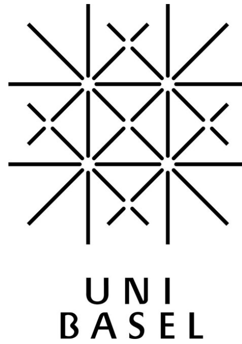

Killer Cell Immunoglobulin-like Receptors and their Ligands

Assessment of Potential Diagnostic and Immunotherapeutic Value

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie vorgelegt

der

Philosophisch-Naturwissenchaftlichen Fakultät

der Universität Basel

von

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aus Poznań, Polen

Basel, 2014

Originaldokument gespeichert auf dem Dokumentenserver der Universität Basel
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ACKNOWLEDGMENTS

I express my sincere gratitude to my supervisor Martin Stern, who gave me the opportunity to work on the fascinating subject concerning functions of NK cells. He introduced me into the field of immunology and infected with his enthusiasm.

I thank Stephan Krähenbühl, Christoph Hess and Radek Skoda for their constructive advices, comments and general support during my time at the Department of Biomedicine in Basel.

Asensio Gonzalez for teaching me, help during planning and performance of experiments and all the effort he made for me.

Laurent Schmied for interesting scientific and non-scientific discussions, and co-operation. I was always impressed by his research spirit and enthusiasm even at very late time in the Lab, Karin Schmitter for her technical support and very positive attitude.

I also thank all present and past members of the Immunotherapy Laboratory for their fruitful collaboration and nice atmosphere for four years of my scientific work.

I am grateful Emmanuel Traunecker and Toni Krebs for their support in flow cytometric analysis and cell sorting.

Laurent Brault, Pontus Lundberg, Alexey Veligodsky, Magdalena Krzyzaniak, Irena Burmann, Adrian Duek, Glenn Bantug, Thorsten Schäfer I thank for sharing with me their experience and being for me examples of successful scientists.

Danielle, Maria, Gideon, Céline, Patrick, Leyla, Anne-Valerie, Katharina, Robert, Zuzanna, Zeinab, Daniela, Kapil, Annalisa, Giacomo, Elham, Sarah, Melanie and all friends who are not mentioned here. With them my life in Basel, far away from home, was much easier and colorful. I will never forget our numerous common activities.

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LIST OF ABBREVIATIONS

aa	Amino acid
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ADCC	Antibody dependent cellular cytotoxicity
BKV	BK virus
CD	Cluster of differentiation
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CMV	Cytomegalovirus
DC	Dendritic cell
EBV	Epstein-Barr virus
ER	Endoplasmatic reticulum
GvHD	Graft versus host disease
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
HSV	Herpes-simplex virus
IL	Interleukin
IFN	Interferon
KIR	Killer-cell immunoglobulin-like receptor
KLRD	Killer cell lectin-like receptor subfamily D
LAT	Linker of activation of T-cells
LRC	Leukocyte receptor complex
LFA	Lymphocyte function-associated antigen
LGL	Large granular lymphocytes
Ly49	Killer cell lectin-like receptor
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
NK	Natural killer
NKG2A	Natural killer cell group antigen 2A

PBMC	Peripheral blood mononuclear cells
SIT	SHP-2 interacting transmembrane adaptor protein
SLP-76	SH-2 containing leukocyte protein of 76kDa
STI	Structured treatment interruption
TGF-β	Transforming growth factor β1
ULBP	UL16 binding protein
ZNRD1	Zinc ribbon domain containing 1

SUMMARY

Human natural killer (NK) cells constitute an important arm of the innate immune system, which participates in protection against viral infections and elimination of malignant diseases. They carry variegated receptors on their surface, with killer cell immunoglobulin-like receptors (KIR) playing a main role in the regulation of their activity. The KIR family involves inhibitory and activating members. The former interact with HLA class I molecules. Decreased or absent HLA class I expression abrogates inhibition. Binding specificities of activating members are mostly unknown (except for KIR2DS1 and KIR2DS4). However, there are abundant clinical data indirectly indicating a role for activating KIRs in antiviral and antileukemic NK cell activity.

Our studies aimed to dissect the role of activating KIRs in antiviral and antileukemic activity, and to assess their potential diagnostic and immunotherapeutic value. They were performed on the level of genotype associations, by evaluation of KIR repertoire after infectious challenge, and by assessing functionality in vitro.

In the first part, we searched for predictive markers allowing identification of HIV-infected patients able to control viral replication after structured treatment interruption (STI). Candidate factors were genes known to associate with HIV viral load in untreated patients. Therefore we studied the correlation of KIR3DL1 and its ligand HLA-Bw4, but also KIR3DS1 and SNPs (HLA-C-35 and HCP5) with the evolution of viral load after STI. Only the presence of HLA-Bw4 was significantly associated with control of viral replication. However, the predictive power of this genotype was modest.

In further studies we investigated differences of KIR expression in resting NK cells in CMV-seronegative and –seropositive individuals, and changes in KIR repertoire after challenging NK cells with CMV-infected fibroblasts *in vitro*. While resting NK cells did not differ in their KIR expression, *in vitro* exposure to CMV caused expansion of KIR2DL1, KIR2DL3, and KIR3DS1 uniquely in CMV seropositive donors. For KIR2DL1 and KIR2DL3, expansion occurred only in patients carrying the respective KIR ligands. The expansion of KIR3DS1 positive

NK cells confirmed participation of the telomeric part of KIR haplotype B in anti-CMV activity, but was unrelated to presence of its putative ligand - HLA-Bw4.

These results encouraged us to broader studies of the function of activating KIRs. We therefore expressed a selected panel of activating KIRs separately in the NK cell line NKL, generated soluble forms of each activating KIR by connecting their extracellular domains to IgG-Fc fragments, and using these reagents to control specificity, we established a novel staining method for KIR2DS5 using commercially available antibodies. Despite functionality of expressed receptors against a mouse cell line, the presence of the KIR for which some HLA class I ligands are known did produce no detectable enhancement of cytotoxicity in cytotoxicity experiments against 721.221 cells transfected with these HLA class I ligands. Soluble forms of KIR-Fc were used to screen a panel of leukemic cell lines for the presence of potential KIR ligands by flow cytometry. The HLA class I deficient cell line K562 bound KIR2DS3-Fc and KIR2DS5-Fc, and the HLA class I expressing cell lines HEL and Namalwa bound also both these KIR-Fc on their surfaces. The binding of the latter was blockable by anti-HLA antibodies and - based on the HLA configuration of both cell lines - was independent on HLA-C. The participation of the KIR2DS5 receptor in killing of K562 cells by primary NK cells was indirectly confirmed, but requires a further investigation.

Collectively, these data suggest a potential antiviral effect of KIR3DS1 and a possible antileukemic effect of KIR2DS5. Their presence could predict individual immune responses, and taking into account their function could be beneficial in immunotherapeutic settings.

INTRODUCTION

Overview

Natural killer cells constitute a subset of lymphocytes being able to recognize directly and eliminate virus-infected cells and malignant cells without any additional priming. Their function is governed by many receptors present on their surface amongst which the KIR family is one of the most important. These receptors are expressed on NK cells in a clonal manner and are divided into inhibitory and activating members. The main difference between them is the structure of their intracellular domain, which is long and carrying ITIM domains in the former and short, associated with DAP12 adaptor protein ITAM domains in the latter. The extracellular part of KIRs decides on their binding specificities. The inhibitory members bind HLA-C molecules and the binding preference of activating KIRs is except from KIR2DS1, sharing the specificity of its inhibitory counterpart, not known. In contrast to T-lymphocytes becoming unresponsive in the absence or downregulation of HLA class I molecules, that change activates NK cells due to abolishment of inhibition. The additional factor triggering them could be substitution in peptide repertoire in HLA-context that decreases binding affinity of inhibitory KIRs or appearance of a ligand bound by an activating KIR. The result of NK cells activation is their degranulation and release of perforin and granzyme elucidating a cytotoxic effect on neighboring target cells, but also the production of immunomodulating cytokines. Genes responsible for KIRs are grouped in haplotypes and depending on presence of only one or more activating KIRs are divided into haplotype A and B respectively. The individuals carrying the latter were shown to have advantage in terms of antiviral protection and also their bone marrow exerted better antileukemic effect if transplanted to patients. Therefore, family of KIRs and especially the activating members become a subject of numerous studies concerning their immunotherapeutic and diagnostic potential.

Natural killer cells – historical view and general characteristics

A new lymphocyte subset with particular features

Natural killer cells were discovered in 1975 as particular lymphocytes, devoid of markers typical for B- and T-cells, and able to recognize and rapidly kill mouse T-cell lymphoma cells without prior stimulation *in vitro* by unknown mechanisms [1, 2]. Research during the following decade revealed more about human NK cell features, and they were therefore classified as a distinct subset of non-adherent, non-phagocytic, large, granular lymphocytes (LGL), comprising 10-15% of circulating lymphocytes. It was noted that, unlike T-lymphocytes, they neither express T-cell receptors or co-receptors (CD3), and hence their mechanism of target cell recognition must be different. The early characterization of NK cells indicated surface markers of which Fc-gamma receptor (CD16) and neural cell-adhesion molecule (NCAM or CD56) are the most important. Nowadays for their identification the CD3(-), CD16(+) and CD56(+) antigens are mainly used. The expression levels of CD16 and CD56 differ among NK cells. They are therefore classified into two groups: CD56^{bright}CD16+ and CD56^{dim}CD16-. The former are presumably precursors of the latter, and the two subsets seem to play different roles in innate immunity. It also emerged that NK cells are able to spontaneously kill virus-infected cells, leaving uninfected cells untouched. This was demonstrated in mice, where transplantation of bone marrow from virus-resistant mouse strains to lethally irradiated virus-sensitive individuals conferred resistance against cytomegalovirus (CMV). Their antiviral role was confirmed in a human adolescent with NK cell deficiency, who was susceptible to severe viral infections [3]. Additionally, NK cells are able to kill a wide spectrum of pathogens *in vitro*. They combat gram-positive and gram-negative bacteria, *Cryptococcus neoformans*, *Trypanosoma cruzi* and *Toxoplasma gondii* [4, 5].

