrington, USA) according to the manufacturer`s instructions. The mean fluorescence intensity of the five bead populations was used to calculate a calibration curve by plotting the MFI for each population against its known units of molecules of equivalent soluble fluorophores (MESF). Staining intensity of DT9 is therefore expressed as MESF units. Samples were acquired on a DAKO CyAn ADP nine-colour flow cytometer (Beckman Coulter). For all analyses of NK cell subsets, we gated on the CD56+/CD3− subset. For quantification of HLA-C expression, we gated on total lymphocytes. Fluorescence-activated cell sorting (FACS) plots were analyzed with FlowJo software version 9.2.

CD107a and IFN-γ assay

Peripheral blood mononuclear cells (PBMCs) from healthy donors were collected under an ethical committee-approved protocol, isolated by density gradient centrifugation, and cryopreserved. After thawing, cells were cultured overnight in the presence of 150 U/mL recombinant human IL-2 (Proleukin, Novartis), after which PBMCs (0.5×10e6) were mixed with the HLA-deficient 721.221 cell line at a ratio of 5:1 in a final volume of

200 µL in flat-bottom 96-well plates and incubated for 5 h at 37 °C/5 % CO2. NK cell response to 721.221 was measured in terms of degranulation (CD107a surface expression) and cytokine production (intracellular cytokine staining for IFN-γ; Alter et al. 2004). Anti-CD107a PE was added to wells at the beginning of the incubation. For the IFN-γ assay, brefeldin A (Golgi Plug, BD Biosciences) was added after 1 h of incubation at a concentration of 1:200, after which cells remained incubated for another 4 h. For intracellular staining of IFN-γ, cells were permeabilized with Cytofix/Cytoperm (BD) and washed with BD Perm/Wash buffer. As positive control, phorbol 12-myristate 13-acetate, and a calcium ionophore (ionomycin) were used. To assess the magnitude of licensing conferred by KIR2DL1 and KIR2DL3, frequencies of IFN-γ+ and CD107a+ cells were assessed in CD56+/CD3−/KIR+ and CD56+/CD3−/KIR− subsets. Ratios of IFN-γ+ cells and CD107a + cells within KIR+ and KIR− NK cells were calculated to estimate the alteration in functional competence derived by the expression of the respective KIR receptor (Fig. 1). We then compared the effect of the presence or absence of HLA C1/C2 group ligands, the HLA-C

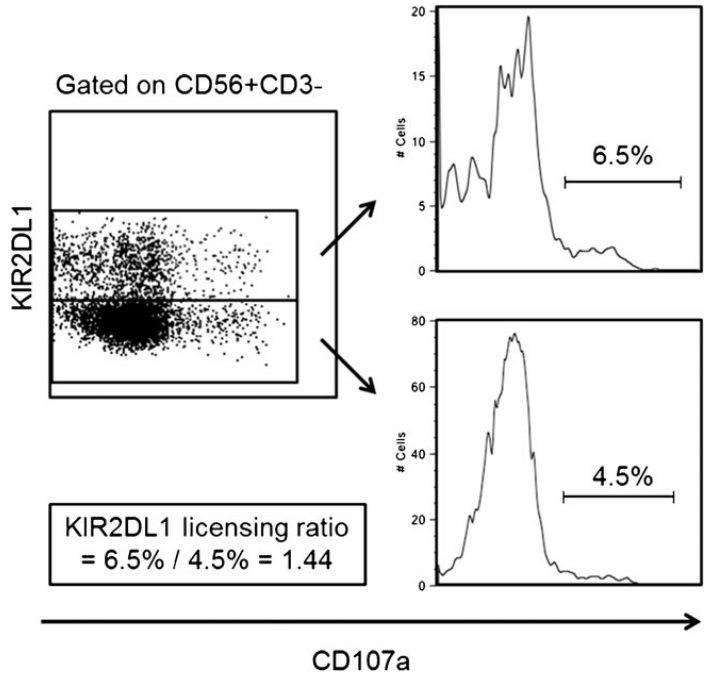

Fig. 1 Increased functional capacity of NK cells expressing inhibitory KIR2D receptors. After gating on CD56+/CD3− NK cells, CD107a expression induced by co-incubation with 721.221 tumor cells is compared in NK cells which do or do not carry the inhibitory KIR2DL1 receptor. The ratio of CD107a expression in KIR2DL1+ and KIR2DL1− NK cells (termed"licensing ratio") quantifies the increase in functional competence derived from expression of KIR2DL1. IFN-γ production and the receptor KIR2DL3 were analyzed in the same fashion

−35 and 3′ UTR HLA-C polymorphisms, and the amount of cell surface HLA-C had on these licensing ratios.

Genotyping and quantitative PCR

The HLA-C −35 SNP was genotyped using a commercial ABI TaqMan allelic discrimination kit on the ABI7500 Sequence Detection System according to manufacturer's guidelines. The rs67384697 polymorphism located within the 3′ UTR was typed by direct sequencing (Kulkarni et al. 2011). Genotyping for HLA-C1/C2 group KIR ligands and for the inhibitory KIR2DL1 and KIR2DL3 was performed using previously established sequence-specific primer protocols (Alves et al. 2009; Frohn et al. 1998). HLA-C was typed by applying a reverse sequence-specific oligonucleotide method according to the manufacturer’s instructions (One Lambda Inc., Canoga Park, CA).

Statistics

Functional and phenotypic NK cell-related characteristics were compared with nonparametric tests (Mann‐Whitney U and Kruskal‐Wallis). Correlations were assessed by calculating Spearman’s rank correlation coefficient. Factors affecting NK cell function were analyzed in a multivariable fashion using generalized linear models. All p values are two-sided and were considered significant if ≤ 0.05.

Results

Function of KIR2DL1+ and KIR2DL3+ NK cells is regulated by the number of HLA-C KIR ligands

Phenotypic and functional characteristics of 66 healthy donors were analyzed. At the HLA-C locus, 20 donors (30 %) were homozygous for C1 (C1C1), 15 donors were homozygous for C2 (C2C2), and the remaining 31 donors were heterozygous (C1C2). All donors in this study carried and expressed the KIR2DL1 gene, and eight donors were negative for KIR2DL3 and were excluded from the analyses focusing on this receptor.

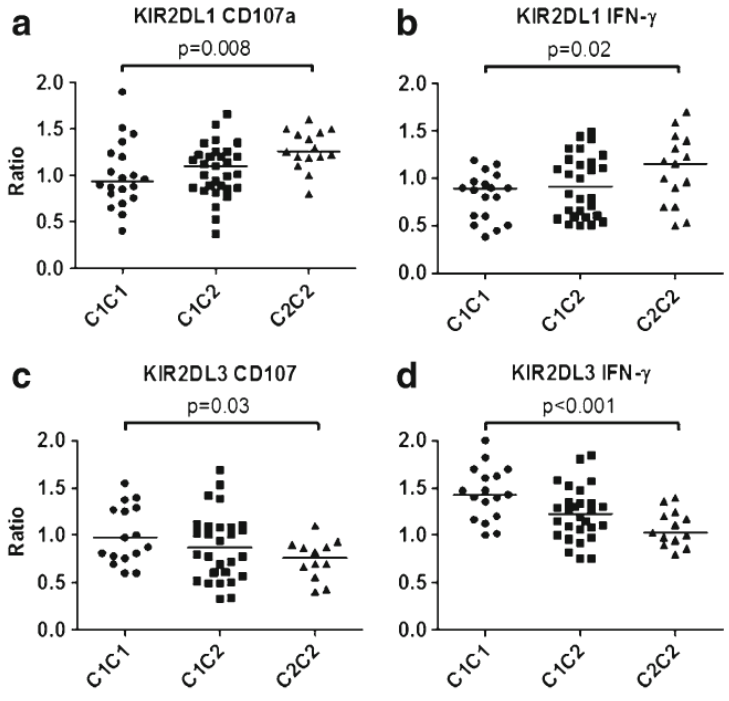

Fig. 2 Degranulation and cytokine production of KIR2DL1+ and KIR2DL3 NK+ cells in relation to the number of KIR ligands. CD107a and IFN expression are evaluated in NK cells derived from 66 donors after co-incubation with the HLA-deficient 721.221 cell line. CD107a expression and IFN-γ production of KIR2DL1+ NK cells significantly increase with growing numbers of C2 ligands (a and b). CD107a expression and IFN-γ production of KIR2DL3+ NK cells significantly increase with growing number of C1 ligands (c and d)

Analysis of NK cell function in correlation with KIR ligand status confirmed and extended previously published data on the functional consequence of co-expression of the inhibitory KIR3DL1 with its ligand, HLA with the Bw4 motif (Kimet al. 2008). Both cytokine production and degranulation significantly increased in NK cells carrying inhibitory KIR2D with HLA-C specificity if

the corresponding ligand was present (Fig. 2). KIR2DL1-positive NK cells produced more cytokine and degranulated at higher frequency in donors carrying one or two copies of the KIR2DL1 ligand HLA group C2 (Fig. 2a,b). In contrast, NK cells expressing KIR2DL3 were most functional in donors carrying the C1 group ligand for this receptor (Fig. 2c, d).

HLA-C-related polymorphisms and quantity of HLA-C do not correlate with NK cell function

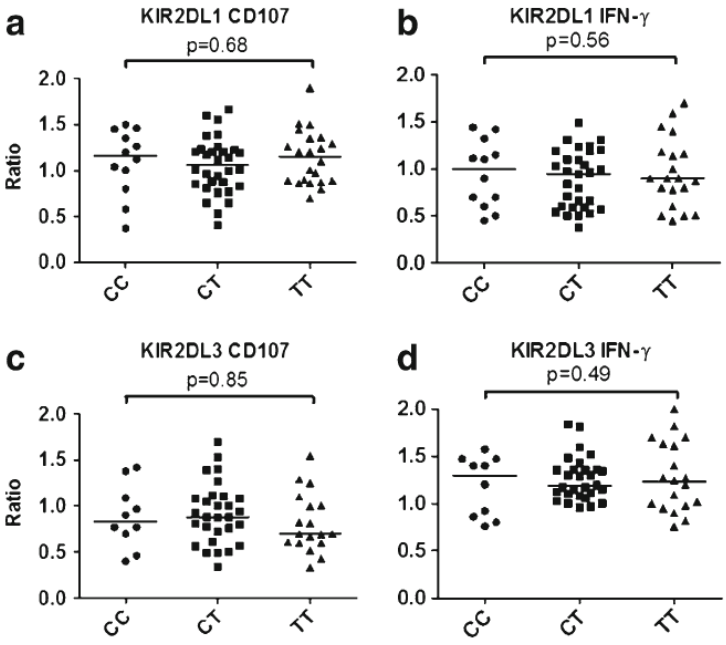

Fig. 3 Correlation of degranulation and cytokine production of KIR2DL1 and KIR2DL3 NK subsets with the HLA-C −35 polymorphism. The HLA-C −35 genotype does not significantly influence NK licensing of KIR2DL1+ and KIR2DL3+ NK cells as assessed by CD107a expression and IFN-γ production after exposure to the HLA class I-negative cell line 721.221

At the HLA-C −35 SNP locus, 12 donors (18 %) carried two C alleles (CC), 22 donors (33 %) carried two T alleles (TT), and the remaining 32 donors (49 %) were heterozygous (CT). In univariate analysis, no difference in CD107a expression and IFN-γ production of KIR2DL1+ and KIR2DL3+ NK cell subsets were detected between CC, CT, or TT donors (Fig. 3).

We next evaluated HLA-C surface expression with the pan-HLA-C antibody DT9 on lymphoid cells by FACS. In agreement with published data (Thomas et al. 2009), we found a significant correlation between HLA-C −35 polymorphism and HLA-C surface

expression (Fig. 4a). As expected, correlation of HLA-C surface expression with the 3′ UTR SNP showed a trend towards higher surface HLA-C for carriers of the deleted variant; however, this correlation did not reach the level of statistical significance (Fig. 4b). As the quantity of surface HLA-C might affect the licensing process independently from the HLA-C −35 and 3′ UTR SNP status, we next correlated NK cell licensing ratios to surface HLA-C quantity. Neither licensing ratios of CD107a degranulation nor IFN-γ cytokine production was significantly correlated to DT9 expression (r2 0.001, p00.77 for CD107a, Fig. 4c; r200.01, p00.24 for IFN-γ, Fig. 4d).

Table1 Multivariate analysis if factors influencing licensing of KIR2D expressing NK cells

	CD107a			**IFN-γ**		
	Effect size	**95% CI**	***p* value**	**Effect size**	**95% CI**	***p* value**
Number of HLA-C ligands	+0.14	+0.05 to +0.23	0.002	+0.17	+0.08 to +0.26	<0.001
HLA-C (increase per 10’000 MESF)	-0.002	-0.02 to +0.02	0.85	-0.01	-0.04 to +0.01	0.26
HLA-C -35						
TT	0.00	-	-	0.00	-	-
CT	-0.01	-0.15 to +0.14	0.95	-0.07	-0.22 to +0.08	0.37
CC	-0.03	-0.22 to +0.17	0.78	-0.04	-0.24 to +0.17	0.71
3′UTR SNP						
Non-del/non-del	0.00	-	-	0.00	-	-
Non-del/del	+0.01	-0.01 to +0.19	0.95	-0.03	-0.37 to +0.25	0.70
Del/del	+0.07	-0.22 to +0.17	0.40	-0.06	-0.21 to +0.15	0.77

Finally, we used multivariable generalized linear models to account for potential confounding between variables influencing licensing status. Table 1 shows the results of the analysis accounting for number of HLA-C ligands, HLA-C quantity (DT9 expression), and the HLA-C-related polymorphisms. The only variable significantly influencing NK cell licensing by KIR2DL1 and KIR2DL3 was the number of HLA-C ligands, whereas both

HLA-C-related polymorphisms and HLA-C quantity failed to significantly impact NK cell licensing.

HLA-Cw7 strongly licenses KIR2DL3+ NK cells despite being expressed at low quantities at the cell surface

A previous study by Corrah et al. investigating the relationship between HLA-C −35 and cell surface HLA-C described low surface expression of HLA-Cw7, but failed to replicate a correlation of HLA-C −35 status with surface HLA-C regarding alleles other than Cw7 (Corrah et al. 2011). We therefore performed HLA-C typing on our cohort, which revealed that 20 donors (30 %) carried one Cw7 allele, and an additional 3 donors (5 %) carried two HLA-Cw7 alleles (in all cases either Cw*0701 or Cw*0702, which are both in linkage disequilibrium with HLA-C −35T).