Introduction of the "missing-self" theory and discovery of KIR

Further investigations concerning the mode of activation of NK cells introduced the "missing-self" hypothesis. It was demonstrated using YAC-1 lymphoma cells that malignant cells with either downregulated or defective expression of MHC class I molecules are more sensitive to killing by NK cells. Similarly, F1 mice hybrids reject parental (MHC-mismatched) hematopoietic cells (hybrid resistance) [6]. This rejection is dependent on the absence of self-MHC antigens [7, 8]. In the early 1990s, research provided more information about the mechanism underlying the recognition by NK cells of "non-self" or rather of lack of "self" antigens. NK cell clones used for immunizing mice delivered a panel of antibodies directed against NK cell surface proteins. One of those antibodies (GL183) not only divided NK cells into subtypes, but also pointed to interesting functions of its cognate ligand protein. NK cells carrying that antigen, a 58kDa protein, were less efficient in killing some tumor target cells (e.g. IGROV I). Addition of the GL183 mAb to NK cells increased the cytolytic activity against a panel of human tumor cells significantly. Based on that, the molecule recognized by GL183 was considered a regulatory molecule [9]. Similarly, in mice, NK cells expressing Ly49 receptors (the accepted equivalent of the KIR family in mice) exhibited reduced killing of tumor target cells possessing H-2^d or H-2^k MHC class I molecules on their surface [10]. Soon thereafter, another protein recognized by the EB6 antibody and with similar characteristics and molecular mass as the antigen recognized by GL183 was discovered [11]. Based on the presence or absence of these two antigens, human NK cells were divided into four subsets, differing in their capability to kill a variety of tumor cell lines (e.g. U937, A549, M14 and IGROV-1). Studies of genetic factors in humans and mice demonstrated the linkage of resistance of certain tumor cell lines to NK cell killing to the MHC locus [12, 13]. It was therefore hypothesized and then confirmed that MHC class I molecules (HLA-C) are ligands for receptors recognized by the GL183 and EB6 antibodies [14]. Later, both proteins were defined as receptors providing inhibitory function. Studying heterogeneity in HLA recognition of NK cells, another receptor called NKB1 was discovered by the DX9 antibody and proved to bind ligands of the HLA-B group [15]. It became clear that receptors in both species interact with MHC class I molecules, getting educated or

licensed and drive the recognition of “self” from “non-self” antigens. The lack of their own cognate ligands during development causes anergy of KIR-expressing NK cell through a mechanism called ‘licensing’ [16]. Further investigations revealed the existence of activating counterparts [17]. Cloning of the newly discovered molecules showed their immunoglobulin-like structure, providing the reason for their name: killer cell immunoglobulin-like receptors (KIR), and also demonstrated diversity in their extra- and intracellular structure [18]. In the next few years, all KIR were identified and their coding genes, located in chromosome 19q13.4 in a region named leukocyte receptor complex (LCR) became known [19]. Similarly to HLA-haplotypes, KIR-haplotypes constituting different combinations of KIR genes were defined [20]. KIR family coexists, however, with many other receptors on the surface of NK cells, by which their function is regulated.

NK cells exhibit variegated functionality

NK cell function is regulated by signals delivered from their repertoire of inhibitory and activating receptors [21]. Moreover, NK cell response can be also modified by cytokine stimulation (IL-2, IL-12, IL-15 and IL-18), cross-talk with other immune cells, and other micro-environmental factors [22].

NK cell receptors of the C-type lectin receptor family are among the most important in regulating NK cell function. Of this group - the activating NKG2D receptor binds as a homodimer mainly stress-induced (e.g. by DNA damage or virus infection) MHC class I chain related A and B proteins (MICA and MICB) and ligands from UL-16 binding proteins group (ULBP 1-4). This allows NK cells to distinguish and lyse malignant or virus-infected cells while sparing healthy tissue [23]. The anti-tumoral role of the NKG2D was confirmed in mouse models, where the receptor was shown to be involved in rejection of tumors carrying its cognate ligands Rae1β and H60 [24]. However, many tumor cells are able to produce TGF-β and L-kynurenine or release soluble ligands, leading to decreased expression of NKG2D and inhibition of NK cell activity [25]. Three remaining receptors from the NKG group: NKG2A, NKG2C and NKG2E in contrast to NKG2D require heterodimerisation with KLRD1 (CD94) and recognize the non-classical MHC class I molecule - HLA-E in human and Qa-1^b in mice. The former inhibits NK cells, whereas the latter two have activating function, since they associate with the

DAP12 adaptor protein [26]. Among them, worth mentioning is the potential antiviral role of the NKG2C receptor, which increases in expression on NK cells in response to CMV reactivation in patients after either hematopoietic cell or solid organ transplants. The expanded population persists after clearance of virus replication as a long-lasting and self-renewing subset with high functional competence [27].

Another group of receptors present on the surface of NK cells is a family of natural cytotoxicity receptors (NCR) belonging to the Ig-superfamily. One representative of the group, the NKp44 receptor, needs stimulation of NK cells by IL-2 to be expressed and is known to bind proliferating cell nuclear antigen (PCNA) on rapidly proliferating cancer cells. Additionally, it was demonstrated that the HIV-1 envelope protein gp41 induces the expression of the recently identified mixed-lineage leukemia 5 (MLL5) protein, a ligand for NKp44 on the surface of non-infected $CD4^+$ T-lymphocytes [28]. This process causes activation of NK cells and killing of autologous $CD4^+$ T-lymphocytes, resulting in their depletion and an increase in viral load. The NKp44 receptor also binds viral hemagglutinins triggering NK cell cytotoxicity. The remaining family members (NKp30 and NKp46) are found on the surface of resting NK cells. The NKp30 receptor seems to have wide functionality. It was shown to bind HLA-B associated transcript 3 (BAT3), which is a stress factor appearing on the membrane of transformed cells or immature dendritic cells (iDC); and B7 homolog (B7-H6) expressed by K562 cells. In the case of CMV infection, the viral tegument protein pp65 binds NKp30 and allows viral escape by dissociating the CD3ζ-chain from the NKp30 complex thereby abolishing the activating signaling capacity of NKp30. Additionally, heparin sulfate proteoglycans present on tumor cells are common ligands to NKp30 and NKp46 [26, 28, 29]. The NCRs are responsible for eliminating many tumors and viral infections, since deletion of single NCR genes or functional blocking by antibodies significantly decreased NK cell activity *in vivo* and *in vitro* [30].

In contrast to NCRs, the leukocyte inhibitory receptors (LIR) family fulfill mostly inhibitory functions by interacting with the low-polymorphic α_3 domain of HLA class I molecules [31]. One of its members, LILRB2, was shown to associate with slower progression to AIDS in individuals carrying HLA-B*35, an HLA antigen preferentially presenting peptides with a tyrosine at position 8 to the LILRB2 receptor. Similarly, the weak binding of some other HLA-B subtypes (B*57 and

B*27) to LILRB2 delays AIDS progression [32, 33]. The most important finding in the interaction with CMV was that another family member, LILRB1, binds the viral protein UL18, a structural viral homolog of the MHC class I molecule with a much higher affinity, protecting the virus from an immune response [34].

NK cells possess the ability to recognize and bind Fc fragments of IgG1 antibodies coating a target cell by virtue of the FcγIIIa receptor (CD16), what leads to target cell lysis by antibody dependent cellular cytotoxicity (ADCC) [35, 36]. Since they do not possess inhibitory FcγIIb receptor, like the others Fc receptor expressing cells, they seem to be main mediators of that mode of action. Activation by antibodies was demonstrated to overcome inhibitory signals resulting in cytotoxic effect and cytokine production [37].

The DNAM-1 receptor (CD226) also belongs to the Ig-superfamily. It acts as a co-stimulatory and activating receptor, is expressed constitutively on approximately 50% of NK cells and binds Polio virus receptor (PVR or CD155) and Nectin-2 (CD112), which are expressed on some tumor cells (e.g. colorectal carcinoma) [38, 39]. The receptor has an additional role in co-stimulation of T-lymphocytes, and in cooperation with the lymphocyte function-associated antigen-1 binds intercellular adhesion molecules (ICAMs) fulfilling an important function in NK cell migration [40].

These receptors comprise the most important but do not represent all receptors present on the surface of NK cells. The variety of receptors, their different repertoires and expression levels shows, from one side, the wide ability of NK cells to detect either malignant or virus-infected cells and hence their importance as an arm of innate immune system, but also the complexity of modulation of their activity from another side. However, for the purpose of this thesis the remaining part of the introduction focuses on characteristics of the KIR family, its role in the function of NK cells and its potential clinical significance.

KIR gene structure and regulation of expression

KIR genes family and its subgroups

The human KIR gene family occupies a 100-200 kbp stretch on chromosome 19 (19q13.4) within the leukocyte receptor complex and consists of fifteen KIR gene

loci (2DL1, 2DL2/L3, 2DL4, 2DL5A, 2DL5B, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1/S1, 3DL2, 3DL3, 2DP1 and 3DP1). The last two members are pseudogenes. These genes are 14 – 16 kbp long and most possess nine exons, which separate fragments responsible for expression of different functional regions of KIR proteins [19, 41]. Aligned KIR genes differ only in single nucleotides and therefore exhibit high sequence similarity. They most probably evolved through duplications, mutations, deletions, recombination and unequal crossing-over form an ancestral, prototypical gene 3DL0, which is highly conserved in primates [42]. The KIR genes are divided into three groups [43]:

I) Type I KIR2D encode extracellular protein immunoglobulin-like domains D1 and D2 and include the following members: KIR2DP1, KIR2DL1-3 and KIR2DS1-5. All are composed of eight exons and one pseudoexon 3 sequence, which is inactive. KIR2DL1 and KIR2DL2 possess a common deletion in exon 7. KIR2DL1-3 also differ from KIR2DS1-5 in the length of exon 9 encoding the cytoplasmic tail.

II) Type II KIR2D include KIR2DL4 and KIR2DL5 containing fragments encoding D0 and D2 domains and are characterized by the presence of a translated exon 3, and deletion of the region corresponding to exon 4 in Type I KIR genes. Additionally, KIR2DL4 differs from all the other genes by having a longer exon 1.

III) KIR3D genes are comprised of 4 members: KIR3DL1-3 and KIR3DS1 possessing D0, D1 and D2 domains, and the non-coding pseudogene KIR3DP1. All of them vary in terms of length of exon 9 encoding the cytoplasmic tail. KIR3DS1 possesses a shorter exon 8 sequence than the others, whereas KIR3DL3 lacks exon 6. The pseudogene KIR3DP1 lacks exons 6-9 and in some alleles also exon 2.

The biggest difference in the length of KIR genes is notable in the fragment encoding the intracellular tail varying from 14 to 108 amino acids [18, 44, 45]. Their domain organization is schematically illustrated in Figure 1.

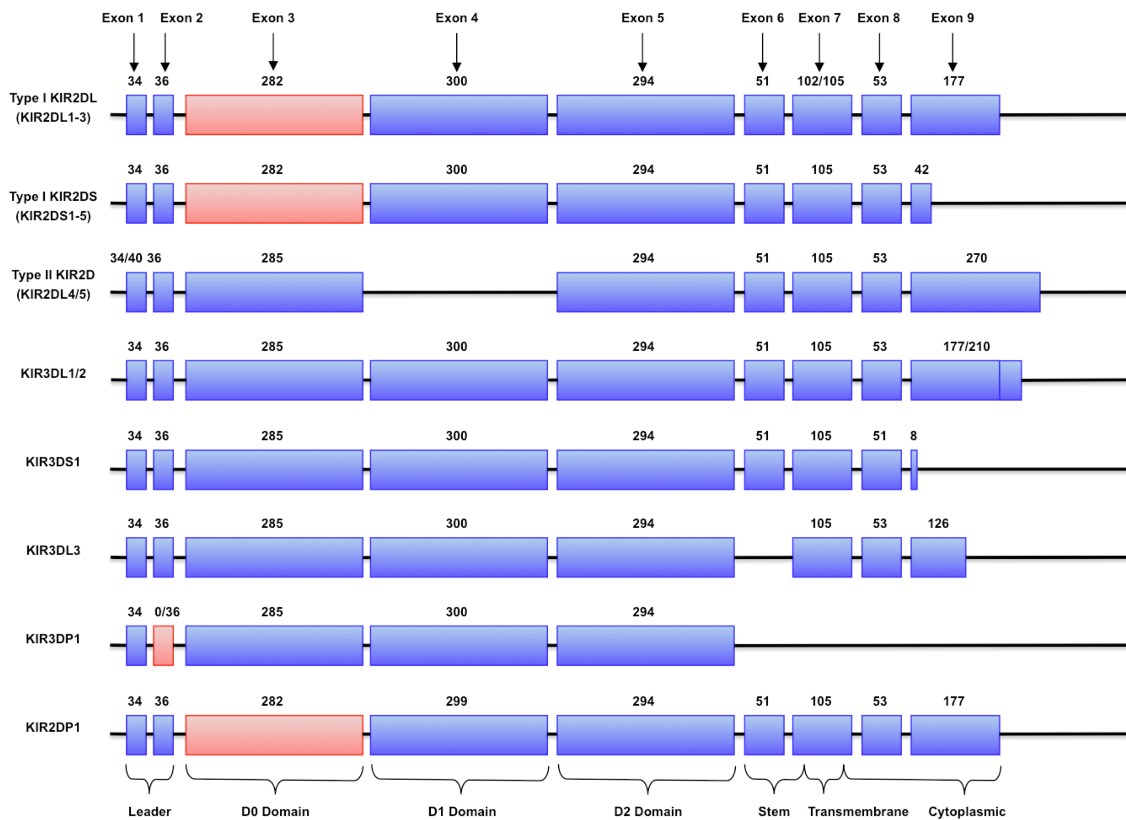

Figure 1. KIR loci structure (modified from [44]).

KIR haplotypes

KIR genes in humans are organized into at least 40 distinct haplotypes, which have been entirely sequenced [46]. Each haplotype possesses four framework genes: KIR3DL3 is located at the centromeric end, KIR3DP1 and KIR2DL4 are present in the middle and KIR3DL2 flanks the telomeric end. Between those constant framework genes there is a varying content of inhibitory and activating KIR genes; all of them arranged at distances of about 2.4 kbp from each other [41, 45, 47]. This variability is the basis for the classification of KIR haplotypes to A and B groups. The former is less variable and contains mainly inhibitory KIR genes (2DL1, 2DL3, 3DL1, 2DS4 and 2DP1), whereas the latter additionally involves activating KIR genes, which vary in number and combinations. Additionally, in these configurations KIR2DL2 and KIR2DL5 may be present. KIR2DL2/L3 and KIR3DL1/S1 occupy the same locus and constitute a kind of framework genes, because in each individual at least one from them is found. The total KIR receptor repertoire is the result of the combination of products expressed by KIR genes located in both centromeric and telomeric parts of the haplotype [44, 48].

KIR expression regulation

The expression of KIR genes is heterogeneously regulated. Gene regulation involves a complex layer of epigenetic modifications such as methylation or deacetylation [49, 50]. Further analysis of intergenic regions of KIR revealed and characterized more precisely promoters, which regulate and coordinate the KIR gene transcription:

- Distal promoters, devoid of cytosine-phospho-guanine and therefore independent on DNA methylation, possess binding sites for the transcription factor c-Myc located 1150 bases upstream from their translational sites for KIR2DL1/2DS1/2DS3/2DS5 and KIR3DL1/3DL2/3DS1 and 1190 bases upstream from the location for KIR2DL2/DS2/2DL3/3DL2. An important player inducing the c-Myc transcription factor is IL-15, which then drives distal transcription [51]. During NK cell development this process starts at CD56^{bright} stage and persists in CD56^{dim} NK cells. The acquisition of IL-15 responsiveness is therefore a key feature required for their final function [52]. The transcription from the distal promoter triggers activation of proximal promoter [51].
- Proximal promoters are about 270 bases long and include many overlapping transcriptional binding sites [53]. The function of these promoters is dependent on DNA methylation, which correlates inversely with transcription level of a particular allele. They exhibit a bidirectional activity, i.e. both sense (forward) and antisense (reverse) transcription is possible. The higher frequency of the former is proportional to the increase in gene expression. The antisense activity correlates negatively with amount of transcripts. The distal and the antisense proximal transcripts fragments overlap. That leads to production of double-stranded RNA (dsRNA), which is then processed to a small 28-base RNA product, found in NK cells to be mediator of maintenance the methylated state of the proximal promoter. As confirmation, the presence of that dsRNA transcript is correlated to silencing of KIR gene expression [51, 54].
- KIR intron 2 promoters whose antisense transcript is found in development stages of NK cells and is responsible for maintenance of DNA methylation

within the proximal promoter. It was shown to be bound by the transcription factor - myeloid zinc finger 1, which is responsible for the start of transcription [51].

Summarizing the regulation of KIR genes expression at the epigenetic level, it appears that during NK cell development the intron 2 promoter produces an antisense transcript. It blocks KIR expression by establishing the methylation of proximal KIR promoters [55]. During further development, the myeloid zinc finger 1 is downregulated, and in consequence transcription from the intron 2 promoter decreases. Upon reaching the $CD56^{bright}$ stage, NK cells become responsive to IL-15 and as a consequence the c-Myc transcription factor is bound to the distal promoter. That, in turn, starts chromatin modifications and initiates demethylation of proximal promoters, so that the KIR expression goes along with the transformation from $CD56^{bright}$ to $CD56^{dim}$ NK cells. At that stage the bidirectional transcription decides, whether sense transcript allows expression, or the antisense transcripts causes de novo methylation of proximal promoter, blocking expression (Figure 2) [51].

Besides the epigenetic regulation process, there exist other internal factors influencing the expression and transport of KIR proteins to the cellular surface. The high polymorphism of KIR exons was shown to influence their expression levels on the cell surface. Based on the binding of the DX9 antibody recognizing KIR3DL1, it was noted that there are three surface expression variants: high (*001, *002, *008, *009, *015, and *020), low (*005, *006, and *007), or null (*004). The reason for the lack of expression the *004 allele lies in amino acid substitution at positions 86 and 182 [56]. Surprisingly, absence of cell surface expression does not imply loss of functionality, as for *004 a delaying effect regarding progression to AIDS was demonstrated [57]. In analogy, KIR2DS4*003 is not expressed on the cell surface, representing a so-called “deleted” phenotype. This subtype is the most prevalent allele, making some individuals of haplotype A totally devoid of activating KIRs [58]. Similar studies concerning the influence of gene polymorphisms on the surface expression were done for KIR2DS3. This protein is always expressed intracellularly. Mutation at crucial locations causes it externalization in vitro [59].

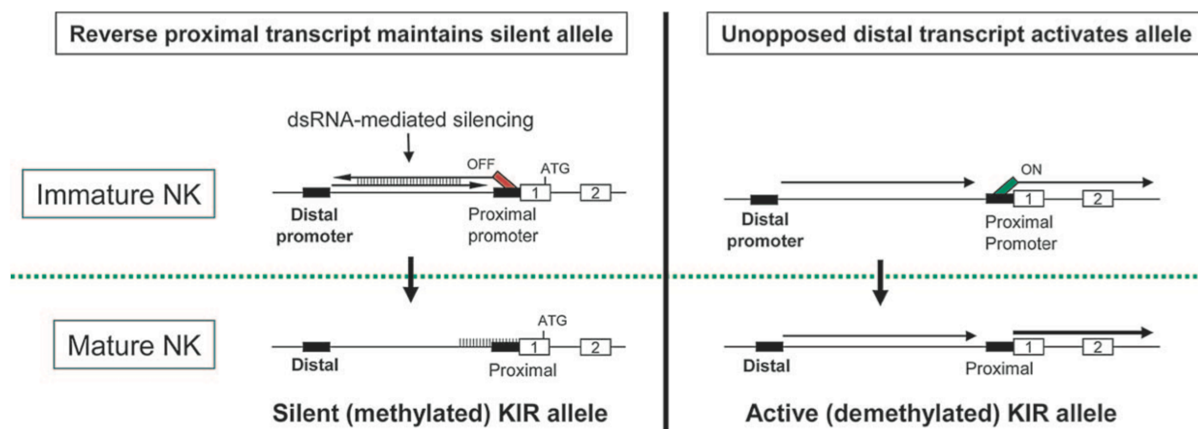

Figure 2. Regulation of KIR gene expression. Black bars indicate promoter regions and numbered rectangles indicate exons (from [60]).