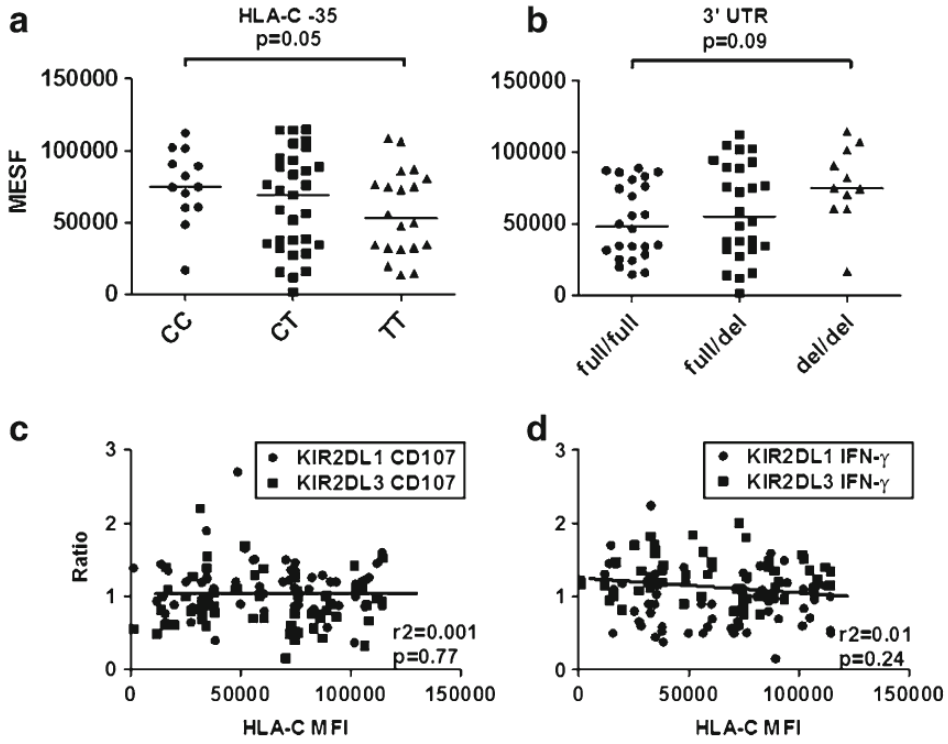

Fig. 4 Correlation of HLA-C expression HLA-C −35 genotype and NK cell function. The HLA-C −35 genotype influences the expression of HLA-C as detected by the DT9 antibody in flow cytometry (panel a). A trend towards higher expression of HLA-C is detected for patients carrying the deletetion variant in the 3'UTR of HLA-C (panel b). In contrast, no significant correlation was detected between CD107a licensing ratios (panel c) and IFN-γ licensing ratios (panel d), respectively, and surface expression of HLA-C

Correlation of Cw7 status with cell surface HLA-C quantity showed a dose-dependent reduction of HLA C quantity with increasing number of HLA-Cw7 alleles (Fig. 5a), thus confirming the data by Corrah et al.. Also in agreement with this study, we found that after exclusion of donors carrying HLA-Cw7 alleles, the HLA-C −35 status no longer significantly affected the HLA-C cell surface expression (median MESF 78590, 77797, and 74665, for CC, CT, and TT donors, respectively, p=0.65).

Despite being expressed at low quantity, HLA-Cw7 proved to strongly license KIR2DL3+ NK cells (Fig. 5b, c). With an average increase per Cw7 ligand in the KIR2DL3 licensing ratios of +0.17 for CD107a and +0.19 for IFN-γ (p00.02 and <0.001), Cw7 licensed KIR2DL3 more strongly than other frequently expressed group C1 ligands: Cw1 +0.12/+0.07; Cw3 +0.08/+0.10, Cw8 0.09/+0.12 (for CD107a and IFN-γ, respectively).

The strongest KIR2DL1-licensing ligand was HLA-Cw5 (+0.24/+0.22 for CD107a and IFN-γ, respectively).

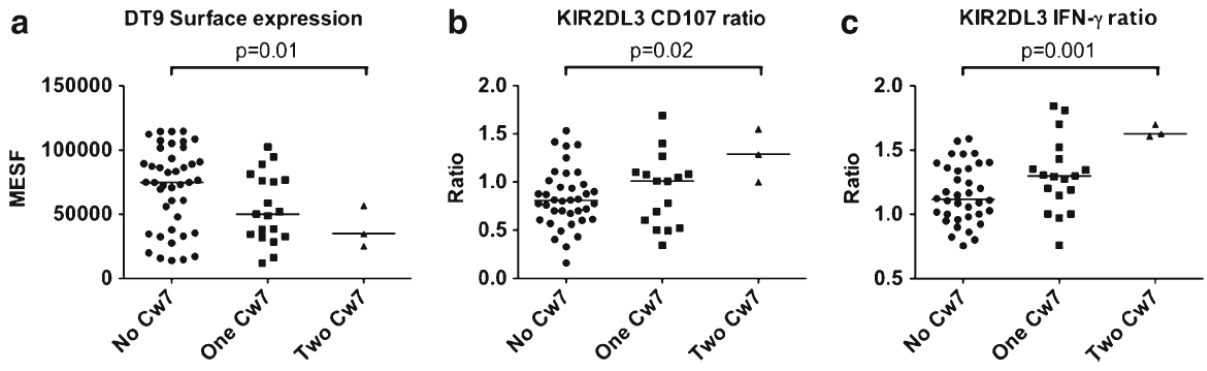

Fig. 5 Correlation of HLA-Cw7 status and cell surface HLA-C quantity and NK cell function. HLA-C surface expression—as measured with the pan-HLA-C antibody DT9—progressively decreases in donors carrying 0, 1, or 2 HLA-Cw7 alleles (a). Despite low surface expression, HLC-Cw7 strongly licenses KIR2DL3+ NK cells as assessed by CD107a and IFN-γ production (b and c)

Discussion

In the present study, we addressed a potential impact of the surface expression quantity of HLA-C on NK cell licensing. We chose to study licensing by KIR2DL1 and KIR2DL3 because these receptors have defined and mutually exclusive ligands and because antibodies to both receptors are available which do not cross-react with the respective counterpart activating KIR. In agreement with previous studies (Anfossi et al. 2006), we show that the HLA-C-specific inhibitory receptors KIR2DL1 and KIR2DL3 confer increased functional capacity to NK cells in donors that carry the respective KIR ligands. Both IFN-γ production and degranulation dose-dependently increased with the number of KIR ligands in NKcells expressing KIR2DL1 and KIR2DL3. No statistically significant effect on NKcell licensing could be attributed to the HLA-C −35, the 3′ UTR HLA-C polymorphism, or the amount of cell surface HLA-C. One possible explanation for the failure of cell surface HLA to influence the licensing process is that the threshold quantity required for NK cell licensing lies below the level of even low-expressing alleles. Recent studies have estimated the number of HLA-C molecules necessary to inhibit NK cell activation via interaction with KIR2DL1 receptor. These analyses have suggested that inhibition increases with growing number of HLA-C molecules with a saturation threshold at approximately 20,000 molecules/cell, above which inhibition reaches a plateau (Almeida et al. 2011). It is conceivable that a similar threshold exists for the KIR/HLA interaction during the licensing process, and that even low-expressing alleles are expressed at quantities above this threshold. In agreement with previous data (Thomas et al. 2009), we found a significant correlation of HLA-C −35with the amount of HLA-C as measured by flow cytometry. Another recently published study only partially confirmed the association of HLA-C −35 with cell surface HLA-C. Corrah et al. showed in their analysis of 25 HIV-infected patients that the correlation of HLA-C −35 with cell surface HLA-C quantity was exclusively due to the linkage disequilibrium of HLA-Cw7

with HLA-C −35T (Corrah et al. 2011). In our population of healthy donors, we confirm that Cw7 shows significantly lower surface expression than non-Cw7 alleles, but unexpectedly found that HLA-Cw7 more strongly licenses KIR2DL3+ NK cells than any other C1 group ligand. These data are in agreement with the notion that allelic polymorphism within HLA-C rather than quantity of cell surface expression influences the NK cell licensing process. An issue that remains unresolved by this study is how homozygosity for class I KIR ligands increases licensing (Elliott and Yokoyama 2011). KIR ligand homozygosity is expected to increase the number of HLA molecules expressed on a cell, but the data shown here suggest that the resulting increased effector capacity might not be due to an increase in quantity of KIR ligands alone. Interestingly, many studies in humans have shown that homozygous presence of KIR ligands is necessary to see licensing effects (Khakoo et al. 2004; Kim et al. 2008), whereas in mouse models heterozygous for the major histocompatibility complex (MHC)-I ligand for a given Ly49 receptor, licensing could be documented (Jonsson et al. 2010). So far, it remains open whether licensing in humans and in mice is differentially regulated regarding the requirement of MHC-I ligand zygosity. In conclusion, we found no evidence that the HLA-C related polymorphisms or quantity of cell surface HLA-C regulates the NK cell licensing process. Regarding the association of HLA-C −35 and of the 3′ UTR SNP with the course of HIV infection, our data do not support a role of NK cell licensing in the improved disease control of carriers of HLA-C −35 C alleles or 3′ UTR deletion variants. However, a major limitation of this study lies in the facts that experiments were carried out with NK cells derived from healthy donors rather than HIV patients. Chronic HIV infection leads to profound changes in NK cell phenotype and function (Mavilio et al. 2003), which might also affect the licensing process. Alternative explanations to be investigated in the future include an association of HLA-C −35 and of 3′ UTR deletion variants with HLA-C alleles endowed with increased function

regarding their interaction with CD8+ T cells, or associations that are completely independent from HLA-C surface expression.

Aknowledgement

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Project 2

Reconstitution of NK cell licensing after autologous and allogeneic HSCT.

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Contribution to the study:

Establishing the cohort, design of the research, design and performance of experiments, analysis and interpretation of the data, writing of the manuscript.

Abstract

Background

NK cells mediate graft-versus-leukemia effects (GVL) after mismatched allogeneic haematopoietic stem cell transplantation (HSCT) when NK cell tolerance is permanently broken in case of KIR ligand mismatch. The existence of GVL induced by temporarily broken NK cell tolerance early after fully HLA/KIR ligand matched HSCT is controversial.

Methods

NK cell function was assessed in 56 patients receiving HSCT (23 autologous and 33 allogeneic) before and one, two, three, and six months after HSCT. Function was measured as degranulation (CD107a expression) and IFN-γ production after exposure to the HLA-deficient cell-line 721.221.

Results

We found that a licensing effect on NK cell function is maintained after both autologous and allogeneic HSCT. However, unlicensed NK cells (expressing non-self KIR; NKG2A-) are equipped with more functional competence and are more similar to licensed NK cells (expressing self KIR; NKG2A-) after allogeneic transplantation than after autologous HSCT ($p<0.05$ in interaction analyses of autologous versus allogeneic patients for all post-transplant timepoints). In contrast, KIR-/NKG2A-, KIR-/NKG2A+ and self-KIR+/NKG2A- NK cells showed comparable function after the two types of transplantation. Further, our data suggest that conditioning with ATG and the development of GvHD influence the licensing effect on NK cell function.

Conclusions

NK cell function is predicted by the presence of KIR-ligands after HSCT. Nevertheless, unlicensed NK cells are equipped with higher functional capacity after allogeneic than autologous HSCT, which corresponds to previous studies showing a survival advantage in patients lacking KIR ligands after allogeneic transplantation.

Introduction

In patients with hematologic malignancies treated by allogeneic HSCT, the reconstituting donor immune system plays an important role in eradicating residual disease. While transplant immunology has traditionally focused on T-lymphocytes as effectors of both graft-versus-leukemia (GVL) and graft-versus-host disease (GvHD), more recent research indicates that natural killer (NK) cells can also exert powerful antitumor immunity after allogeneic transplantation without causing GvHD (1-5).

NK cell regulation depends on germ-line encoded activating and inhibitory receptors, including inhibitory Killer cell immunoglobulin-like Receptors (KIR). Inhibitory KIR bind to ubiquitously expressed Human Leukocyte Antigen (HLA) class I molecules, providing the main mechanism of NK cell tolerance. The interaction between inhibitory KIR and HLA is also important during NK cell development: In a process termed 'licensing', NK cells expressing inhibitory KIR acquire full functional competence through interaction of their inhibitory KIR with cognate HLA ligands (6,7).

NK cell driven graft-versus-leukemia effects were first demonstrated after haploidentical HSCT. In case of a KIR ligand mismatch between donor and patient, NK cells are licensed according to donor HLA and tolerance is permanently broken (4,8-10). More recent studies demonstrated a reduced relapse incidence also after fully HLA- and therefore KIR ligand-matched transplantation, in case the donor recipient pair lacks one or more KIR ligands (11,12). A recent study investigating the reconstitution of NK cell function after allogeneic HSCT offered a possible explanation for this observation, by showing that NK cell tolerance may be temporarily disturbed in the first months after HSCT. As a consequence, NK cells were unselectively equipped with functional capacity, whereas inhibition of NK cells through KIR/HLA interaction remained intact (13). However, several confirmatory studies have failed

to replicate either the survival advantage or the aberrant NK cell function during the early post-transplant period. A number of possible explanations for contradicting results between studies have been discussed, as e.g. the impact of GvHD, its treatment or prophylaxis as well as the graft quality (amount of T-cell depletion), which vary between centers (9,14,15). Most of these factors have not been taken into consideration when investigating licensing in reconstituting NK cells after HSCST.

In parallel, retrospective cohort studies in pediatric patients provide evidence for a survival advantage after autologous HSCT for leukaemia or neuroblastoma, if patients lack a KIR ligand (16,17). Autologous HSCT provides a unique setting to study reconstitution of NK cell licensing, as many of the factors potentially affecting NK cell function after allogeneic HSCT such as pharmacological immunosuppression and graft-versus-host disease are absent. We therefore aimed to compare functional reconstitution of NK cells in two cohorts of patients treated with autologous or allogeneic HSCT.

Patients & Methods

Patients

Fifty-six patients were included in this study, 23 recipients of autologous HSCT and 33 patients treated with allogeneic HSCT. Blood was drawn from participants before, and at one, two, three and six months after HSCT. Underlying diagnoses, conditioning regimens and GvHD prophylaxis are summarized along with demographic characteristics in Table 1. Data on GvHD occurrence and CMV replication were prospectively collected. Written informed consent was obtained from all study participants and the study was approved by the local institutional review board (EKBB 317/10).

Table 1: Patient characteristics

Type of transplantation	autologous (n=23)	allogeneic (n=33)
Diagnosis, n (%)		
- Acute leukemia	1 (4.3)	24 (72.7)
- Lymphoproliferative disease	7 (30.4)	5 (15.2)
- Myeloproliferative neoplasm	-	3 (9.1)
- Plasma cell disorder	15 (65.2)	1 (3.0)
Origin of stem cells, n (%)		
- PBSC (n,%)	23 (100)	32 (97.0)
- BM (n,%)	-	1 (3.0)
Conditioning, n (%)		
- Melphalan	15 (65.2)	-
- Cyclophosphamide, TBI	-	11 (33.3)
- Cyclophosphamide, Busulfan	1 (4.3)	17 (51.5)
- BEAM	7 (30.4)	-
- BEAM, Fludarabine, TBI	-	5 (15.2)
ATG, n (%)		
- Yes	-	21 (63.6)
- No	23(100)	12 (36.4)
GvHD prophylaxis, n (%)		
- CyA, MTX	-	28 (84.8)
- CyA, MMF	-	4 (12.1)
- Sirolimus, MMF	-	1 (3.0)
Recipient donor sex, n (%)		
- M/M (n,%)	14 (60.9)	11 (33.3)
- M/F (n,%)	-	4 (12.1)
- F/F (n,%)	9 (39.1)	10 (30.3)
- F/M (n,%)	-	8 (24.2)
CMV serology, n (%)		
- D-/R-	8 (34.8)	14 (42.4)
- D-/R+	-	9 (27.3)
- D+/R-	-	5 (15.1)
- D+/R+	15 (65.2)	5 (15.1)
Age recipient, median (range)		
- Donor (n,%)	-	35.4 (20 – 61)
- Recipient (n,%)	62.3 (28 - 69)	48.1 (27 – 63)
KIR ligands, n (%)		
- Missing HLA-C1	5 (21.7)	4 (12.1)
- Missing HLA-C2	6 (26.1)	16 (48.5)
- Missing HLA-Bw4	8 (34.8)	11 (33.3)

PBSC: Peripheral blood stem cells, BM: Bone marrow, ATG: Antithymocyte globulin, GvHD: Graft-versus-host disease, CyA: Cyclosporin A, MTX: Methotrexate, MMF: Mycophenolate mofetil, CMV: Cytomegalovirus

Assessment on NK cell function

NK cell degranulation and cytokine production was assessed as previously described (18). Briefly, full PBMCs were thawed and cultured over-night (16h) in full medium (RPMI supplemented with 10% fetal calf serum and penicillin/streptomycin) in a humidified incubator at 37°C in 5% CO2. Thereafter PBMCs were washed and stimulated by co-incubation with the HLA-deficient target cell line 721.221 in an effector to target ratio of 5:1 (PBMC: 721.221) in the presence of anti-CD107a-PE mAb (clone H4A3; Becton Dickinson) for staining. After 1h, Brefeldin A (BD Bioscience) was added and after 6 h the cells were harvested for FACS analysis.