Another internal factor playing a role in trafficking and surface stability of activating KIRs is the adapter protein DAP12. The interaction between immature activating KIR proteins and DAP12 starts in the endoplasmatic reticulum (ER), what not only improves the transport efficiency towards cellular membrane, but also a proper glycosylation pattern. The interaction with the adapter protein promotes correct folding of the KIR and protects its binding by factors causing internalization of the receptor [61].

KIR repertoire formation

First observations concerning Ly49 and KIR repertoire in mice and humans suggested that the probability to find an NK cell clone expressing two or more receptors is equal to the mathematical product the total frequency of cells carrying these receptors ("product rule": $P_{all\ KIR} = P_{KIR1} \times P_{KIR2} \times P_{KIR3}$ where P is equal to probability of expressing a particular KIR receptor) [62-64]. A verification of that theory and conclusions about KIR repertoire formation came initially from studies on mouse Ly49 receptors. MHC molecules were shown to influence the Ly49 repertoire. β2-microblobulin deficient (hence MHC negative) mice exhibited a higher frequency of Ly49 positive NK cells in comparison to wild-type mice. Conversely, there was less frequent co-expression of two Ly49 receptors in mice carrying their cognate ligands [65, 66]. Parham et al. confirmed the dependence of KIR repertoire on HLA configuration indicating a bias towards the optimal missing-self response [67]. A segregation of KIRs to self- and non-self in other studies demonstrates a ligand-instructed model of KIR acquisition. It was shown that in

haplotype A, the frequency of KIR2DL1 increases in homozygous individuals bearing its ligand HLA-C2, and similarly a higher frequency of KIR2DL3 is observed in HLA-C1 homozygous individuals. That difference disappears, if an individual possesses KIR2DL2, which is explained by partial overlap of specificity and function of KIR2DL2 and KIR2DL1/2DL3 [68]. The recent complex studies support dependence of KIR repertoire not only on their cognate ligands but also on other KIRs. It was stated that the presence of KIR2DL2, but also KIR2DS1 and KIR2DS2 restrict KIR2DL1 bearing NK cell subpopulation [69]. Noteworthy, the KIR repertoire is also dependent on the presence of receptors from other families. NKG2A expression was inversely correlated with expression of inhibitory KIRs [67]. Summarizing, the acquisition of the KIR repertoire is not explainable exclusively by a “product rule” and is complex process, which requires further investigation. A great help here could be knowledge of ligands for all the inhibitory and activating receptors. That would give a possibility to better predict behavior of NK cells in physiologic and pathologic states.

KIR nomenclature, structure and signaling

KIRs consist of 306 to 456 amino acids, depending on the number of extracellular domains and the length of the intracellular tail. The extracellular domain of a KIR comprises either 2 or 3 IgG-fold (like) domains, as indicated by the first digit in the respective receptor index name. As previously discussed, there exist three types of receptors having the domains D0 (96 aa), D1 (102 aa) and D2 (98 aa) in different configurations: KIR2DL1-3 and KIR2DS1-5 (D1+D2) due to lack of exon 3 encoding the D0 domain; KIR2DL4-5 (D0+D2) devoid of exon 4 encoding the D1 domain; and all KIR3D (D0+D1+D2). All KIRs share thus the D2 domain. The domains form angles between each other. Each newly biosynthesized KIR protein possesses a leader peptide, which is 21 aa long (23 aa long in case of KIR2DL4). This is then cut off during transport of the protein to the cellular membrane. The domains are located on a short stem (24 aa long with the exception of KIR3DL3 having only 7 aa) forming together the extracellular part, connected to a transmembrane part (usually 20 aa, but 19 aa for KIR2DL1 and KIR2DL2) [43, 44, 70]. The structure of this part forms the ligand recognition specificity. Since KIRs evolved in parallel with HLA molecules, they are also highly polymorphic,

especially in their extracellular part, and it was necessary to introduce a 7-digit nomenclature system similar to that used in HLA (Figure 3) [71].

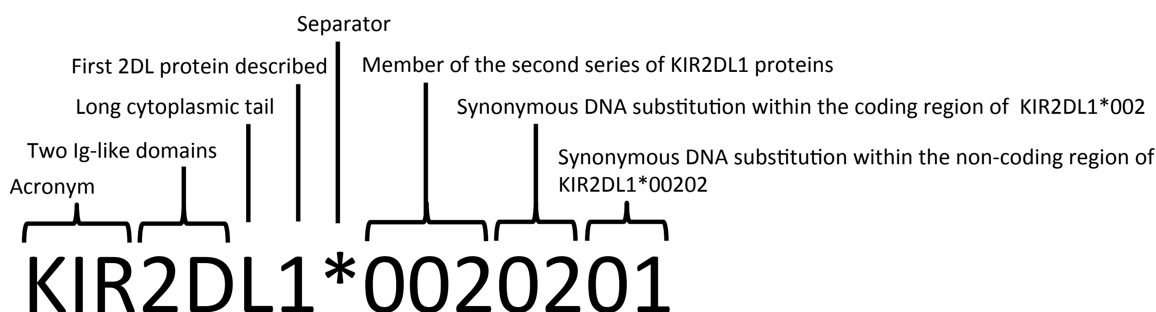

Figure 3. KIR nomenclature system (modified from [71]).

The intracellular tail of KIRs may be short (marked by an S) or long (marked by an L). The structure of this part specifies whether a KIR is activating or inhibiting. The short-tail KIRs recruit the DAP12 adaptor protein already in the ER, prior to transport to the cell membrane. DAP12 carries an immunoreceptor tyrosine-based activation motif (ITAM). The KIR-DAP12 complex, upon binding of a ligand, induces activation of protein tyrosine kinases (e.g. Syk, ZAP-70) and a further downstream phosphorylation of adaptor proteins, like LAT, SLP-76 or Shc. This results in cytoskeleton remodeling and mobilization of lytic granules containing perforin and granzymes. The long cytoplasmic tail contains two immunoreceptor tyrosine-based inhibitory motifs (ITIM) and upon ligand binding becomes phosphorylated, resulting in recruitment of SHP-1 or SHP-2 phosphatases. Both phosphatases target phosphorylated Syk-family protein tyrosine kinases, src-family protein tyrosine kinases and adaptor proteins interrupting the activating signal [72]. Therefore, the function of NK cells is regulated by the balance between activation and inhibition (Figure 4).

The KIR2DL4 constitutes an exception to this rule. This is due to presence of only one ITIM and a charged arginine residue in transmembrane part, which associates with FcεR-Iγ [73]. Engagement of that receptor, which is mostly located in endosomes, causes activation of NK cells resulting in cytokine secretion. Its ligand is a soluble form of HLA-G coming from trophoblast. The KIR2DL4 in therefore involved in remodeling of maternal vasculature in early period of pregnancy [74].

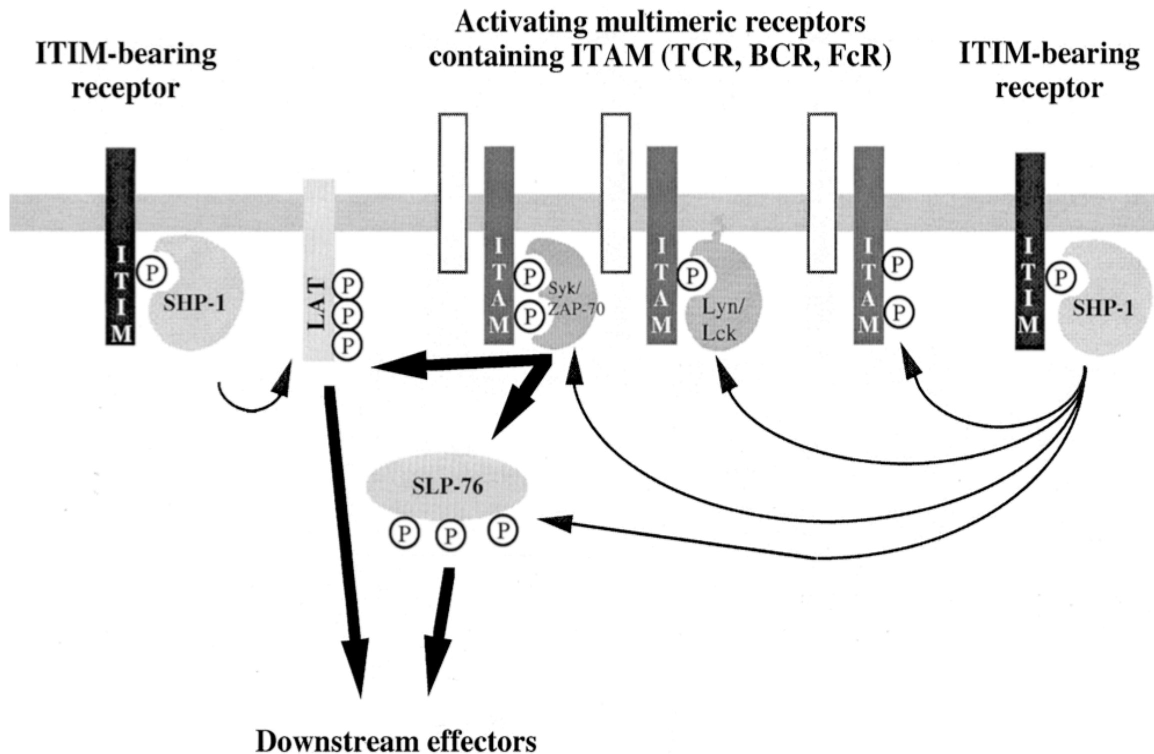

Figure 4. Coupling of inhibitory and activating signaling in NK cells (modified from [75]).

KIR ligands and function

Protection from killing by NK cells was found to be conferred by HLA class I molecules [76]. Antibody blocking of KIRs abolished the inhibitory effect and restored lysis of P815 cells transfected with HLA [14]. The interaction between KIR and HLA is based on hydrogen bonds and charge complementarity. In general, KIR2DL1 recognizes HLA-C (group 2) and KIR2DL2/3 detects HLA-C (group 1). Differential binding is based on a polymorphism at position 80 of the α1-domain in the HLA molecule, with specificities based on asparagine (group 1) or lysine (group 2) [77]. KIR2DL2/L3 shares some binding specificity with KIR2DL1, recognizing HLA-C*0501 and -*0202 and additionally HLA-B*4301 and -*7301. The binding region also covers the C-terminal end (positions 7 and 8) of peptides presented in the HLA context. By crystal structure, it was shown that there are 16 residues in KIR2DL receptors participating in the contact with α1- and α2-domains of HLA-C molecules. Differences in these residues result in changes in specificity and strength of binding to particular ligands. The activating counterparts KIR2DS1 and KIR2DS2 bind group 2 and group 1 ligands from HLA-C respectively, but with lower affinity [69, 70]. However, the peptide presented in complex also influences

the binding, and it was shown that EBV infection shifts the affinity from inhibitory receptor KIR2DL1 towards its activating counterpart [78].

The crystal structure of KIR3D receptors was not elucidated yet, but it was demonstrated that the D1 and D2 domains are responsible for binding and that the D0 domain, which is the most polymorphic, may enhance contact with a ligand or also contribute to binding [79, 80]. The D0 domain influences surface expression. The substitution of valine by leucine in position 18 enhances expression, whereas substitution of leucine by serine in position 86 abolishes the surface expression (e.g.: 3DL1*004 or 3DL1*053) [56]. The KIR3D specificity is directed towards HLA-B allotypes possessing the Bw4 epitope, which is encoded by amino acids in locations 80-83 (IALR, TALR, TLLR) in the α2-domain [70, 81]. The remaining HLA-B allotypes, like most HLA-A antigens, contain the Bw6 epitope, and do not bind to KIR3DL1. The exceptions are the following HLA-A antigens: A*23, A*24 and A*32 having also the Bw4 motif (IALR) in the mentioned location [82]. Within the Bw4 epitope, KIR3DL1 differentially binds Thr80 and Ile80, with the latter displaying a stronger interaction [83]. Additionally, the interaction between KIR3DL1 and HLA-Bw4 can be modified by the repertoire of peptides presented, as shown by the example of HLA-B*2507 [41, 84]. Despite a high structural similarity, no interaction between common KIR3DS1 alleles and HLA-Bw4 could be detected.

Further search of candidates for HLA class I ligands to KIRs led to discovery of an interaction between KIR3DL2 and HLA-A*03 and A*11 [85]. The KIR2DL4 receptor was shown to bind HLA-G molecules and play a role in maternal tolerance to trophoblast cells devoid of HLA-A and HLA-B molecules [86]. The ligands to KIR2DL5 and KIR3DL3 remain unknown. KIR2DS4 demonstrates the highest structural similarity to KIR2DL2, but does not share binding specificity. Using conjugates of the extracellular domains of KIRs with immunoglobulin Fc parts (KIR-Fc) it was demonstrated that KIR2DS4 reacts with HLA-A*1102, HLA-C*1602, -*0202, and -*0501 [87]. However, the functional consequences of this interaction were not confirmed yet *in vitro*. The ligands for KIR2DS3 and KIR2DS5 are at present unknown. All KIRs and their known ligands are summarized in Table 1.

As described before, the principal function of inhibitory KIRs seems to have been identified in protection from NK cell killing. Downregulation of the expression

of their ligands causes a malignant or virus-infected cell to become susceptible to NK cell attack. For the activating KIRs the function is more elusive. Only for KIR2DS1 more efficient killing of dendritic cells and T-cell blasts as functional confirmation of binding to HLA-C2 molecules was demonstrated [88]. The KIR2DS4 was shown to enhance killing of melanoma cell lines, but since the cell lines used did not express any HLA class I molecules, it is supposed that activating KIRs might recognize ligands outside the HLA context.

Receptor	No. of alleles	Ligand
KIR2DL1	43	HLA-C2: C*02, C*04, C*05, C*06
KIR2DL2	28	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
KIR2DL3	34	HLA-C1: C*01, C*03, C*07, C*08 Some HLA-C2: C*0501, C*0202 Some HLA-B: B*4601, B*7301
KIR2DL4	46	HLA-G
KIR2DL5A/B	41	unknown
KIR3DL1	73	HLA-B expressing Bw4 epitope Some HLA-A: A*23, A*24, A*32
KIR3DL2	84	Some HLA-A: A*03, A*11
KIR3DL3	107	unknown
KIR2DS1	15	HLA-C2: C*02, C*04, C*05, C*06
KIR2DS2	22	HLA-C1?
KIR2DS3	14	unknown
KIR2DS4	30	HLA-C: C*0501, C*1601, C*0202
KIR2DS5	16	unknown
KIR3DS1	16	unknown

Table 1. KIRs and their known ligands; number of alleles is given for April 2011 (modified from [89]).

NK cells may respond in several ways to activating signals. The release of lytic granules containing perforin and granzymes causes disruption of the cell membrane in target cells. This promotes apoptosis in target cells leading to their

death [22]. Moreover, NK cells produce chemokines and cytokines after target cell recognition, orchestrating the innate and adaptive immune response [90]. This immunoregulatory function is mainly attributed to the $CD56^{bright}$ subset of NK cells, whereas $CD56^{dim}$ NK cells exhibit predominantly cytotoxic function. IFNγ released by activated NK cells inhibits the viral replication and has immunostimuatory effects. TNF-α induces apoptosis by cell-death receptors on target cells [22, 91]. NK cells are also able to stimulate monocytes to extravasation, and induce progenitor cell development towards granulocytes by secretion of granulocyte/macrophage colony stimulating factor (GM-CSF) [92].

Interactions (or lack of them) between KIRs and their ligands determine in high extent the antiviral and antileukemic function of NK cells. There are clinical data indicating indirectly their role. In some cases these correlations, observed in humans are confirmed in mouse models.

KIR-mediated NK cell function in viral infections

Natural killer cells are able to recognize a variety of viruses with the broad spectrum of receptors they carry. The complex role of the most important receptors was characterized above. The focus of this part lies in the antiviral role of KIRs.

A role for inhibitory KIR in viral infections was demonstrated in the case of HIV infection, where the presence of KIR3DL1 with its ligand HLA-Bw4 was shown to be protective regarding progression to AIDS [93]. That is supposedly due to downregulation of HLA class I molecules by the virus, which triggers a strong "missing-self" effect [94]. However, some viruses avoid that response by expression of surrogate HLA proteins as it was shown e.g. for the mCMV m157 protein, which binds to inhibitory Ly49 receptors [95]. On the other hand, weak interactions between inhibitory KIRs and their ligands are easier to overcome by activating signals during viral infection. This was shown to be relevant in hepatitis C infections. Of the two receptor-ligand pairs: KIR2DL2-HLA-C1 and KIR2DL3-HLA-C1, the latter has a lower affinity leading to decreased inhibition, and resulting in better resolution of infection [96]. This is similar to the role of the LILRB2 receptor, whose weaker affinity to some HLA class I molecules causes delayed HIV progression. This decreases the NK cell activation threshold, which

may be additionally induced by unknown activating ligands upon infection or by change of the peptide repertoire in the HLA context, shifting NK cell signaling from inhibition towards activation. An argument supporting the latter theory could be KIR2DS1 binding to HLA-C2 ligands in complex with peptides of EBV origin on target cells [97]. Besides, some viral infections influence the expression of NK cell receptors. Murine CMV shapes the mouse Ly49 repertoire. That might suggest the antiviral role of KIRs, which are their analogues [98, 99]. The influence of human CMV on KIRs repertoire is subject of this thesis.

Little is known about an antiviral role of activating KIRs. Genetic analysis of HIV-positive patients has shown that the presence of KIR3DS1, in combination with HLA-B Bw480Ile, delays the progression to AIDS. This association and also the high structural similarity to the inhibitory counterpart suggest a receptor-ligand relation between these two molecules [100]. There are limited data suggesting that aKIRs contribute significantly to the control of CMV infection. Studies in HSCT recipients clearly show the effect that the aKIR genotype has on decreased CMV reactivation incidence and, consequently, improved survival rates [101, 102]. Other studies have shown that the most protective effect against the virus reactivation in HSCT patients is achieved if donors carry either KIR2DS2 and KIR2DS4, or more than five aKIR genes [103]. This correlation supports a possible role of activating KIR in anti-CMV immunity. Our own analysis, performed in patients after kidney transplants, has shown a significant decrease in CMV reactivation in patients carrying more than one aKIR (individuals with at least one B haplotype) compared to individual homozygous for the haplotype A [104]. Remarkably, the protective effect is limited to CMV infection and not seen for EBV, HSV, or BKV. An additional analysis enabled us to map the locus of resistance to the telomeric part of the B haplotype KIR gene complex, which may contain three aKIRs: -2DS1, 2DS5, and 3DS1 [105]. Unlike the studies on HSCT patients, we were unable to confirm the role of KIR2DS2.

These data suggest the variegated antiviral role of KIRs on NK cells. The deeper explanation of mechanisms and interaction between them and the infected cells could be useful to predict the individual immune response. As earlier mentioned, that is not only beneficial function of KIRs.

KIR-mediated NK cell function against leukemias

As discussed, the antitumoral role of NK cells was discovered more than 30 years ago. A correlation between increased risk of cancer with low cytotoxicity of peripheral NK cells has been shown in several studies [106-108]. Conversely, infiltration of tumors by NK cells is beneficial, especially if supported by cytokine-mediated activation [109].

The inhibitory KIRs participate in elimination of tumor and leukemic cells in accordance to ‘missing-self’ hypothesis, if they are not able to detect their cognate HLA ligand. The lack of inhibition causes triggering of their cytotoxicity. This feature is used in hematopoietic stem cell transplantation, where a mismatch between KIRs and ligands increases therapeutic efficacy [110]. Additional studies showed an impact of KIRs on the risk to develop malignant diseases. For example, in a pediatric cohort, carrying KIR2DS2 was shown to be associated with decreased risk of B-ALL. In general the higher number of activating KIRs had a statistically significant protective influence against ALL in children [111]. Another role for KIR was found regarding reduced relapse risk of AML after HLA-matched sibling transplants, if the donor for hematopoietic stem cell transplantation (HSCT) carried all three KIR2DL5A, KIR2DS1 and KIR3DS1 together [112]. A beneficial effect regarding leukemia relapse and mortality was confirmed in other studies of patients transplanted for AML, with a protective role of the telomeric part of the haplotype B [113]. In contrast, a similar analysis of AML patients receiving T-replete transplantation showed a benefit if at least one haplotype B of KIRs in unrelated donor was present [114]. Further investigation of the impact of activating KIRs more precisely pointed out the protective role of the centromeric part of the haplotype B [115]. Unfortunately, due to high linkage disequilibrium between KIR genes, identification of a single protective locus is difficult. Besides, it would require functional confirmation, which was only shown for KIR2DS1. This receptor was demonstrated to be implicated in recognition of C1/C2 and C2/C2 T-cell blasts, patients’ leukemia blasts, and myelomonocytic DC [88, 116]. The antileukemic function of the remaining activating KIRs is a subject of that thesis.