FACS analysis and antibody staining

Full PBMC were washed twice after the functional assay and then labelled using the following monoclonal antibodies: anti-CD3 APC-eFluor780 (clone SK7, eBioscience); anti-CD14 APC-eFluor780 (clone 61D3, eBioscience); anti-CD19 APC-eFluor780 (clone HIB19, eBioscience); anti-CD56 PE-C7 (clone HCD56, Biolegend); anti KIR2DL1-FITC (clone 143211; R&D systems); anti-KIR2DL2/DL3-PerCP (clone DX27; Miltenyi Biotec); anti-KIR3DL1-Biotin (clone DX9; Biolegend); anti-NKG2A-APC (clone Z199; Beckman Coulter). After staining with antibodies against surface molecules, cells were washed twice, and co-incubated for 30 minutes at 4°C in staining buffer containing Streptavidin BV-570 (Biolegend). Thereafter, cells were fixed (Fixation Buffer, Biolegend) and permeabilized (Permeabilization Buffer, Biolegend) for subsequent intracellular staining with anti-IFN-γ Violet 450 (clone B27, Becton Dickinson) for 30 minutes at room temperature. Afterwards, cells were again washed twice and analysed on a nine-color flow cytometer (CyAn ADP, Beckman Coulter, Pasadena, CA). For analysis of FACS data, FlowJo v. 9.4.11 (Tree Star, Ashland, OR) was used.

KIR-ligand status & PCR

In patients receiving unrelated donor allogeneic HSCT, the ligand status to KIR2DL1, KIR2DL2/3 and KIR3DL1 was derived from high-resolution HLA-typing of the recipient. In patients receiving allogeneic sibling or autologous grafts, ligands to KIR2DL1, KIR2DL2/3 and KIR3DL1 were identified by direct DNA typing for HLA-Cw group 1/2 polymorphisms, whereas Bw4 ligand status was deduced from low-resolution HLA typing (19,20). NK cells exclusively expressing one inhibitory KIR receptor and no NKG2A are henceforward called self-KIR (S-KIR) NK cells, if the corresponding HLA ligand is present and non-self KIR (NS-KIR) NK cells, if the corresponding HLA-ligand is missing. KIR2DL2/3 positive NK cells were not considered in the analysis due to recent findings suggesting that besides HLA-C1 also several HLA molecules belonging to the HLA-C2 subset can be a ligand to KIR2DL2/3, as well as due to lack of commercial antibodies discriminating these inhibitory receptors from the activating KIR2DS2 (21,22). If in a patient both HLA-ligands to KIR2DL1 and KIR3DL1 were present or absent, the assessed functional values were averaged.

Statistical analysis

Patient characteristics are provided as numbers and percent, or as median and range. All CD107a and IFN-γ values obtained from stimulated NK cells were corrected for background activation, which was measured in unstimulated NK cells. Student's T-test and generalized linear models (GLM) incorporating a patient identification variable to adjust for inter-individual variation in NK cell function were used to compare CD107a and IFN-γ values in licensed and unlicensed NK cells at different timepoints. An interaction term was added to GLM to assess potential differences in licensing between patients in the autologous and allogeneic cohorts in respective analyses. P-values are two sided and values $p \leq 0.05$ were

considered statistically significant. All analyses were performed using SPSS v.21 (IBM Corp, Armonk, NY).

Results

First, we investigated if functional separation of S-KIR and NS-KIR expressing NK cells is maintained after both allogeneic and autologous HSCT. Comparative analysis between S-KIR and NS-KIR NK cells revealed consistent differences in degranulation and IFN-γ production in response to co-incubation with HLA deficient target cells across all post-transplant timepoints, with licensed NK cells showing greater degranulation and cytokine production than un-licensed NK cells (Fig. 1A&B and Table 2). In parallel, however, we observed that the functional difference between licensed and unlicensed NK cells is more distinct after autologous than allogeneic HSCT (difference in licensing effect for CD107a: 4.5-8.2% and IFN-γ: 9.1-18.9%, $p<0.05$ for all timepoints, Fig.1A&B and Table 2).

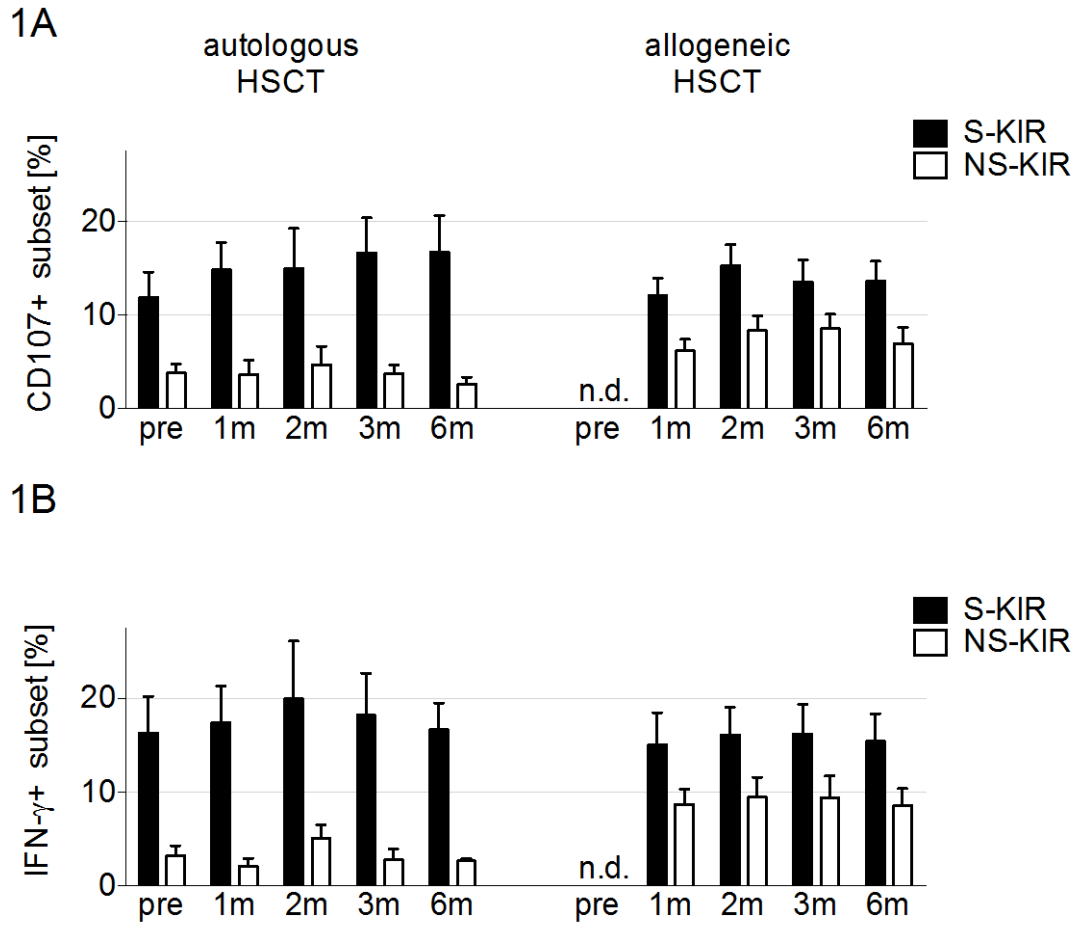

Figure 1 Functional reconstitution of S-KIR and NS-KIR NK cells after autologous and allogeneic HSCT.

Side by side comparison of degranulation (CD107a) and cytokine production (IFN-γ) between S-KIR or NS-KIR NK-cells after autologous and allogeneic HSCT. Depicted are mean and SE.

Little variation was observed between the different post-transplant timepoints: on average, degranulation and cytokine production of unlicensed NK cells amounted to between 15-36% (CD107a) and 16-28% (IFN-γ) of the respective readouts obtained in licensed NK cells after autologous transplantation, and to 46-61% (CD107a) and 55-61% (IFN-γ) of licensed NK cells after allogeneic HSCT (Fig. 2A and 2B). These data differ from a previous report showing a temporary increase in NK cell function of NS-KIR NK cells after allogenic matched HSCT, which is restored six months after transplantation (13).

Table 2: Evolution of licensing impact during post-transplant period

		Auto	CI	p-value	Allo	CI	p-value	Auto vs allo	CI	p-value
1mo	CD107a	8.2	5.2 – 11.2	< 0.01	3.7	2.0 – 5.5	< 0.01	4.5	1.1 - 7.8	0.01
	IFN-γ	16.4	9.1 – 23.7	< 0.01	7.3	2.7 – 12.0	< 0.01	9.1	0.6 – 17.6	0.04
2mo	CD107a	10.2*	*	*	4.6	2.1 – 7.1	< 0.01	*	*	*
	IFN-γ	25.9	13.4 – 38.4	< 0.01	6.4	3.5 – 9.4	< 0.01	18.9	9.1 – 28.7	< 0.01
3mo	CD107a	9.9	4.9 – 14.9	< 0.01	2.5	0.7 – 4.3	< 0.01	7.4	2.5 - 12.3	< 0.01
	IFN-γ	15.0	7.5 – 22.5	< 0.01	5.2	3.4 – 7.0	< 0.01	9.2	3.2 – 15.2	< 0.01
6mo	CD107a	11.8	10.3 – 13.2	< 0.01	3.6	2.0 – 5.2	< 0.01	8.2	5.2 – 11.1	< 0.01
	IFN-γ	13.5	11.6 – 15.4	< 0.01	2.9	1.3 – 4.4	< 0.01	10.6	6.8 – 14.4	< 0.01

PBSC: Peripheral blood stem cells, BM: Bone marrow, ATG: Antithymocyte globulin, GvHD: Graft-versus-host disease, CyA: Cyclosporin A, MTX: Methotrexate, MMF: Mycophenolate mofetil, CMV: Cytomegalovirus

We next asked if besides NS-KIR NK cells further NK cell subsets exhibit functional differences between autologous and allogeneic HSCT. In particular, we investigated if the function of NKG2A+KIR- and NKG2A-KIR- NK cells is different after allogeneic and autologous HSCT at any timepoint. In contrast to HLA-KIR dependent licensing, we found here no significant differences between autologous and allogeneic transplanted patients, neither in CD107a expression nor IFN-γ production (Fig. 3).

Subsequently, we investigated if secondary effects or treatments, specifically related to allogeneic HSCT, may be responsible for the increased functionality of unlicensed NK cells.

First, we explored if CMV replication has an influence on the licensing process, as it was previously shown that CMV replication can interfere with NK cell maturation as well as functionality (23,24). CMV replication was detected in twelve allogeneic HSCT patients (36.4%) during the first six months after transplantation. Thereby functional differences between licensed and unlicensed NK cells remained comparably distinct in patients with CMV replication (CD107a: 7.9±1.8% vs 3.6±1.0%; IFN-γ: 12.5±2.9% vs 7.6±2.0%) and without CMV replication (CD107a: 15.4±1.2% vs 9.5±0.9%; IFN-γ: 16.6±1.8% vs 9.9±1.1%) (Fig.4A&B).

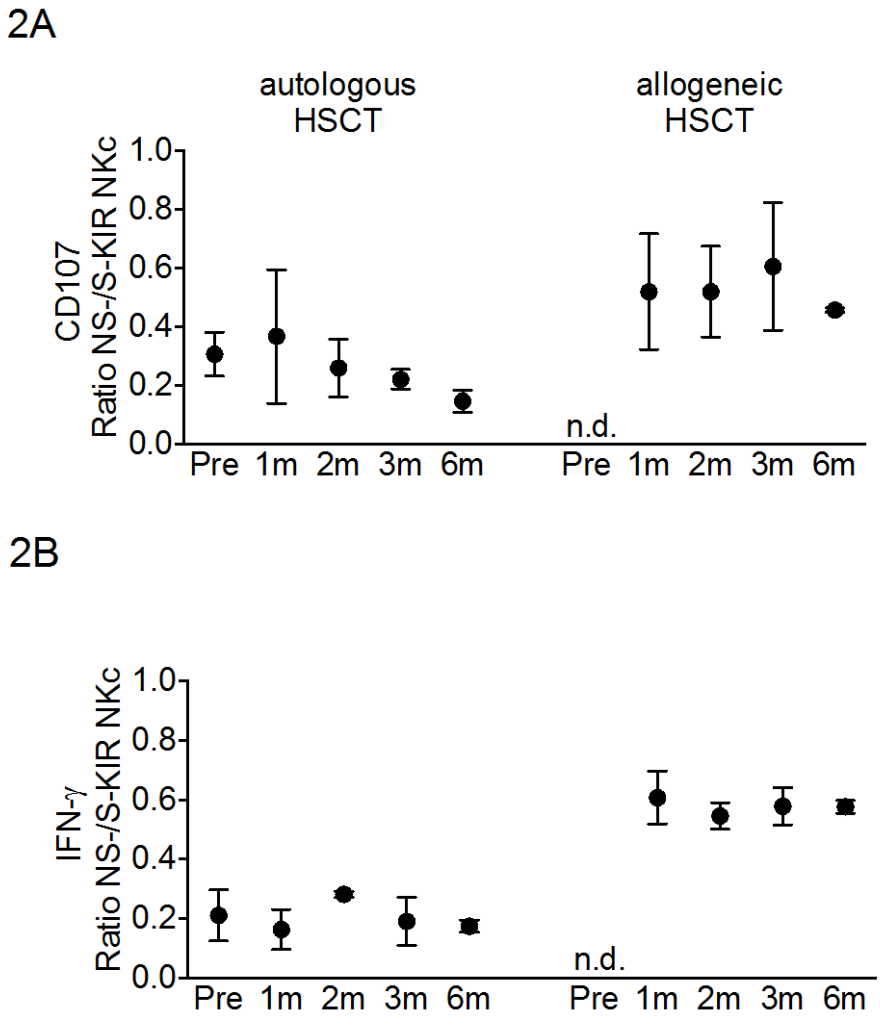

Figure 2 NK cell function as ratio of S-KIR and NS-KIR NK cells. Comparison of the licensing effect after allogeneic and autologous transplantation. The ratio indicates how much function NS-KIR NK cells exhibit taking S-KIR NK cells as reference. Depicted are mean and SE.

Next, we investigated if the occurrence of acute graft versus host disease (aGvHD) or its treatment interfere with NK cell licensing. In twenty-two recipients of allografts (66.7%) no or grade I aGvHD developed, and in eleven patients (33.3%) grade II-IV aGvHD was diagnosed and treated with systemic corticosteroids. While patients with no or grade I GvHD exhibited a clear functional difference of S-KIR and NS-KIR (CD107a: 13.7±1.3% vs 7.1±0.8%, p<0.01; IFN-γ: 19.1±2.0% vs 9.2±1.0%, p<0.01), the functional difference between S-KIR and NS-KIR expressing NK cells is smaller in patients who developed GvHD grade II-IV (CD107a: 13.4±1.8% vs 10.5±2.4%, p=0.40; IFN-γ: 8.9±1.1% vs 6.8±3.7%, p=0.48) (Fig. 4C&D).