Similarly to viruses, malignant cells develop mechanisms to evade the activation of NK cells by missing-self mechanism or stimulating receptors. In the

example of melanoma the immunoediting process was shown. The initial contact of NK cells with cancer cells caused killing of the latter. However, long-term equilibrium between effector and target cells was established, and melanoma cells became NK cell resistant. This was due to increased HLA expression and also a consequence of IFNγ production by NK cells. On the other hand, IFNγ release was proportional to NK cell stimulation. Additional activation of the effector cells by IL-15 partially overrode the effect [117]. In ALL, the resistance to NK cell lysis results from lack of activating signals [118].

Importantly, the tumoral microenvironment may also influence NK cell function. For example, release of lactate from cells as a reduction product of cumulating pyruvate, and hypoxia cause impairment of NK cell function, mainly by decrease of perforin and granzyme production. Prostaglandin overproduction and secretion also produce a similar effect. Cancer cells possess an ability to release exosomes containing ligands for NKG2D, decreasing expression of that receptor by NK cells and their activity [119]. Additionally, by production of cytokines (e.g. TNFα, stromal-cell derived factor-1 - SDF-1 and CCL2) they attract myeloid-derived suppressor cells (MDSC) producing IL-10, which is anti-inflammatory cytokine and decreases production by macrophages of NK activity-triggering IL-12 [119].

These facts collectively confirm an antileukemic and antitumoral role of NK cells, and encourage further investigations to optimize their role in elimination of the malignant diseases.

NK cells in clinical applications

The capabilities of NK cells to distinguish “self” and “non-self” by KIRs encouraged clinical studies to manipulate receptor – ligand configurations in order to more efficiently combat malignant diseases. In AML or MM these differences in HLA ligands between donor and recipient resulted in lack of inhibition to subsets of transplanted NK cells missing their cognate ligands. Hence, these ‘alloreactive’ NK cells provide advantages in HSCT. Their application allows elimination of leukemic blasts, and protects from rejection and graft versus host disease [110]. The preliminary success of that therapy encouraged clinicians to inject mature KIR-mismatched NK cells to children suffering from AML, which resulted in successful

engraftment and good tolerance [120]. Furthermore, in MM patients the transfer of purified and IL-2 stimulated NK cells before autologous HSCT didn't result in graft rejection. The activated NK cells survived *in vivo* and displayed increased killing capacity of leukemic blasts *in vitro* [121]. NK cell transfer was also successfully applied after mild conditioning preventing rejection without HSCT in case of AML and other malignancies [89, 122]. The systemic toxicity of IL-2, and its role in expanding regulatory T-cells encouraged clinicians to search for an alternative. Preliminary data suggest that it might be substituted by IL-15, IL-12 or IL-18 to enhance NK cell activity [123]. The adoptive transfer of pre-activated NK cells is also applied in order to support HCST of CD34+ hematopoietic stem cells, since the development of functional KIR-expressing NK cells takes 6-8 weeks. That time period exposes a patient to a risk of relapse without alloreactive NK cell infusion [124].

Another way to provoke a 'missing-self' reaction is to apply a monoclonal antibody blocking inhibitory KIRs. Such an antibody (IPH2101) binds KIR2DL1/DL2/DL3 receptors expressed on about 50% of NK cells and is in clinical development. Its biggest advantage lies in the possibility to apply it universally, i.e. independently on KIR and HLA configuration. The phase I trials in MM patients confirmed its safety at full inhibitory KIR saturating concentration [125]. The literature data shows even better efficacy of the IPH2101 antibody if applied with chemotherapy, bortezomib or lenalidomide, due to additive therapeutic effects against MM [126]. The antileukemic application of homologous anti-Ly49 antibodies was confirmed in mice. An additional advantage of that therapy was persistence of the receptor inhibition for several weeks without interference in NK cells reactivity [127].

Monoclonal antibodies directed against antigens expressed by particular tumors induce ADCC in NK cells. One of them is rituximab, recognizing CD20 molecule on the surface of B-cells and therefore applied in therapy against CLL and non-Hodgkin lymphomas. Nowadays, another improved antibody recognizing the same antigen with higher affinity, named GA101 is in preclinical phase [128]. Studies of our group show that the antibody is able to override KIR inhibitory signal counterbalancing ADCC, especially if more than one cognate HLA ligands for KIRs are present in an individual. Similar findings emerged from studies with bispecific

antibodies CD16x33, which in AML were also able to overcome the KIR inhibitory signaling [129].

Taken together, NK cells constitute a highly valuable therapeutic tool and further optimization of their more efficient activation or abolishing their inhibitory signals is a proper way for improving outcome of leukemia patients. A more precise role of activating KIRs, from which only the function of KIR2DS1 positive NK cell clones was confirmed in clinical settings and *in vitro* [88, 113], in the elimination of leukemic cells remains still to be evaluated.

AIMS OF THE THESIS

Referring to literature data indicating antiviral and antileukemic activity of human NK cells mediated by activating killer cell immunoglobulin-like receptors, the general aim of this thesis is to dissect more precisely their biological function and evaluate their diagnostic and immunotherapeutic potential.

In the first project (Project 1), we search for factors predicting maintenance of a low viral load in HIV patients undergoing structured treatment interruption. Here, certain genes responsible for expression of KIRs and their ligands, and also single nucleotide polymorphisms linked to a qualitative or quantitative repertoire of expressed HLA molecules, which were shown to delay HIV progress in untreated patients, are taken into consideration.

Further studies (Project 2) take a closer look on the antiviral function of KIRs. We perform an assessment of their frequency in CMV-infected healthy donors and compare it to uninfected individuals. Additionally, we analyze changes in KIR receptor repertoire of both subgroups after challenging them with CMV-infected fibroblasts in functional assays and assess underlying molecular mechanism.

A broader understanding of the function of KIRs requires development of adequate research tools (Project 3). We produce soluble forms of KIRs and create NK cell lines expressing each of them separately. Moreover, we revise anti-KIR antibodies specificity and introduce a new flow cytometric sample-analysis strategy.

We use soluble forms of KIRs in studies concerning antileukemic significance of individual KIRs (Project 4). We perform binding screening of KIR-Fc to a panel of leukemic cell lines of different origin. We test binding affinity and check the blocking of binding by antibodies in order to identify potential ligands. We also try to use KIR-transduced cell lines to confirm their binding consequences. We apply the new flow cytometric sample staining strategy to assess functional consequences of activating receptor binding.

LIST OF PUBLICATIONS

This doctoral thesis is based on three publications and one manuscript:

1. Martin Stern*, **Karol Czaja***, Andri Rauch, Martin Rickenbach, Hundrych Günthard, Manuel Battegay, Jacques Fellay, Bernard Hirschel, Christoph Hess and the Swiss HIV Cohort Study Group.
   HLA-Bw4 identifies a population of HIV-infected patients with an increased capacity to control viral replication after structured treatment interruption.
   HIV Med. 2012 Nov;13(10):589-95.

2. Hojjatollah Nozad Charoudeh*, Grzegorz Terszowski*, **Karol Czaja**, Asensio Gonzalez, Karin Schmitter, Martin Stern.
   Modulation of natural killer cell KIR repertoire by cytomegalovirus infection.
   Eur J Immunol. 2013 Feb;43(2):480-7.

3. **Karol Czaja**, Aline Borer, Laurent Schmied, Grzegorz Terszowski, Martin Stern and Asensio Gonzalez.
   A comprehensive analysis of the binding of anti-KIR antibodies to activating KIRs.
   Genes and Immunity 2013 Jan;15(1):33-7.

4. **Karol Czaja***, Asensio Gonzalez*, Laurent Schmied, Jasmin Grählert, Irena Burmann, Karin Schmitter, Martin Stern
   Characterization of novel binding specificities of KIR2DS3 and KIR2DS5 to leukemic cell lines.
   Manuscript

* - equal contribution

PROJECT 1.

HLA-Bw4 identifies a population of HIV-infected patients with an increased capacity to control viral replication after structured treatment interruption

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Journal: HIV Med. 2012 Nov;13(10):589-95.

My contribution to work:
Sample preparation, participation in experimental design, performance of experiments, participation in data analysis and interpretation, participation in manuscript writing.

Abstract

After structured treatment interruption (STI) of treatment for HIV-1, a fraction of patients maintain suppressed viral loads. Prospective identification of such patients might improve HIV-1 treatment, if selected patients are offered STI.

We analyzed previously identified genetic modulators of HIV-1 disease progression on patients' ability to suppress viral replication after STI. Polymorphisms in the genes KIR3DL1/KIR3DS1, HLA-B, HCP5, and a polymorphism affecting HLA-C surface expression were analyzed in 130 Swiss HIV Cohort Study patients undergoing STI. Genotypes were correlated with viral load levels after STI.

We observed a statistically significant reduction in viral load after STI in carriers of *HLA-B* alleles containing either the Bw480Thr or the Bw480Ile epitope (mean adjusted effect on post-STI viral load: -0.82 log, p<0.001; and -1.12 log, p<0.001, respectively). No significant effects were detected for the other polymorphisms analyzed. The likelihood of being able to control HIV-1 replication using a pre-specified cutoff (viral load increase <1000 copies/ml) increased from 39% in Bw4-negative patients to 53% in patients carrying Bw4-80Thr, and to 65% in patients carrying Bw4-80Ile (p=0.02).

These data establish a significant impact of HLA-Bw4 on the control of viral replication after STI.

Introduction

Antiretroviral therapy (ART) enables long-term control of HIV-1 infection through suppression of viral replication in the majority of treated individuals. This leads to substantial immune reconstitution, significantly delays morbidity and mortality, and transforms HIV infection into a chronic disease [1]. However, ART is not curative and life-long pharmacological treatment is required, which can lead to numerous adverse effects. Depending on the drug combination applied, cardiovascular events, hepatotoxicity, neuropathy, renal dysfunction and lipodystrophy may occur [2]. Additional concerns regarding continuous ART are the induction of drug resistance, high costs, and treatment fatigue in patients. Structured treatment interruption (STI) strategies have therefore been explored in patients with viral replication suppressed under ART [3–6]. Overall, results were disappointing, with a significant proportion of patients showing rapid increases in viral load, declining CD4 T-cell counts, and an increased risk for disease progression [4, 7]. However, a subgroup of patients is able to suppress HIV replication for prolonged periods of time after STI [8]. A marker identifying such patients would be of great practical value and might renew interest in STI.

Several studies have identified genetic factors influencing the pretreatment set-point viral load and time to progression to AIDS in untreated patients: the first locus identified was human leucocyte antigen (HLA)-B [9]. The natural killer (NK) cell receptor pair killer cell immunoglobulin-like receptor 3DL1 (KIR3DL1)/KIR3DS1 followed [10]. More recently, whole-genome association studies provided the information that two single nucleotide polymorphisms, found in or close to the HLA complex, both correlate with HIV viral load in untreated individuals [11]. The first (rs9264942) is located 35 kbp upstream of *HLA-C* (HLA-C -35 C/T) and governs the level of surface expression of HLA-C [12]. The second (rs2395029) lies in the HLA complex P5 (*HCP5*) and is in strong linkage disequilibrium with HLA B*5701, the *HLA-B* allele associated with the strongest protection from disease progression. Interestingly, all of these polymorphisms are potentially associated with the function of NK cells, a subgroup of lymphocytes important in defense against viral infection: KIR3DL1 is an inhibitory receptor binding HLA-B antigens that carry the Bw4 epitope [13]. HLA-C is the ligand for

the NK cell receptors KIR2DL1, KIR2DL2, KIR2DL3 and KIR2DS1 [14]. KIR–HLA interactions are important during NK cell development, as only NK cells carrying inhibitory KIR and their HLA ligands acquire full functional competence [15]. As antiviral effects of NK cells have been shown to operate most effectively in states of low viral load [16], we hypothesized that these polymorphisms may have a role in predicting which patients are able to maintain suppressed viral load after STI. We therefore studied the association of these polymorphisms with the evolution of viral load after STI in 130 Swiss HIV Cohort Study patients.

Patients and methods

Study subjects

Patients were recruited from Swiss HIV Cohort Study participants of the Strategies for Management of Anti-Retroviral Therapy (SMART), Swiss Spanish Treatment Interruption Trial (SSITT)/2nd SSITT, and STACCATO (A Trial of CD4 Guided Treatment Interruption, Compared to Continuous Treatment, for HIV Infection) trials [3–6]. Of note, patients enrolled in SSITT/2nd SSITT treated with granulocyte macrophage colony-stimulating factor were excluded from the present analysis. All patients gave written informed consent to their study treatment and to having their data analyzed. One-hundred and thirty patients with viral load data available at set point, and before and after treatment interruption lasting at least 7 days, were included in the study. Mean pretreatment set- point viral loads were calculated, if more than one value was available within 6 months before initiation of antiretroviral treatment. If patients underwent more than one STI, only the first interruption was considered in the analysis. Demographic data for the patient population are summarized in Table 1.

Patient age	
– Median (Range)	41 (22-71)
Gender (n,%)	
–Male	87 (67)
– Female	43 (33)
Source of infection (n, %)	
– Blood products	1 (1)
– Injection drug user	13 (10)
– Heterosexual contact	57 (44)
– Men who have sex with men	55 (42)
– Other/unknown	4 (3)
Ethnicity (n, %)	
– White	110 (85)
– Black	14 (11)
– Hispano-American	2 (2)
– Asian	4 (3)
Number of treatment interruptions	
– Median (range)	2 (1-5)
Trial (n, %)	
– SITT*	76 (58)
– 2nd SSITT*	18 (14)
– SMART*	13 (10)
– STACCATO *	25 (19)

Table 1. Patient population. *SSITT (5,6), Swiss Spanish Intermittent Therapy Trial; SMART (4), Strategies for Management of Anti-Retroviral Therapy; STACCATO (3), A Trial of CD4 Guided Treatment Interruption, Compared to Continuous Treatment, for HIV Infection.

Genotyping

The *KIR* genotype was assessed using sequence-specific primer (SSP) polymerase chain reaction (PCR) [17]. Alleles of *KIR3DL1* differing in cell surface expression were discriminated by intermediate resolution allele-specific PCR into those carrying alleles with high (*h; 3DL1*001, *002, *003, *006, *008, *009, *015 and *020), low (*l; 3DL1*005 and *007), or no surface expression (3DL1*004) using a combination of PCR SSP protocols previously described [18–20]. Patients were grouped into those expressing two high expression alleles (*h/*y) and those expressing at least one low-expressing allele (*l/*x) [21]. The KIR3DL1*004 allele, which is not expressed on the cell surface, was analyzed separately.

KIR3DL1 ligands were typed using a real-time PCR method that

discriminates between the two types of HLA- Bw4, Bw4-80Thr and Bw4-80Ile [22]. The HLA-C35 single nucleotide polymorphism (SNP) (rs9264942) was typed using a pre-designed custom assay using TaqMan chemistry (Applied Biosystems, Foster City, CA). The SNP in *HCP5* (rs2395029) was typed by direct sequencing (forward primer 5′-3′ ACG ATT CTC CTC ACA CTT ACA; backward primer 5′-3′ TCT CTC CCA AAA CCA CAC TC).

Statistical analysis

Viral load data were compared using nonparametric tests (the Mann–Whitney *U*-test and the Kruskal–Wallis test). Values are reported as medians and interquartile ranges (IQRs). Correlations between variables were assessed by calculating Spearman's rho. Generalized linear models were used to test the impact of the polymorphisms on the control of viral replication in multivariate fashion. All *P*-values reported are two-sided. To account for multiple testing, we considered associations of $P \leq 0.01$ as significant.

Results

HIV viral load at set point, on and off ART

The distribution of pretreatment set-point viral loads, as well as viral loads before and after STI, is shown in Figure 1. The median pretreatment viral load was 4.73 log HIV-1 RNA copies/ml (interquartile range 4.14–5.74 copies/ml). Immediately before treatment interruption, viral load was below the detection level in the majority (71%) of patients. Viral load increased after STI to a median of 3.06 log copies/ml (IQR 1.46–4.61 log copies/ml; Fig. 1a).

The median interval between treatment interruption and viral load assessment off ART was 21 days (IQR 18–43 days). A significant correlation was found between this interval and the rise in HIV copy number (P=0.002; Fig. 1b). In addition – and as previously demonstrated [6, 23] – the pretreatment set-point viral load correlated significantly with the post-STI viral load ($P < 0.001$). The duration of STI and viral load at pretreatment set point were therefore included in multivariable analyses.

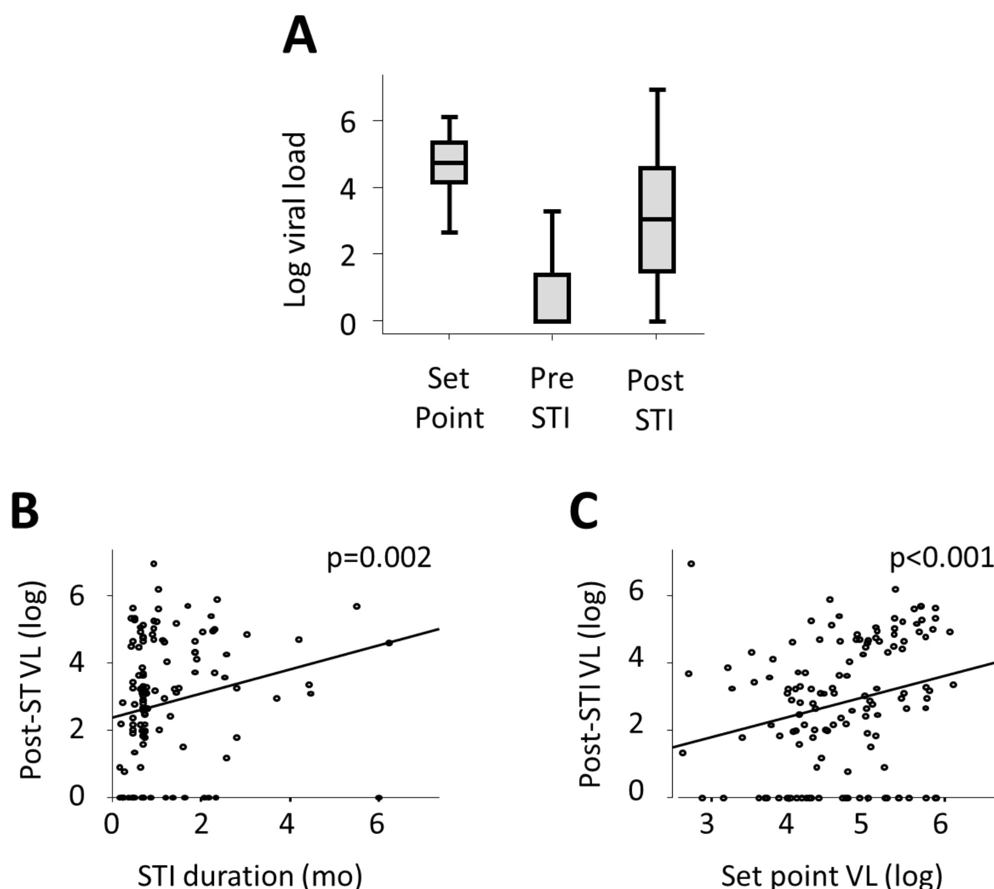

Figure 1. HIV viral load in patients at pre-treatment set point, before and after structured treatment interruption (STI). Horizontal lines represent medians, the box represents the interquartile range, and whiskers represent the 2.5 and 97.5 percentiles (panel A). A significant correlation was detected between the post-STI HIV viral load and the interval since interruption of ART (p=0.002, panel B), and between the set point viral load and the post-STI viral load (p<0.001, panel C).