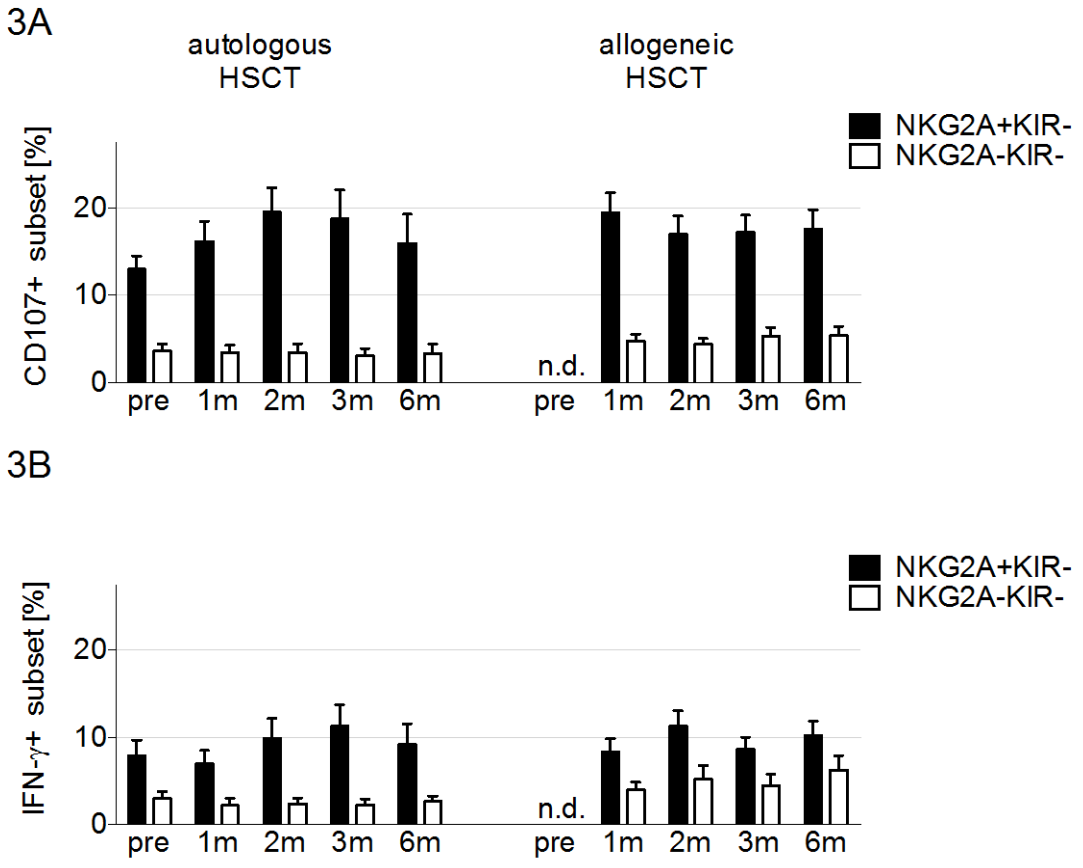

Figure 3 Functional reconstitution of NKG2A+KIR- and NKG2A-KIR- NK cells after autologous and allogenic HSCT. Comparison of degranulation (CD107a) and cytokine production (IFN-γ) between NKG2A+KIR- and NKG2A-KIR- NK cells after autologous and allogeneic HSCT. Depicted are mean and SE.

Finally, we assessed if pre-transplant ATG administration modulates NK cell licensing. It has previously been described that ATG remains for weeks in the patient serum and may exert cytotoxic effects on NK cells (25-27). When grouping recipients of allogeneic grafts into those whose conditioning did or did not include ATG, our data revealed that patients receiving ATG display more pronounced functional differences between licensed and unlicensed NK cells (CD107a: 14.9±1.5% vs 6.7±1.0%, $p<0.01$; IFN-γ: 18.4±2.2% vs 8.9±1,5% $p<0.01$), than patients whose conditioning did not include ATG (CD107a: 11.3±1.0% vs 8.7±1.0%, $p=0.16$; IFN-γ: 11.2±1.3% vs 9.4±1.1%, $p=0.64$) (Fig. 4E&F). The incidence of acute GvHD (grade II-IV) was similar in patients with and without ATG conditioning (30% in both groups).

Discussion

In this prospective study, we performed longitudinal analyses of the reconstituting NK cell function at a single cell level in 23 and 33 recipients of autologous and allogeneic HSCT. We aimed to investigate if NK cells with potential GVL function emerge as a consequence of irregularities of the licensing process after allogeneic and autologous transplantation.

We found that after both types of transplantation, the functional difference between licensed (S-KIR) and unlicensed (NS-KIR) NK cells is maintained. This is in line with previously published work (14). Further, we observed no dynamic changes in NK cell function between different timepoints assessed during six months of follow up. In particular, we found no temporary increase of NK cell function, neither in NS-KIR NK cells nor in any other subset. This stands in some contrast to a previous study finding a transient increase in NK cell function after allogeneic stem cell transplantation (13). Interestingly, however, both degranulation (CD107a) and cytokine production (IFN-γ) of NS-KIR NK cells appears roughly two fold higher after allogeneic HSCT compared to NS-KIR NK cells after autologous transplantation during the whole six months of follow up (Fig. 1). In contrast, all other NK cell subsets investigated (S-KIR+, NKG2A+/KIR- and NKG2A-/KIR- NK cells) show comparable functionality after autologous and allogeneic HSCT, suggesting that the increase in NK cell function after allogeneic HSCT is specific to NK cells expressing NS KIR. An enhanced functional competence of NS-KIR NK cells might allow for an antitumor effect driven by NS-KIR NK cells after allogeneic transplantation. This finding supports and may moreover explain the survival benefit documented for patients after HLA-matched allogeneic HSCT with lacking KIR ligands (7,11,13). Investigations in pediatric patients have shown a similar missing ligand effect after autologous transplantation, whereas in adult patients no such

effect was found (15,16). No missing ligand effect in adult patients after HSCT is in agreement with the pronounced NK cell licensing, we observed after autologous HSCT at all timepoints. Whether NK cell licensing works different after HSCT in pediatric patients remains to be investigated.

Subsequently we asked if characteristics that differentiate allogeneic from autologous HSCT may explain the different post-transplant NK cell licensing. In particular, we elaborated the influence of pre-transplant ATG, GvHD with its treatment and CMV replication as a common opportunistic infection and result of GvHD prophylaxis. Replication of CMV has recently been shown to interfere with phenotypical and functional NK cell reconstitution after HSCT (23,24,28). Nevertheless, we found no evidence in the present study for a CMV related alteration of the licensing process. Further, we found no increased NK cell function after allogeneic HSCT in case of CMV replication as it has been reported before (23).

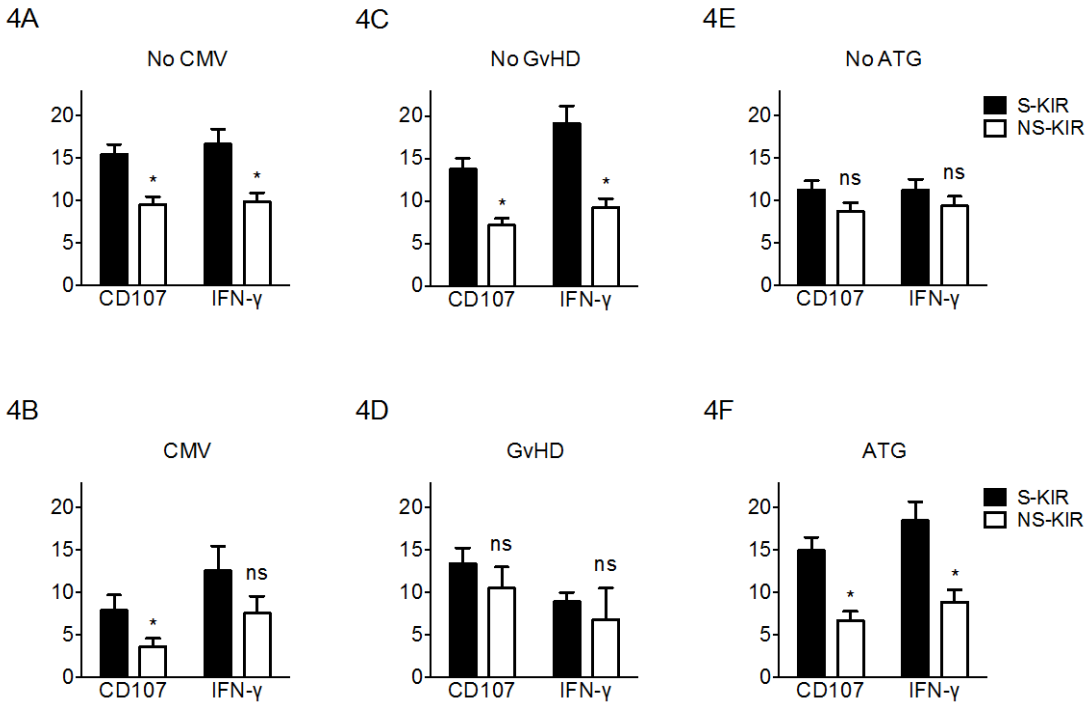

Figure 4 Impact of CMV, GvHD and pre-transplant ATG on NK cell licensing.

Figure 4A&B Post-transplant function of allogeneic transplant recipients grouped by CMV replication status. S-KIR and NS-KIR CD107 and IFN-γ response to target cell stimulation are compared in both subgroups. **Figure 4C&D** Post-transplant functional data of allogeneic transplant recipients grouped by GvHD. S-KIR and NS-KIR CD107 and IFN-γ response to target cell stimulation are compared in both subgroups. **Figure 4E&F** Post-transplant functional data of allogeneic transplant recipients grouped upon

While so far several studies have reported protective influence of NK cells against GvHD in mice and humans, little is known regarding a potential effect of GvHD or its treatment on NK cell function (29-31). In the present study we found GvHD to be associated with reduced functional differences between S-KIR and NS-KIR NK cells. During GvHD serum-levels of several pro- and anti-inflammatory cytokines reach exceptionally high levels and local concentrations may be even higher (32,33). In parallel previous studies show that pre-

stimulation with cytokines can reduce the differential response of licensed and unlicensed NK cells in both mice and humans (7,34). Finally we also assessed the impact of pre-transplant ATG on NK cell licensing. We found, that patients which had received conditioning containing ATG, showed more pronounced licensing effects than patients not treated with ATG. The reason for this difference remains unclear.

In summary, we found that single KIR positive NK cells after HSCT are equipped with functional capacity according to commonly acknowledged licensing concepts. However, our data suggest that the licensing effect is less distinct after allogeneic transplantation when compared to autologous HSCT, or in other words, that the licensing mechanism assuring tolerance of unlicensed NK cells might be disrupted after allogeneic HSCT. In direct comparison, the functional competence of NS-KIR NK cells is higher in allogeneic transplant patients. Consequently, the latter may profit from increased function of NS-KIR expressing NK cells, possibly representing the cellular correlate of studies finding survival benefits of patients with NS-KIR NK cells after HSCT. By analyzing subgroups of allogeneic transplant patients, we found ATG treatment associated with more, and GvHD associated with less distinct NK cell licensing, whereas CMV replication appeared to have no effect.

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Project 3

Protection From Varicella Zoster in Solid Organ Transplant Recipients Carrying Killer Cell Immunoglobulin-Like Receptor B Haplotypes.

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Contribution to the study:

Analysis and interpretation of the data, writing of the manuscript.

Abstract

Background

Natural killer cell function is regulated by inhibitory and activating killer cell immunoglobulin-like receptors (KIR). Previous studies have documented associations of KIR genotype with the risk of cytomegalovirus (CMV) replication after solid organ transplantation (SOT).

Methods

In this study of 649 solid organ transplant recipients followed prospectively for infectious disease events within the Swiss Transplant Cohort Study, we were interested to see if KIR genotype associated with virus infections other than CMV.

Result

We found that KIR B haplotypes (which have previously been linked to protection from CMV replication) were associated with protection from varicella zoster (VZV) infection (hazard ratio 0.43, 95% confidence interval 0.21-0.91, p=0.03). No significant associations were detected regarding the risk of herpes simplex, Epstein-Barr Virus or BK polyomavirus infections.

Conclusion

In conclusion, these data provide evidence that the relative protection of KIR haplotype B from viral replication after SOT may extend beyond CMV to other herpes viruses such as VZV and possibly EBV.

Introduction

Natural killer cells are important in the early response against many pathogens including viruses. In contrast to B- and T-lymphocytes they do not express rearranged surface receptors. Instead, diversity in the NK cell repertoire is a function of expression of varied combinations of inhibitory and activating surface receptors. It has been shown recently that thousands of different NK cell phenotypes exist within the polyclonal pool of healthy donor NK cells (1).

Among the most polymorphic NK cell receptors are killer-cell immunoglobulin-like receptors (KIR). KIR can be either activating or inhibitory in nature, depending on the structure of their intracellular tail containing an immunoreceptor tyrosine-based inhibitory or activating motif (ITIM/ITAM) (2). Inhibitory KIR mediate NK cell tolerance by binding to self-HLA class I molecules. By contrast, the ligands and physiological relevance of activating KIRs are only poorly understood; binding to HLA class I has only been documented for KIR2DS1, KIR2DS2, and KIR2DS4 (3-5). KIR genes segregate as haplotypes, which are classified as "A" if they contain a canonical set of six inhibitory receptors and one single activating KIR gene (KIR2DS4), whereas the remaining haplotypes, which may contain up to 5 additional activating receptors, are collectively classified as "B". In Caucasians, A and B haplotypes are found at similar frequencies. Recipient B haplotype and high numbers of activating KIR genes have been linked with protection from cytomegalovirus replication after solid organ transplantation in a number of studies (6, 7). More recently, several studies have suggested that KIR genes may also associate with the risk for other viral infections such as hepatitis C (8), herpes simplex (9), BK polyomavirus (BKPyV) (10), and influenza (11). For Epstein-Barr and varicella zoster virus, two further members of the human herpes virus family, evidence for involvement of NK cells in disease control recently emerged, but no possible correlation with KIR genotype has been investigated yet (12, 13). For several of the

above-mentioned viruses, different receptors were shown to be involved in the innate immune response (14-16). At the same time, all these receptors are present in every individual. In contrast, KIR receptors vary in number and composition between individuals. A possible influence of single KIR receptors might therefore operate as a prognostic marker. As virus infection/replication is a frequent complication of immunosuppression after solid organ transplantation, we were interested to analyze the correlation of KIR genotype and virus replication after solid organ transplantation (SOT).

We made use of the Swiss Transplant Cohort Study, a large prospective multicenter effort collecting data and bio samples on all solid organ transplants performed in Switzerland (17), to address whether recipient KIR genotype associates with the risk of viral infections in recipients of solid organ transplantation.

Patients and materials

Patients

Six hundred and forty-nine patients undergoing single solid organ transplantation at six transplant centers in Switzerland between May 2008 and December 2010 were combined in this analysis (Inselspital Bern, n=99; Centre Hospitalier Universitaire Vaudois, n=102; Hôpitaux Universitaires de Genève, n=95; Kantonsspital St. Gallen, n=15; Universitätsspital Basel, n=106; Universitätsspital Zürich, n=232). Induction and maintenance immunosuppressive regimens in patients grouped by KIR haplotype are summarized in Table 1 along with demographic characteristics.

Data on transplant characteristics and transplant outcome including infectious complications were prospectively collected and retrieved using an electronic database (17). Written informed consent was obtained from all study participants and the study was approved by the institutional review boards in all centers.

KIR genotyping

KIR genotyping was performed using a reverse sequence-specific oligonucleotide method (OneLambda, Canoga Park, CA) according to the manufacturer's instructions (7), these results were in part reconfirmed by qPCR(18). KIR genotypes were grouped into AA if they contained only the canonical group A haplotype genes (KIR2DL1, KIR2DL3, KIR2DL4, KIR3DL1, KIR3DL2, KIR3DL3, and KIR2DS4). Any genotype containing additional KIR genes is referred to as a BX, as it contains at least one group B haplotype (19). BX patients were further subdivided into imputed BB haplotypes based on the absence of the A haplotype genes KIR2DL3, KIR2DL1 and KIR3DL1. Finally, genotypes were dichotomized into telomeric and centromeric A and B haplotype motifs according to published algorithms (20).

Table 1: Patient characteristics

KIR haplotype	AA (n=176)	BX (n=473)	p-value
Patient age at transplantation – Median (Range)	50 (1-75)	50 (0-79)	0.91
Gender (n, %)			
– Male	116 (66)	309 (65)	0.89
– Female	60 (34)	164 (35)	
Center (n, %)			
– Basel	23 (13)	83 (18)	0.26
– Bern	29 (17)	70 (15)	
– Geneva	28 (16)	67 (14)	
– Lausanne	36 (21)	66 (14)	
– St. Gallen	4 (2)	11 (2)	
– Zürich	56 (32)	176 (37)	
Nr of HLA-A/B/DR-mismatches (n, %)			
– 0-2	13 (7)	47 (10)	0.10
– 3-4	53 (30)	150 (32)	
– 5-6	94 (53)	208 (44)	
– missing	16 (9)	68 (14)	
Antibody induction (n, %)			
– Anti-CD25 MAb	104 (59)	282 (60)	0.98
– ATG	32 (18)	87 (18)	
– none	40 (23)	62 (13)	
Maintenance immunosuppression (n, %)			
– Tacrolimus	120 (68)	317 (67)	0.78
– Cyclosporine	65 (37)	176 (37)	0.95
– Prednisone	161 (92)	425 (90)	0.53
– MMF	163 (93)	437 (92)	0.92
– mTOR	22 (13)	62 (13)	0.84
Antiviral prophylaxis (n, %)			
– Ganciclovir/Valgancyclovir	68 (39)	179 (38)	0.85
– Acyclovir/Valacyclovir	23 (13)	36 (8)	0.03
Organ Transplant (n, %)			
– Heart	23 (31)	51 (69)	0.88
– Kidney	78 (27)	213 (73)	
– Liver	50 (27)	138 (73)	
– Lung	25 (26)	71 (74)	

MMF = mycophenolate mofetil;
mTOR = mammalian target of rapamycin inhibitor (i.e. sirolimus or everolimus)

Diagnosis of viral infections

Recipients of kidney transplants were screened for BKPyV replication by quantitative polymerase chain reaction (qPCR) in blood and urine at minimum once every three months in the first year after transplantation. Additionally, BKPyV replication was assessed by qPCR when clinically indicated. Patients receiving organ transplants other than kidney were not regularly screened for BKPyV. EBV screening guidelines differ between the involved study centers. In 121 patients, EBV replication was assessed six and twelve months after transplantation. The remaining patients were tested for EBV based on clinical suspicion. In both cases EBV replication was detected by qPCR from the blood. Infection with HSV was diagnosed clinically and confirmed by qPCR only exceptionally in single cases. Infection with Varicella Zoster was clinically diagnosed in totally 28 patients. Of these, diagnosis was reassessed and confirmed by qPCR in 17 patients. The serological status for EBV, HSV and VZV of donors and recipients prior to transplantation is indicated in Table 2.

Statistical analysis

Patient characteristics were compared by Mann-Whitney U test or Pearson's chi square test, where appropriate. The cumulative incidence of viral infection/replication events (+/- standard error) was estimated using death and graft loss as competing risks. Patients were grouped into those carrying two KIR A haplotypes (AA) versus those carrying one or two KIR B haplotypes (BX). Only the first episode of infection with each pathogen was considered in each patient. Cox regression was used for multivariable analyses, adjusting for induction immunosuppression, number of HLA-mismatches (HLA-A/B/DR), graft-rejection and respective therapy, type of organ transplanted, for recipient pre-transplant serology, and antiviral prophylaxis (val-/ganciclovir and val-

/acyclovir) in the case of herpes virus infections. All statistical analyses were performed with SPSS v.21. (IBM Corp, Armonk, NY).

Table 2: Infection status patients and donors

KIR Haplotype	AA (n=176)		BX (n=473)		p-value
EBV serology (n,%)					
- D-/R-	2	(1)	5	(1)	0.95
- D-/R+	7	(4)	26	(6)	
- D+/R-	11	(6)	29	(6)	
- D+/R+	145	(82)	387	(82)	
- missing	11	(6)	26	(6)	
HSV serology (n,%)					
- D-/R-	3	(2)	16	(3)	0.22
- D-/R+	14	(8)	19	(4)	
- D+/R-	12	(7)	35	(7)	
- D+/R+	50	(28)	150	(32)	
- missing	97	(55)	253	(54)	
VZV serology (n,%)					
- D-/R-	3	(2)	2	(0)	0.33
- D-/R+	10	(6)	24	(5)	
- D+/R-	3	(2)	14	(3)	
- D+/R+	77	(44)	198	(42)	
- missing	83	(47)	235	(50)	

Results

The following numbers of infectious episodes were recorded in this cohort: cytomegalovirus 289, Epstein Barr virus (EBV) 98, herpes simplex (HSV) 69, BK virus (BKV) 63, rhinovirus 32, hepatitis C virus 29, varicella zoster virus (VZV) 28, influenza virus 20, respiratory syncytial virus 16. Since viral infections of the respiratory tract are frequent, and only in a minority of cases identification of the pathogen responsible is attempted, we excluded rhinovirus, influenza virus, and respiratory syncytial virus from the analysis. Cytomegalovirus and hepatitis C virus were also excluded, since the effect of KIR genotype on virus replication/infection has been described previously in previous studies (6-8).

Figure 1

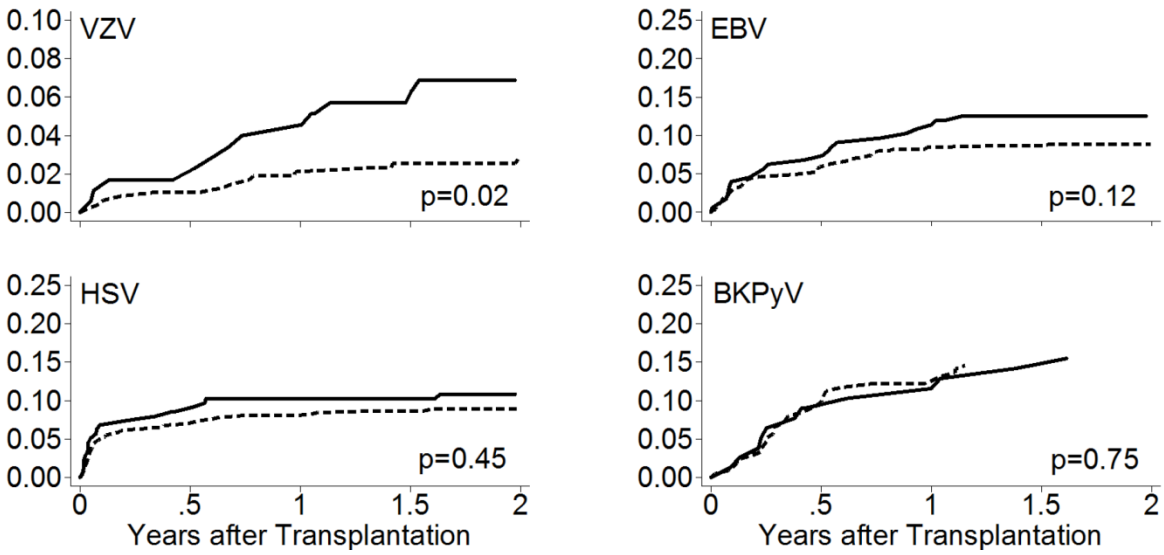

FIGURE 1. Cumulative incidence of varicella zoster (VZV), EBV, HSV, and BKPyV. Solid lines represent patients homozygous for the KIR-A haplotype (AA), whereas dashed lines represent patients carrying 1 or 2 KIR B haplotypes (BX). P value derived from Gray test.

Analysis of the cumulative incidences of viral infections revealed that presence of a KIR-BX haplotype in the patient provided relative protection from VZV replication. The two-

year cumulative incidence of VZV infection was significantly lower in KIR-BX haplotype patients (3 ± 1%) compared to AA individuals (7 ± 2%, p=0.02, Figure 1). A trend towards lower rates of EBV replication was also observed (two-year CI 9 ± 1% versus 13 ± 3%), however, this difference did not reach the level of statistical significance (p=0.12, Figure 1). For the remaining viruses, no effect of KIR haplotype was observed: HSV 9 ± 1% versus 11 ± 2% (p=0.45); and BKPyV 15 ± 2% versus 15 ± 4% (p=0.75, Figure 1) for BX and AA haplotype patients, respectively.

Figure 2

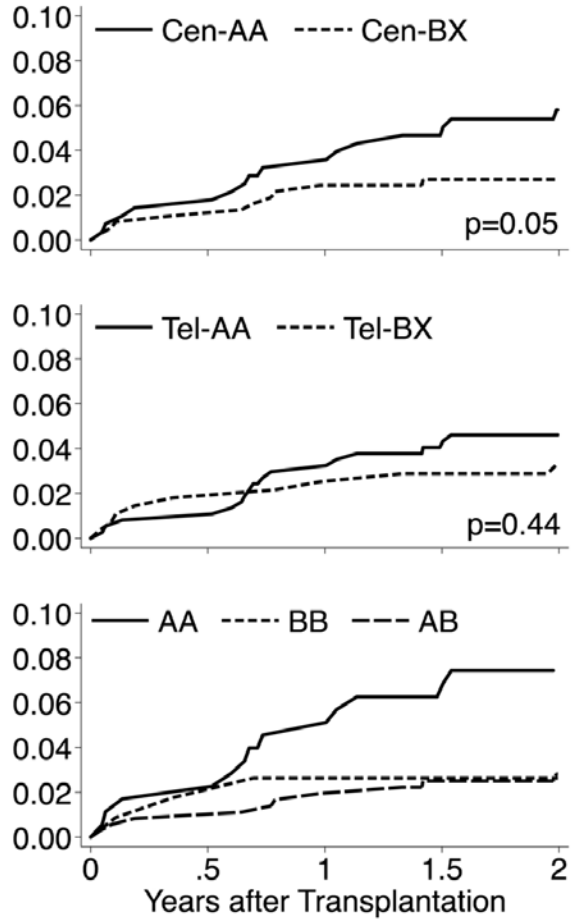

FIGURE 2. Cumulative incidence of VZV events in patients grouped by telomeric and centromeric KIR-B haplotype content (upper and middle panel) and by imputed haplotypes (lower panel). For the upper and middle panels, solid lines represent patients homozygous for the KIR-A haplotype (AA), whereas dashed lines represent patients carrying 1 or 2 KIR B haplotypes (BX). P value derived from Gray test.

To further address the correlation of KIR haplotype and protection from VZV, we grouped patients by imputed haplotypes (AA versus AB versus BB), and by centromeric and telomeric KIR A and B motifs. This analysis revealed that protection from VZV reactivation was related to centromeric (p=0.05) rather than telomeric (p=0.44) KIR B motifs (Figure 2, upper and middle panel). Furthermore, we found that one haplotype B was associated with relative protection from VZV reactivation, as rates in AB and in BB patients were comparable (6 ± 2% versus 7± 3%, respectively, Figure 2, lower panel). Finally, multivariable analysis confirmed a statistically significant protective effect of KIR BX

status regarding post-transplant VZV infection (hazard rate versus AA haplotype: 0.43, 95% confidence interval 0.21-0.91, p=0.03). Similarly, the risk for EBV replication after adjustment for covariates was reduced still without reaching the level of statistical significance (HR 0.68, 95% CI=0.41-1.12, p=0.13).

Discussion

In this study, we report that patients carrying KIR B haplotypes benefit from relative protection regarding VZV reactivation during the first two years after SOT. This effect appeared to be specifically linked to VZV as no effect was seen on HSV and BKV replication. EBV replication was less frequent among carriers of B haplotypes, but this difference did not reach statistical significance, presumably in part due to the low rate of events for this type of complication. It is therefore notable that the data presented here suggest susceptibility to activating KIRs for one or possibly two further members of the herpes virus family besides CMV. To further disclose which activating KIRs may be involved in immunological control of VZV, we divided activating KIRs in relation to KIR2DS4 as previously described in centromeric and telomeric genes (20). These analyses illustrate that centromeric rather than telomeric KIR loci are associated with protection from VZV, reducing the number of potential candidate loci mainly to three: the inhibitory KIR2DL2 and the activating receptors KIR2DS2 and KIR2DS3. KIR2DS3 is an unlikely candidate, as the most prevalent alleles are not expressed on the cell surface. Unfortunately, further genotype/phenotype correlations will add little to discriminate between the effect of these two genes, as they are in almost perfect linkage disequilibrium (21). Another limitation of this study derives from the fact that diagnosis of viral replication was based on clinical suspicion for some of the viruses analyzed, implying that subclinical replication may have been missed in some instances.

Our findings stand in line with previous work exploring the physiological relevance of activating KIR. Several recent studies have described a protective effect of activating KIRs against CMV replication in different settings of immunosuppression (6, 7). After SOT or hematopoietic stem cell transplantation (HSCT), when the adaptive immune system is pharmacologically suppressed, patients with a higher number of (haplotype B)

activating KIR genes experience relative protection from CMV replication (22, 23). Further clinical conditions associated with KIR B haplotypes are reduced relapse rates in AML patients after allogeneic HSCT (if KIR B genes are present in the donor); and a reduced risk of preeclampsia in women carrying activating KIR genes (24, 25). These associations indicate that activating KIR receptors might recognize patterns on wide range of different potential target cells. We therefore explored the possibility that KIR haplotype associates with the susceptibility to other opportunistic infections in patients under pharmacological immunosuppression. Apart from CMV and hepatitis C (6-8), limited knowledge regarding potential roles for KIR and NK cells in antiviral immunity exists. No effect of KIR haplotype was seen regarding replication of HSV and BKPyV, which stands somewhat in contradiction to previous studies partly done in healthy donors (9, 10). However this may reflect the different role for NK cells in patients with compromised adaptive immunity and the sites of reactivation. In summary, we find that telomeric B-haplotype KIR genes are associated with protection from VZV reactivation after SOT. If confirmed, these data may have an impact on VZV prophylaxis in these patients.

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Project 4

Platelet derived Ectosomes Suppress NK Cells, submitted to Journal of Immunology.

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Contribution to the study:

Design and performance of experiments, analysis and interpretation of the data writing parts of the manuscript.

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Abstract

Platelet (PLT) transfusions are potentially life-saving for individuals with low PLT numbers. However, previous work revealed that PLT transfusions are associated with increased infection risks. During storage, PLT intended for transfusion, continuously shed ectosomes (Ecto) from their surface, expressing immunomodulatory molecules like phosphatidylserine or TGF-β1. Recently, PLT-Ecto have been shown to reduce pro-inflammatory cytokine release by macrophages, and to favor the differentiation of naïve T cells towards regulatory T cells. Whether PLT-Ecto modify NK cells remains unclear. We exposed both purified NK cells and full peripheral blood mononuclear cells (PBMC) from healthy blood donors to PLT-Ecto. We found a reduced expression of several activating surface receptors (NKG2D, NKp30 and DNAM-1) and decreased NK cell function as measured by CD107a expression and IFN-γ production. Pre-treatment of PLT-Ecto with anti-TGF-β1 (aTGF-β1) neutralizing Ab restored surface receptor expression and NK cell function. We further observed a TGF-β1 mediated upregulation of miR-183, which in turn reduced DAP12, an important protein for stabilization and downstream signaling of several activating NK cell receptors. Again these effects could be partially antagonized when PLT-Ecto were pre-incubated with aTGF-β1 Ab. Erythrocyte Ecto (ERY-Ecto) did not affect the expression of NK cell activating receptors nor NK cell function. Interestingly, polymorphonuclear Ecto (PMN-Ecto) induced the secretion of TGF-β1 by NK cells, which in an auto-/paracrine manner suppressed them. In sum, our study showed that PLT-Ecto could inhibit NK cell effector function in a TGF-β1-dependent manner, suggesting that recipients of PLT transfusions may experience reduced NK cell function.

Introduction

Transfusion of platelets (PLT) is a potentially lifesaving therapy for patients with low PLT count after substantial loss of blood due to severe trauma or extensive surgery. In addition, PLT transfusion is indispensable in patients with congenital PLT disorders, disturbed haematopoiesis, or after myeloablative chemotherapy preceding haematopoietical stem cell transplantation (1-3). Many of these conditions are accompanied by an increased risk of infection related to trauma itself or neutropenia (4-7). However, PLT transfusion per se may induce immunosuppression as suggested by an increased susceptibility for infections as well as evidence for tumor growth after transfusion (8-11). While high PLT numbers have been associated with worse outcome in ovarian and other cancer patients more than 40 years ago, recently mechanisms were unraveled how PLT protect cancer cells from recognition and elimination through the immune system (12, 13). Possible effectors of the immunosuppressive side effects of PLT infusion are the large amounts of PLT-Ectosomes (PLT-Ecto) that are released during storage and transfused concomitantly with PLTs (14, 15). We have previously shown that PLT-Ecto have the ability to modulate functional properties of macrophages and dendritic cells through their phosphatidylserine (PS) expression (16). They induced the release of TGF-β1 and decreased the production of pro-inflammatory molecules by macrophages. Further, they reduced the phagocytic activity and cytokine release of monocyte derived dendritic cells. They interfered with dendritic cells' differentiation as well. Recent evidence shows that PLT-Ecto have the potential to differentiate naïve $CD4_+$ T into functional regulatory T cells (17). Analog to PLT infusions, erythrocytes (ERY) are frequently infused after surgery or to hemato-oncological patients. Prior studies report that ERY infusions are associated with increased infection risk, which correlates with ERY storage time (18). Like PLT, ERY release Ecto during storage, which express PS but no TGF-β1. ERY-Ecto showed capacities to down-modulate macrophage activity through

PS expression, but not through TGF-β1 release (19). Upon activation, PMN release Ecto that can reach comparable concentrations to those found at site of inflammation. These PMN-Ecto are immunosuppressive; their effects are mainly mediated through PS that induces the release of TGF-β1 and the decrease of pro-inflammatory molecules, a phenotype comparable to that of PLT-Ecto (20). Little is known about possible interactions between Ecto and NK cells, except for the recent observations made by Pliyev et al., who have shown that PMN-Ecto can change the cytokine profile of NK cells (21). The main aim of the present study was to define whether PLT-Ecto alter NK cell function.

Material and Methods

Monoclonal Ab staining and FACS analysis

For flow cytometric analysis we used the following mouse anti human mAbs according to manufacturer's instruction: anti-CD3-APC-780 (clone SK7, eBioscience); anti-CD14-APC-780 (clone 61D3, eBioscience); anti-CD19-APC-780 (clone HIB19, eBioscience); anti-CD56-PE/Cy7 (clone HCD56, Biolegend); anti-CD107a-PE (clone H4A3, BD Bioscience); anti-CD226(DNAM-1)-PE (clone DX11, Becton Dickinson); anti-CD314(NKG2D)-PE (clone 1D11, Biolegend); anti-CD335(NKp46)-PE (clone 9E2, Biolegend); anti-CD337(NKp30)-PE (clone P30-15, Biolegend); anti-IFN-γ Violet 450 (clone B27, Becton Dickinson) and anti-DAP12-PE (clone H10E12F4, Beckman Coulter). After staining, cells were analyzed by a nine-color flow cytometer (CyAn ADP, Beckman Coulter, Pasadena, CA). For analysis of the FACS data FlowJo v. 9.4.11 (Tree Star, Ashland, OR) was used. Phenotypic characterization of NK cells was performed on enriched NK cells. NK cell enrichment was ascertained by gating on CD3-CD14-CD19-CD56+ cells. Functionality of NK cells was assessed in full PBMC and enriched NK cells, defining NK cells as CD3-CD14-CD19-CD56+ population.

Preparation of PLT-Ecto

PLT-concentrates were obtained by apheresis as described previously (16); briefly, donor blood was processed by a cell separator equipped with an in-line centrifuge for PLT separation (Blood Transfusion Center Beider Basel). Subsequently PLT were transferred to a collection bag and the remaining blood components are reinfused to the blood donor. Concentrated PLT were stored at room temperature (RT) for maximally 5 days. In order to clear remaining cells several sequential centrifugation steps at RT were performed. For removal of high-density cells (ERY and leukocytes), the PLT-concentrate

was centrifuged for 15 min at 300 x g and for another 15 minutes at 500 x g. Thereafter PLT were pelleted by centrifugation of the suspension for 20 min at 800 x g. Residual PLT and low-density debris were removed by a further centrifugation step of 20 min at 3000 x g. Supernatants (SN) were subdivided in aliquots and preserved at -80°C. For concentration of PLT-Ecto, the SN containing PLT-Ecto were centrifuged for 1 h at 200'000 x g at RT. Before use the pellet was washed by centrifugation at the same speed and time in 0.9% NaCl.

Preparation of PMN-Ecto

PMN were obtained from fresh buffy coats of healthy blood donors, as previously described (22). Briefly, all manipulations were performed at 4°C. First fresh buffy coats were diluted 1/1 (v/v) with 2 mM PBS-EDTA, gently mixed with 0.25 v 4% Dextran T500 and stored for 30 min for ERY sedimentation. The supernatant containing the leucocytes was removed and centrifuged for 10 min at 200 x g. The pellet was re-suspended in 9 ml ultrapure water for 1 min in order to lyse remaining ERY. Subsequently 3 ml 0.6 M KCl and 40 ml 0.15 M NaCl were added to restore isotonicity. Thereafter cells were spun 10 min at 350 x g and re-suspended in 20 ml of 2 mM PBS-EDTA. The cell suspension was carefully layered over 20 ml Ficoll-Hypaque and again centrifuged for 30 min at 350 x g. The PMN-rich pellet was washed twice in 2 mM PBS-EDTA. To stimulate PMN, they (107 cells/ml) were diluted 1/1 (v/v) in warm (37°C) RPMI 1640 (Life Technologies, Basel, Switzerland) and fMLP (Sigma Chemical Co.) was added to a concentration of 1 µM followed by an incubation for 20 min at 37°C. Subsequently PMN were pelleted by centrifugation (4000 x g for 15 min at 4°C), the PMN-Ecto in the SN were concentrated using Centriprep centrifugal filter devices (10'000 m.w. cut-off, Millipore, MA, USA). Aliquots were prepared and stored at −80°C. Before use PMN-Ecto were centrifuged for 45 min at 160'000 x g at 4°C and

resuspended in 0.9% NaCl.

Preparation of ERY-Ecto

Whole blood was drawn from healthy donors and ERY were obtained and stored as previously described (19). Briefly, 450 ml of whole blood was collected and stored in plastic bags (triplicate bag system with an integrated whole blood filter Leucoflex Sang Total 1, Macopharma, Tourcoing, France) containing 63 ml citrate phosphate dextrose. Filtration took place within 3 h after the blood was collected. After centrifugation for 10 min at 1500 x g, 20°C and separation of packed leuko-depleted ERY (LD-E) from plasma, LD-E were transferred to satellite bags containing 100 ml saline adenine-glucose-mannitol. LD-E were stored 25 days before tests. SN were separated through centrifugation of packed LD-E for 10 min at 1000 x g, 4°C. This step was repeated to clear residual ERY. Subsequently ERY-Ecto were concentrated using Centriprep centrifugal filter devices (10'000 m.w. cutoff, Millipore, MA, USA) and aliquots were stored at −80°C. Before use SN were centrifuged for 1 h at 160'000 x g at 4°C and re-suspended in 0.9% NaCl.

Membrane labeling

PLT-Ecto and NK cells were incubated with Dylight amine-reactive dye 633 and 488, respectively, for 30 min at RT. Labeled PLT-Ecto were separated from the remaining unbound dye by ultracentrifugation (45 min, 200'000 x g at RT) and washed with 0.9% NaCl. Labeled NK cells were washed by centrifugation (300 x g) 2 times with NaCl before use.

Live-cell microscopy

Labeled NK cells were incubated on 4-chambered #1.0 Borosilicate Coverglass System (Lab-Tek, Nunc, Thermo Fischer Scientific) with medium alone or fluorescently labeled PLT-Ecto, and time-lapse microscopy was performed.

Confocal microscopy

Confocal images were acquired on an Axiovert confocal laser-scanning microscope (LSM 710) from Zeiss AG (Feldbach, Switzerland) using a 40x oil-immersed objective (Carl Zeiss). For the duration of a given experiment, settings on the microscope were kept constant for all samples, including exposures, pinhole size (2 µm) and photomultiplier tube gain. Images were exported as JPEG files.

Effects of Ecto on NK cells within PBMC

PBMC were freshly isolated from healthy donors using a density gradient medium (Lymphoprep, Fresenius Kabi Norge AS). For culturing and functional assays RPMI 1640 medium supplemented with 10% heat inactivated FCS, L-Glutamine and penicillin/streptomycin was used. Full PBMC were incubated for 16-20 h at 37°C in 5% CO2 in absence or presence of the indicated concentrations of PLT-Ecto or PMN-Ecto. When stated PLT-Ecto were pre-incubated for 30 min with neutralizing Ab to TGF-β1 (aTGF-β1) at a concentration of 1 µg/ml (purified monoclonal mouse IgG1, clone 9016, R&D System) or 1 µg/ml isotype control (purified mouse IgG1, InvivoGen). Additionally aTGF-β1was added to the culture of PBMC directly with PMN-Ecto when indicated, at the respective concentration.

Effects of Ecto on enriched NK cells

NK cells were purified from whole blood by negative selection (RosetteSep, NK cell enrichment cocktail, catalog no. 15025/15065, Stemcell). NK cells were cultured for 16-20 h at 37°C in 5% CO2 in absence or presence of the indicated concentrations of PLT Ecto or PMN-Ecto.

When stated PLT-Ecto were preincubated for 30 min with aTGF-β1 at a concentration of 1 mg/ml (purified monoclonal mouse IgG1, clone 9016, R&D System) or 1 mg/ml isotype control (purified mouse IgG1, InvivoGen). Additionally, when indicated aTGF-β1 was added to the culture of PBMC directly with PMN-Ecto at the respective concentration.

Staining and flow cytometric analysis of Ecto

Flow-cytometric analysis of PLT-Ecto TGF-β1 expression was performed using a FACSCalibur flow cytometer (BD Biosciences). In total 30'000 events per sample were acquired and analyzed using CellQuest software (BD Biosciences). Both, PLT-Ecto and TruCount beads were identified by size, as assessed by the logarithmic amplification of the respective forward scatter (FSC) and side scatter (SSC) signals. PLT-Ecto were localized within R2 and TruCount beads in R1.

Functional NK cell assays

NK cell function was investigated as previously described (23). Briefly, for functional analysis, full PBMC or enriched NK cells were co-incubated (37°C in 5% CO2) with the cell line 721.221 in an effector to target ratio 5:1 (PBMC:721.221) or 1:2 (NKc:721.221) respectively in the presence of anti-CD107a mAb (clone H4A3; Becton Dickinson). After

1 h of co-incubation, Brefeldin A (BD Bioscience) was added, and after 6 h the cells were harvested. Thereafter, the cells were stained at 4°C for surface antigens, fixed (Fixation Buffer, Biolegend), permeabilized (Permeabilization Buffer, Biolegend) and subsequently stained for IFN-γ.

TGF-β1 ELISA

Supernatants of PBMC and purified NK cells exposed or not to PLT-, PMN- and ERY-Ecto were collected and spun for 10 min at 1000 x g at 4°C to remove cell debris. The SN was then assessed in the ELISA duo-set kit for human TGF-β1 (R&D Systems). The assay was performed according to the manufacturer protocol.

Quantitative real-time RT-PCR

NK cells were lysed in Qiazol (QIAGEN), and total RNA including microRNAs was extracted using miRNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. The isolated RNAs were quantified with Nanodrop (Epoch, BioTek Instruments Inc., Switzerland). Expression of miR-183, and the endogenous controls U6 snRNA, RNU44 and RNU6B was assessed using TaqMan miRNA assays (Applied Biosystems) by real-time RT-PCR. RNA was converted to cDNA using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems), and a pre-amplification step was performed using TaqMan PreAmp Master Mix (Applied Biosystems). The data were normalized to U6 snRNA (Applied Biosystems), and are shown as relative fold change. Results were similar when data were normalized to RNU44 or RNU6B.

Statistics

For comparative analysis of differently treated NK cells or PBMC paired two tailed t tests were performed. For calculating the statistics GraphPad Prism (version 6. GraphPad Software, San Diego California USA) was used.

Results

PLT-Ecto interaction with NK cells

In order to analyze whether PLT-Ecto physically interact with NK cells, we first labeled their surface proteins with two different amine-reactive dyes, and subsequently co-incubated them for 18 h. Using time-lapse confocal microscopy, we detected after 20 min of co-culture, short-time contacts (lasting from seconds to few minutes) between PLT-Ecto and NK cells (Fig 1a).

Figure 1

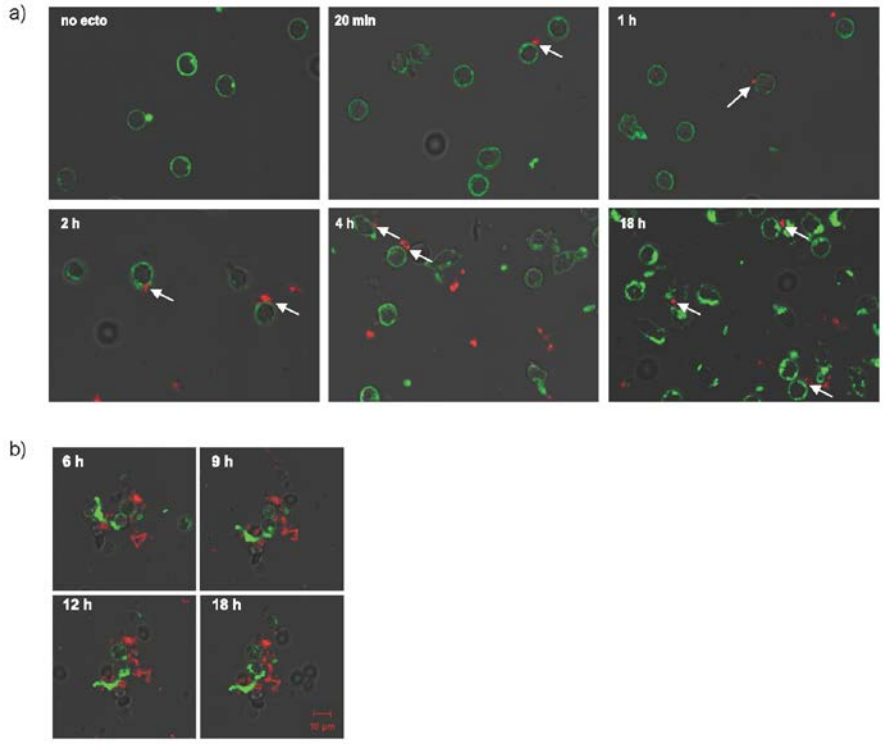

Figure 1: Interactions between PLT-Ecto and NK cells

PLT-Ecto proteins were labeled with DyLight Amine-Reactive Dye 633 (red), and NK cells with DyLight Amine-Reactive Dye 488 (green). Subsequently they were then co-incubated, and followed by time-lapse confocal microscopy for 18 h. Representative images of NK cells alone (no ecto) and NK cells (0.5 x10^6 cells/ml) + PLT-Ecto (15 µg/ml) are shown. (a) Contact between NK cells and PLT-Ecto was followed up for 18 h and different time points are shown (20 min, 1, 2, 4 and 18 h). (b) Fixed contact between the same NK cells and PLT-Ecto showed interactions for up to 12 h.

Interestingly, starting from 6 h, longer contact periods for up to 12 h could be observed (Fig 1b).

PLT-Ecto modulated NK cell surface receptor expression

We therefore investigated the effects of PLT-Ecto (30µg/ml) on NK cell surface receptors that mediate activating signals such as NKG2D, a member of the C lectin-like family, NKp30 and NKp46, members of immunoglobulin superfamily, and the DNAX accessory molecule-1 (DNAM-1). Their surface expressions were analyzed by flow cytometry after an overnight co-incubation of NK cells with PLT-Ecto (Fig 2a). This exposure induced a significant downregulation of NKG2D, NKp30 and DNAM-1, but not NKp46 (Fig 2a-b). As previously known, PLT-Ecto express TGF-β1 (Fig 2c) (17). Downregulation of NK cell surface receptors expression could partially be rescued by adding 1 µg/ml aTGF-β1 Ab (Fig 2b).

PLT-Ecto inhibited NK cell function

Since PLT-Ecto down-modulated surface receptor expression, we analyzed whether NK cell function would be impaired. Purified NK cells were pre-incubated overnight with different concentrations of PLT-Ecto. They were then washed and subsequently co-incubated with target cells for 6 h. The degranulation marker CD107a and the intracellular pro-inflammatory cytokine IFN-γ were measured by flow cytometry as NK cell activation markers. Already with 10 µg/ml PLT-Ecto, we observed a significant decrease in NK cell function represented by a loss of 18±4% CD107a and 32±7 % IFN-γ expressions (Fig 3a). This phenomenon was dose dependent, and was most evident upon exposure of NK cells to 30 µg/ml PLT-Ecto. Next, we evaluated the suppressive activity of PLT-Ecto on NK cells within PBMC, consider

Figure 2

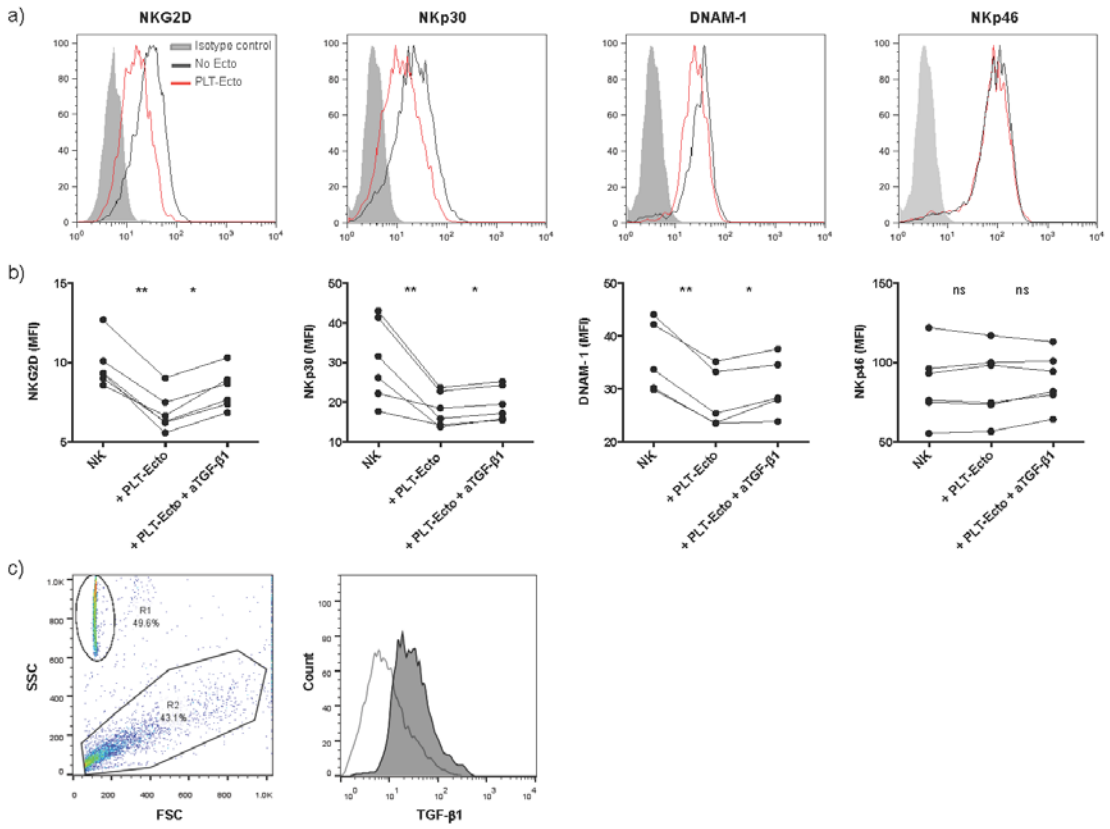

Figure 2: NK cell surface receptors expression after incubation with PLT-Ecto

(a) NK cell expression of NKG2D, NKp30, DNAM-1 and NKp46 after PLT-Ecto (30 µg/ml) overnight co-incubation was analyzed by flow cytometry. Representative flow cytometric histograms of NK cells surface receptors expression is represented by open lines in black is in absence of PLT-Ecto, in red after overnight co-incubation with PLT-Ecto. Filled histogram represents staining with isotype control Ab. (b) NK cell expression of NKG2D, NKp30, DNAM-1 and NKp46 after co-incubation with PLT-Ecto or PLT-Ecto pretreated with aTGF-β1 Ab. The mean fluorescence intensity (MFI) from 6 independent experiments is shown (*p≤0.05; **p≤0.01; ns: not-significant). (c) Representative flow cytometric dot plot of PLT-Ecto. The region R2 represents FSC/SSC light scatter gate of PLT-Ecto. The region R1 represents the known density TruCount beads. On the right, PLT-Ecto expression of TGF-β1 is represented by filled histogram. Open line represents staining with isotype control Ab.

ing that the presence of other white blood cells might modify the effect of PLT-Ecto on NK cells. However, PLT-Ecto had similar effects on functional markers of NK cells' activation in whole PBMC as assessed by membrane and intracellular expression of CD107a and IFN-γ respectively (Fig 3b). As for purified NK cells, this phenomenon was dose dependent and a maximum inhibitory effect was induced upon exposure to 30

µg/ml PLT-Ecto.

PLT-Ecto-derived TGF-β1 played a role in NK cell suppression

PLT-Ecto were pre-incubated with different concentrations of aTGF-β1 Ab (0.1, 1, 10 µg/ml) or a control Ab (10 µg/ml), added to NK cells for 20 h before functional evaluation. The addition of aTGF-β1 Ab reversed the inhibitory effect of PLT-Ecto on the NK cell activity (CD107a and IFN-γ expression) in a dose dependent manner (Fig 3c), indicating that PLT-Ecto derived TGF-β1 played the major role in NK cell suppression.

Figure 3

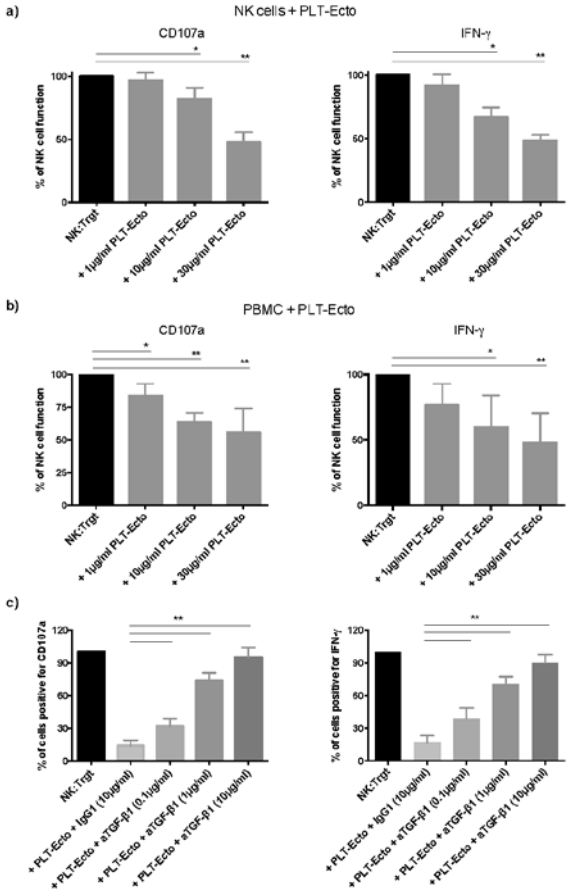

Figure 3:

After overnight exposure to 1, 10 or 30 µg/ml PLT-Ecto, NK cells were washed and co-incubated with target cells (Trgt). Surface staining for CD107a and intracellular staining for IFN-γ were analyzed by FACS. The results were normalized to 100% normal function. Results show mean and SEM of 6 independent experiments. Significance: *p≤0.05, **p≤0.01. (a) Effects of PLT-Ecto on purified NK cells. (b) Effects of PLT-Ecto on NK cells in full PBMC. (c) NK cell functions when incubated with PLT-Ecto, that were pretreated with aTGF-β1 (0.1, 1, 10 µg/ml) or isotype control (10 µg/ml) Abs. (p≤0.01 for all aTGF-β1 Ab concentrations.)

PMN-Ecto modulated NK cells

PMN-Ecto and ERY-Ecto do not express TGF-β1, however they are known to have similar down-modulating activities on macrophages as PLT-Ecto do. Therefore, we investigated whether they would affect NK cells by pathways other than TGF-β1. ERY-Ecto did not modify NK cell function using the same assays for surface receptors and cytotoxic functions (not shown). Intriguingly, PMN-Ecto modified NK cell function. Co-incubation of NK cells with 30 µg/ml PMN–Ecto significantly decreased NKG2D and NKp30 expression (Fig 4), but not NKp46 and DNAM-1 (not shown). The expression of NKG2D, NKp30 could be restored in presence of aTGF-β1 Ab. Interestingly, PMN-Ecto affected cytotoxic functions of NK cells in a dose dependent manner (Fig 5a). aTGF-β1 Ab could reverse these effects as well (Fig 5b). Suppressive effects of PMN-Ecto were also shown on NK cells within PBMC (Fig 5c). We previously have shown that PMN-Ecto have the capacity to induce TGF-β1 release by macrophages, and Ghio et al, have demonstrated that NK cells secrete TGF-β1 (20, 24). Therefore, we analyzed whether PMN-Ecto would modify NK cell TGF-β1 secretion. Indeed, incubation with PMN-Ecto increased significantly the release of TGF-β1 by NK cells (Fig 6).

Figure 4

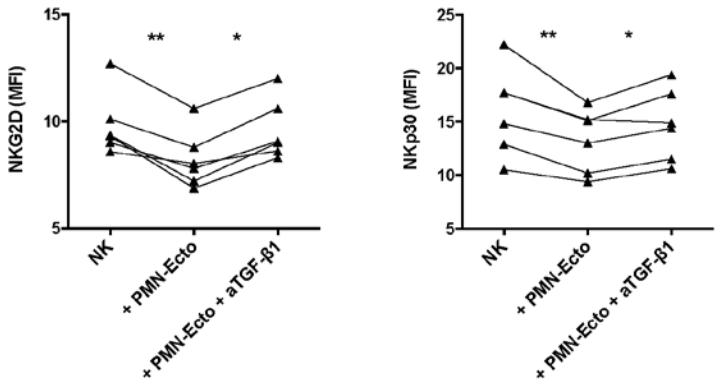

Figure 4: NK cell surface receptors expression after incubation with PMN-Ecto

NK cells' expression of NKG2D, NKp30 receptors were analyzed alone, after PMN-Ecto or PMN-Ecto + aTGF-β1 Ab co-incubations. MFI from 6 independent experiments is shown. Significance: *p≤0.05, **p≤0.01.

PLT- and PMN-Ecto modified miR-183 and DAP12

TGF-β1 has the potential to induce miR-183 expression in NK cells, which in turn decreases DAP12 (a stimulatory signaling adaptor molecule) suppressing thereby NK cells. Thus, we analyzed whether exposure to PLT- or PMN-Ecto would affect miR-183 expression in NK cells. We found that both of PLT- and PMN-Ecto increased miR-183 expression up to 10 fold in NK cells (Fig 7a-b). This increase was accompanied by a significant decrease in DAP12 surface expression (PLT-Ecto: p≤0.01 and PMN-Ecto: p≤0.01) (Fig 7c-d). NK cells co-incubated with recombinant TGF-β1 as control showed a drop in DAP12 expression. Preincubation of PLT-Ecto or PMN-Ecto with aTGF-β1 Ab restored partially the NK cell DAP12 expression (Fig 7c-d), suggesting that TGF-β1 is in part responsible for reducing the DAP12 pathway.

Figure 5

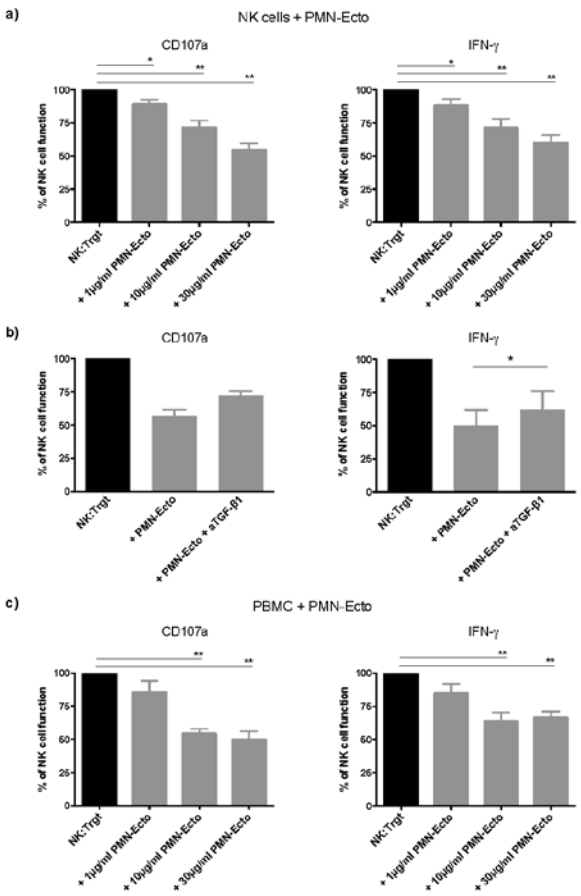

Figure 5: PMN-Ecto suppress NK cell function
After overnight exposure to 1, 10 or 30 µg/ml PMN-Ecto, NK cells were washed and co-incubated with target cells (Trgt). Surface staining for CD107a and intracellular staining for IFN-γ were analyzed by FACS. The mean and SEM of 6 independent experiments are shown; *p≤0.05, **p≤0.01. (a) Effects of PMN-Ecto on purified NK cells. (b) NK cells co-incubated with target cells alone, with 30 µg/ml PMN-Ecto or with PMN-Ecto + aTGF-β1 Ab. (c) Effects of PMN-Ecto on NK cells in full PBMC.

Discussion

In the present study, we assessed the immunomodulatory influence of PLT-Ecto on NK cells. Indeed we found that PLT-Ecto suppressed NK cell function, and reduced surface expression of activating NK cell receptors as well DAP12. TGF-β1 expressed by PLT-Ecto was mainly responsible for these effects. In parallel, we observed that NK cells themselves produced more TGF-β1 when exposed to PMN-Ecto leading to similar effects to those observed with PLT-Ecto.

Figure 6

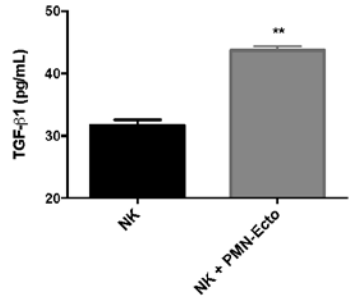

Figure 4: NK cell surface receptors expression after incubation with PMN-Ecto

NK cells' expression of NKG2D, NKp30 receptors were analyzed alone, after PMN-Ecto or PMN-Ecto + aTGF-β1 Ab co-incubations. MFI from 6 independent experiments is shown. Significance: *p≤0.05, **p≤0.01.

PLT-Ecto bound to NK cells with increasing strength over hours in vitro, but we found no evidence for their ingestion. This is different from macrophages, which phagocyte Ecto (16). Thus, for NK cells the signaling from PLT-Ecto probably occurs mainly via ectosomal surface molecules, although we cannot exclude the possibility that some molecules are released by Ecto and taken up by NK cells. PLT-Ecto released during aging tend to assemble into aggregates, which corresponds to their in vivo hemostatic function (25), and would explain the confocal microscopic images, which often showed such aggregates. It might well be that this enhanced the signaling towards NK cells.

Once NK cells have bound PLT-Ecto, there were evident changes on the expression of surface proteins involved in functional activities, i.e. indicating a down-modulation of

NK cells. However, it is worth indicating that not all markers of activations were reduced in a similar way. This suggests that there was a specific modulation of NK cells by PLT-Ecto. Co-incubation of purified NK cells with PLT-Ecto reduced their IFN-γ production and CD107 surface expression upon encountering target cells.

The likelihood of a direct contact between PLT-Ecto and NK cells in vivo might well be low considering the number of NK cells in blood. Thus, we repeated the same experiments using PBMC rather than purified NK cells. The results were similar. Indirect effects were very likely to have occurred as well. More specifically, it is known that other cells including monocytes/macrophages and T cells are modified by PLT-Ecto and may release mediators capable of influencing NK cells. One of the major mediators released immediately upon binding of Ecto to macrophages is TGF-β1. As reported by Kopp et al., TGF-β1 released in a soluble form by PLT activated by thrombin inhibits NK cell functions, and reduces the expression of NKG2D as well (26). In addition, Castriconi et al. have showed that TGF-β1 downregulates the surface expression of NKp30 and NKG2D but not that of NKp46, corresponding to our observation of no change in NKp46 expression (27). The similarities between the two observations suggest that TGF-β1, known to be expressed at the surface of PLT-Ecto, is involved in the inhibition of NK cells. This was confirmed using specific TGF-β1 Abs. Interestingly, the immunomodulatory capacity of PLT-Ecto appeared to be comparable to that of microvesicles derived from AML blasts, which have been previously shown to exert powerful immunosuppressive influence on NK cells, also by the expression of membrane-associated TGF-β1 (28). In addition, Wilson et al. showed that co-incubation of human ovarian cancer cell line with NK cells produced similar phenotypical and functional NK cell modifications to those we observed by exposing NK cells to PLT-Ecto. These changes were also mediated by TGF-β1 (29). In our subsequent analysis, we

showed that we could reverse downregulation of activating receptors NKp30, NKG2D and DNAM-1 and NK cell function when pre-incubating PLT-Ecto with aTGF-β1 Ab.

Figure 7

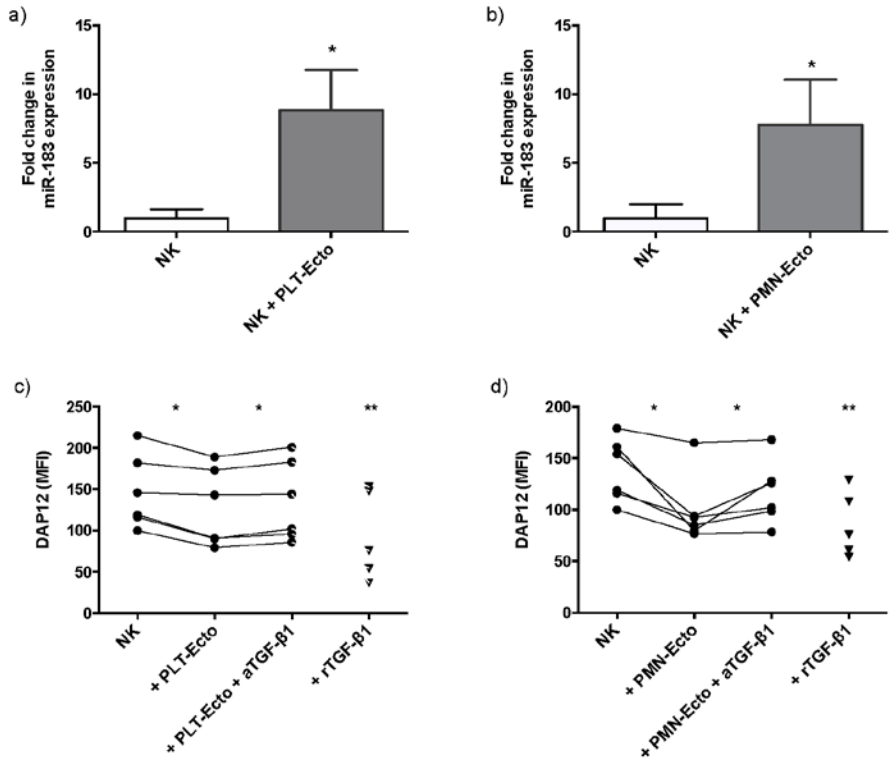

Figure 7: PLT-Ecto and PMN-Ecto modulate NK cell miR-183 and DAP12 expressions

Real time RT-PCR analysis of miR-183 in NK cells exposed to (a) PLT-Ecto and (b) PMN-Ecto. The results given are normalized to U6 and shown as relative fold changes. The mean and SEM of 5 independent experiments are shown. The surface receptor expression of DAP12 on NK cells was analyzed by flow cytometry (c) after co-incubation with PLT-Ecto or PLT-Ecto pretreated with aTGF-β1 Ab, and (d) after co-incubation with PMN-Ecto or PMN-Ecto with aTGF-β1 Ab. MFI from 6 independent experiments. The right column of each graph shows the inhibitory effect of rTGF-β1 (10 ng/ml) on DAP12 expression as control. Significance: *p≤0.05, **p≤0.01.

Recent findings showed that TGF-β1 leads to transcriptional changes in NK cells that induce the downregulation of DNAX accessory protein 12 kDa (DAP12) by inducing miR-183 (30). DAP12 is crucial for the stabilization and signal transduction of a vast amount of activating surface receptors. Among those are NKG2C, NKp44 and all activating KIR receptors (31-36). Here, we showed that TGF-β1 on the surface of PLT-Ecto was able to upregulate miR-183, which in turn downregulated DAP12, thus

reducing the receptors required for NK cell function. These observations do however not exclude other mediators from playing a role in the down-modulation of NK cells, such as Prostaglandin D2, which is abundantly produced by PLT, and inhibits NK cell cytotoxic function (37).

Interestingly, other Ecto, here PMN-Ecto, had many similar effects, which were mediated by TGF-β1 as well. Of note, TGF-β1 is not expressed by PMN and their Ecto. NK cells produce and release some TGF-β1, a release that was enhanced by PMN-Ecto so that an autocrine mechanism may well have been responsible for the downregulation of NK cells. Indeed, preliminary results showed that the activation of NK cells was reduced when the cells were exposed to aTGF-β1 Ab (data not shown). Evidently, other molecules may participate in the inhibition of NK cells. One of the major contenders would be PS, which is highly expressed by all Ecto (of PLT, PMN and ERY). The absence of a modulation of NK cells by ERY-Ecto would exclude PS involvement. Interestingly, Ghio et al. have shown that NK cells from ERY transfused patients, had a decreased killing activity at day 3 compared to before the transfusion. This downregulation was found to be associated with the length of ERY storage period. These effects were related to the presence of TGF-β1 in the ERY SN (24). In our in vitro experiments, ERY-Ecto derived from ERY concentrates did not affect NK cell surface receptor nor function since they were washed before being added onto NK cells. Thus, there was no TGF-β1 in our experimental conditions. The effect observed by Ghio et al. could be explained by an unknown source of TGF-β1 in the SN of stored ERY.

The results presented here are all in vitro, and we have no evidence that a similar inhibitory activity occurs in vivo in humans. Probably this effect is limited in vivo in most cases by the dilution of PLT-Ecto. However, at the time of allogeneic stem cell

transplantation, PLT are transfused often in large amounts exactly at the time when the transplanted patients mostly need NK cell activity to prevent post-transplant viral infections, and to exert an anti-tumoral effect (38-40). To transfuse less PLT-Ecto can be achieved by transfusing very fresh PLT (the release of Ecto increases with storage time) and reducing PLT transfusion. However, the complexity of the management of such patients should not be ignored; indeed PLT transfusion might have positive effects due to other properties not studied here (not only on preventing bleeding). Despite this, it might be of interest to study NK cell functions after massive PLT transfusions.

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Clinical implications and Future perspectives

From description to the first clinical use

After the initial discovery of NK cells in 1975, the antitumor potential of NK cells was quickly uncovered and many in-vitro studies revealed antitumor activity against various different tumor cells and cell lines with low levels of HLA class I molecules on their surface. In consequence, exploiting NK cell driven anti-tumor effects was a long term goal from the beginning of NK cell research. For decades, evidence for clinical relevance of NK cell mediated antitumor effects increased, but no clinical application was found to exploit NK cell antitumor activity in humans. [20-22,128-130] It was only in 1999, when patients receiving haploidentical allogeneic HSCT were found to benefit from increased survival, if the HLA ligands to inhibitory KIR are mismatched and NK cell tolerance is permanently broken.[95] Subsequently, donor selection criteria in haploidentical HSCT were adjusted accordingly and HLA-ligand mismatch became a recommended donor selection criterion. Whether a similar survival advantage also exists after matched HSCT, in case the donor/recipient pair lack one or more KIR-ligands is still discussed controversially.[96,100,102,103] In the present work we found that unlicensed NK cells exhibited more functional competence after matched allogeneic HSCT compared to autologous HSCT as result of less distinct NK cell licensing after allogenic HSCT. Further, we unravelled that reduced functional differences between licensed and unlicensed NK cells are associated with GVHD and/or lacking ATG administration. How NK cell licensing can be modified may be not only of prognostic value but further contribute to improve the understanding of the NK cell licensing process.

After clinically relevant NK cell driven GvL effects were shown after haploidentical inhibitory KIR ligand mismatched HSCT, exploration of adoptive NK cell transfer as antitumor therapy was intensified. Initial studies intended to ameliorate NK cell function and proliferation through cytokines including type I interferons, IL-2, IL-12, IL-

15 and IL-18.[131-133] Clinical improvement after direct administration of IL-2 to patients and ex vivo activation of autologous NK cells with IL-2 was limited. However these first studies showed that the procedure per se was safe.[133,134] Adoptive NK cell transfer from allogeneic source with and without in vitro expansion resulted in more clinical impact than autologous NK cell therapy. In contrast to autologous NK cells, allogeneic NK cells emerge in an environment free from potential tumor induced immunosuppression. Furthermore haploidentical donor selection comes with the possibility of KIR ligand mismatch and consequently increased antitumor reactivity. Apart from hematological malignancies, also solid tumors as e.g. melanoma and renal cell carcinoma become a target of NK cell therapy.[135-137] A precise understanding of the NK cell licensing process, when NK cell functional competence is determined could constitute the basis for future modulation of NK cell function during ex vivo expansion. In this regard, both our present findings concerning the varying licensing capacity between different HLA-ligands and the licensing irregularities we found after allogeneic HSCT may be fundament for studies aiming to manipulate NK cell licensing during NK cell maturation. Once the molecular basis of NK cell licensing has been unravelled, new possibilities for NK cell manipulation may arise.

While, as aforementioned, donor selection according to inhibitory KIR receptors is broadly accepted, activating KIR receptors are not considered during donor selection, neither for HSCT nor for adoptive NK cell transfer. Recent findings suggest though that activating KIR receptors are involved in leukemia cell recognition.[57] In parallel, several recent studies found evidence for activating KIR gene dependent protection from viral infections including our own study, where we present evidence for relative protection of centromeric KIR from varicella zoster reactivation/infection after SOT. Considering activating KIR in donor selection for allogeneic HSCT and adoptive NK cell transfer may

improve NK cell mediated anti-tumor effects on the one hand and reduce the risk of reactivation/infection with opportunistic viral infections. Furthermore, the activating KIR receptor profile may be considered to establish a patient specific risk profile for opportunistic infections during immunosuppression of different cause. Activating KIR based donor selection criteria may be added in future stem cell donor search algorithms. Moreover, identification of activating KIR ligands and their respective antiviral specificity may allow for adoptive antiviral NK cell therapies during immune suppression, as currently CMV specific T-cells are expanded and infused after HSCT. Despite preventive CMV specific T-cell therapy successfully reduced CMV infection/reactivation, and no GVHD has been observed, T-cell infusion comes with potential risks, while direct NK mediated GVHD has not been documented. An exception is a recent study where adoptive transfer of extensively activated NK cells contributed to GVHD possibly augmenting T-cell mediated GVHD.[138-140] Besides increasing NK cell response against specific targets, NK cell expansion may also serve the single purpose to provide sufficient numbers of effector lymphocytes while target identification is left for monoclonal antibodies. NK cells efficiently mediate antibody dependent cytotoxicity.[141] Furthermore bispecific killer cell engager (BIKE) and tri-specific killer cell engager (TRIKE) may in the future increase NK cell therapies.[142-144]

With the recently approved monoclonal antibodies (mAb) targeting the programmed cell death protein 1 (PD-1) (nivolumab and pembrolizumab) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (ipilimumab) two new classes of checkpoint inhibitors for effector T-cells have become available for clinical application. CTLA-4 is a negative regulatory receptor limiting T-cell activation. PD-1 is upregulated after T-cell stimulation and transduces an inhibitory signal upon ligation. Both show impressive clinical benefit e.g. in melanoma therapy and are currently tested in additional malignant diseases and in combination therapies.[145-148] Analogous efforts are currently undertaken to augment NK cell driven anti tumor effects. Based on the survival

advantage that comes with a broken NK cell tolerance after inhibitory KIR ligand mismatched haploidentical HSCT, lirilumab was developed. Lirilumab is a blocking antibody targeting inhibitory KIR receptors breaking NK cell tolerance for the time the inhibitory KIR receptors are blocked. In phase I trials, patient safety and persistent KIR blocking has been assessed and phase II trials are currently ongoing. A similar approach represents the development of a blocking antibody against the inhibitory NKG2A, which currently is assessed in a phase I/II study.[149-151]

In a complex therapy such as the HSCT, interactions of the involved therapies and interventions must not be underestimated. In order to optimize NK cell driven antitumor and antiviral immunity, it is of vital interest to understand treatment-related side effects on NK cell function. It was previously shown that immunosuppressive drugs frequently applied after HSCT or SOT supress NK cell function.[152] Besides immunosuppression, transfusion of cellular blood components is a cornerstone of successful post-transplant management. Foremost PLTs and ERYs are transfused in large amounts in the first months after HSCT, when the newly engrafting bone marrow cannot yet sustain sufficient cell numbers.[108,109] It was previously described that PLTs can reduce NK cell function or that elevated PLT numbers are accompanied by increased tumor growth.[116,117] We found in *in vitro* experiments that PLT not only directly interact with NK cells, but PLT ectosomes, which extensively accumulate during storage and are co-infused with each PLT transfusion, constitute a novel mechanism of PLT derived suppression of NK cell function. In parallel we found no effect of ERY ectosomes on NK cells.

There are currently many different strategies ameliorated to increase clinical benefits of NK cell-based therapies and after all a combination of different approaches may be the way to go.

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