Impact of genetic polymorphisms on control of viral load

Eighty-nine patients (68%) carried at least one HLA-B Bw4 allele. Bw4 alleles can be further separated into those carrying isoleucine or threonine at position 80 (Bw4-80Ile and Bw4-80Thr, respectively). Functionally, alleles with isoleucine act as strong ligands, whereas alleles carrying a threonine act as weak ligands of KIR3DL1 [24]. The former were detected in 52 patients (40%) and the latter in 37 patients (28%), whereas 41 patients carried no Bw4 alleles (32%). Patients not carrying a Bw4 allele showed a median post-STI viral load of 3.24 log copies/ml (IQR 2.21–4.29 log copies/ml), whereas the median post-STI viral load was 2.39 log copies/ml (IQR 0–3.62 log copies/ml) in Bw4- positive patients (*P* = 0.003; Fig. 2a). No difference was found between carriers of 80Thr and 80Ile subgroups of the Bw4 (median increase 2.40 and 2.39 log copies/ml, respectively; *P* = 0.66; Fig. 2b).

We next analyzed the impact of allelic diversity within the *KIR3DL1* locus in Bw4-positive patients. Of 125 *KIR3DL1*-positive patients, 84 tested positive for at least one Bw4 antigen. We found no difference between patients carrying *KIR3DL1* alleles with high (*h/*x) and low (*l/*l) surface expression (median increase 2.91 and 2.71 log copies/ml, respectively; *P* = 0.57; Fig. 2c). Equally, the presence of the KIR3DL1*004 allele – which in conjunction with Bw4 has been shown to delay the progression to AIDS – had no significant impact on post-STI viral loads (median increase 2.65 *vs*. 2.91 log copies/ml, respectively; *P* = 0.58; Fig. 2d).

The activating receptor KIR3DS1 – which segregates as an allele of *KIR3DL1* – was contained in 45 patients' genotypes (35%), of which 13 also carried Bw4Ile. The presence of KIR3DS1 with Bw4Ile has been shown to delay progression to AIDS [25]. In our setting, we found no difference in the rise in viral load between KIR3DS1+/ Bw4-80Ile+ patients (median increase 2.65 log copies/ml) and patients who did not carry either KIR3DS1 or Bw4- 80Ile or both (median increase 2.91 log copies/ml; *P* = 0.81; Fig. 2e).

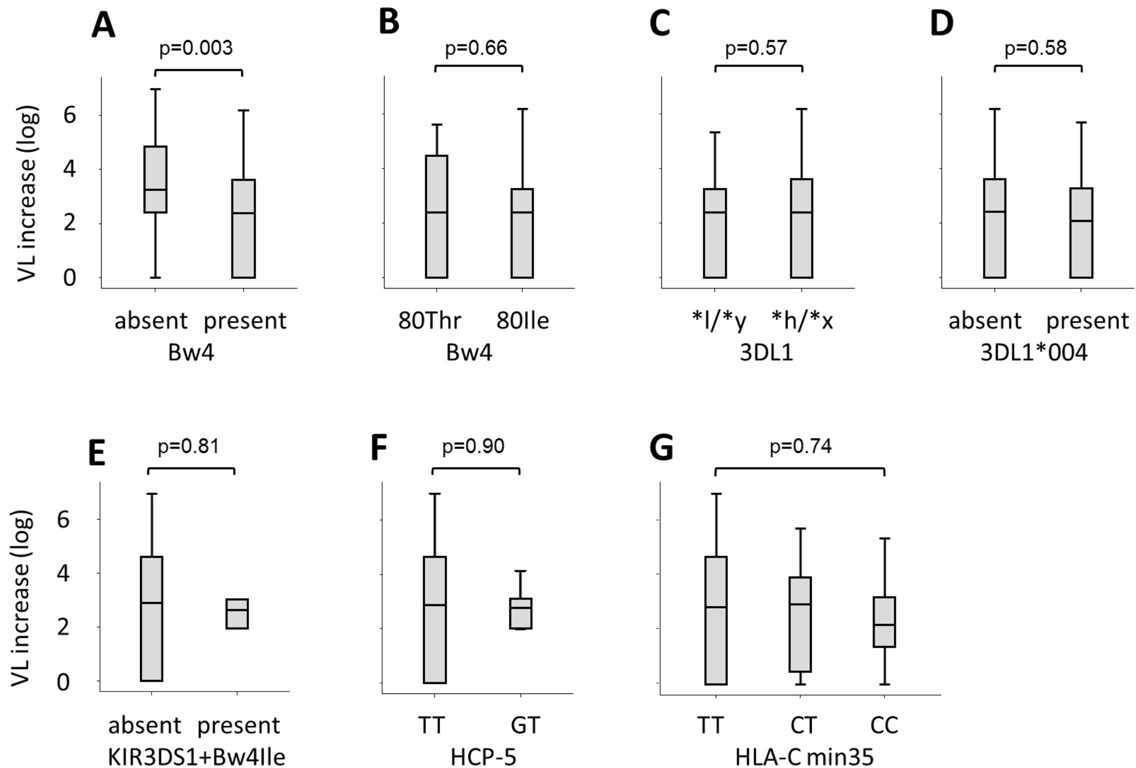

Figure 2. Post-STI log HIV viral is significantly lower in patients carrying the HLA-Bw4 epitope (panel A). Within Bw4-positive patients, no significant differences are seen between patients carrying Bw4-80Thr or Bw4-80Ile (panel B), between patients carrying low-or high surface expression alleles of KIR3DL1 (panel C), or in patients which do or do not carry the KIR3DL1*004 allele (panel D). The compound genotype KIR3DS1+/Bw4-80Ile+ does not significantly influence post-STI viral load (panel E), as so polymorphisms in HCP-5 and HLA-C min35 (panels F and G).

Finally, we analyzed the impact of the SNPs in *HCP5* and in HLA-C -35. Nine patients (7%) carried one G allele in the *HCP5* locus, and all remaining patients were homozygous for the wild-type T-allele. The median viral load was lower in patients with HCP5-G (median 2.76 log copies/ml) compared with HCP5-TT homozygous patients (median 2.85 log copies/ml). This difference was, however, not statistically significant (P = 0.90; Fig. 2f). At the HLA-C -35 locus, 79 patients (61%) were homozygous for the major T-allele and seven patients (5%) were homozygous carriers of the protective C allele, whereas the remaining 44 patients (34%) carried one copy of each allele. We found a lower post-STI viral load in homozygous carriers of the protective C allele (median 2.33 log copies/ml) compared with heterozygous patients (median 2.91 log copies/ml), and homozygous carriers of the T allele (median 2.81 log copies/ml). However, this difference did not reach statistical significance (P = 0.74; Fig. 2g).

Multivariable and categorical analysis

To account for the possibility of an interaction between variables predicting HIV viral load evolution after STI, we used multivariable generalized linear models to analyze the impact of pretreatment viral load, the duration of STI and genotype. Results are summarized in Table 2. Importantly, the protective effects of both Bw4-80Thr and Bw4-80Ile were maintained in the analyses adjusted for other covariates including time of STI and pretreatment set-point viral load.

Risk factor	Change in log post STI viral load	95% CI	P
HLA Bw4			
No Bw4 (baseline)	-	-	-
Bw4-80Thr	- 0.82	-1.27 to -0.36	<0.001
Bw4-80Ile	- 1.15	-1.61 to -0.70	<0.001
HCP-5			
Wild type (baseline)	-	-	-
Mutated	- 0.79	-1.52 to -0.06	0.04
HLA-C -35			
TT (baseline)	-	-	-
CT	- 0.02	-0.37 to +0.42	0.90
CC	- 0.12	-0.91 to +0.66	0.74
Pretreatment viral load			
Increase per log	+0.19	+0.02 to 0.35	0.03
STI duration			
Increase per day	+0.01	+0.002 to 0.014	0.006
KIR3DL1 (Bw4+ only)			
*h/*y versus *l/*x	+ 0.43	-0.05 to +0.90	0.08
*004 present versus absent	- 0.22	-0.76 to -0.11	0.41
KIR3DS1 (Bw4-80Ile+ only)			
Present versus absent	+0.00	-0.48 to +0.48	0.99

Table 2. Multivariable analysis. CI, confidence interval; HLA, human leucocyte antigen; HCP-5, HLA complex P5; KIR, killer cell immunoglobulin-like receptor; STI, structured treatment interruption.

Using a predefined cut-off of a post-STI viral load copy number of 1000 copies/ml, the frequency of patients able to control viral replication increased from 39% of Bw4- negative patients to 53% of Bw4-80Thr patients to 65% of Bw4-80Ile

patients (*P* = 0.02). None of the other polymorphisms analyzed showed any significant impact in this analysis.

Discussion

Previous studies have identified a number of genetic factors affecting viral load at diagnosis of HIV infection and the interval from seroconversion to the development of AIDS [10, 11, 26]. STI has been advocated as a therapeutic strategy in HIV-infected patients. Although a minority of patients in STI trials were able to suppress viral replication off ART, this approach has largely been abandoned, after randomized studies had shown increases in complications following STI when compared with patients treated continuously [4]. A genetic profile identifying patients with a higher likelihood of being able to suppress viral replication might point towards pathways involved in the control of viral replication and may renew interest in STI.

Our study found that an *HLA-B* allele containing the Bw4 public epitope conferred statistically significant protection regarding the rise in viral load after treatment interruption. No effect of *KIR3DL1* alleles – which act as receptors for HLA-Bw4 – on post-STI viral load was detected. This may be a consequence of the relatively small sample size or be an indication that HLA-Bw4-related effects are the results of T-cell- rather than NK-cell-mediated immunity to HIV-1. Similarly, polymorphisms in *HCP5* and in *HLA-C -35* did not significantly influence post-STI viral loads in this analysis. However, the number of patients carrying the respective protective alleles was low in this study, which may preclude a definitive appraisal. One further drawback inherent to the design of this study is that only patients requiring treatment were included, which may select against HIV ‘elite suppressors’.

Importantly, the impact of Bw4 on viral load after STI operated independently from pretreatment viral loads, indicating a prognostic power additional to that of pretreatment set-point viral load. Other factors already known to influence viral replication during and after treatment of HIV infection include viral ‘fitness’ parameters such as its sensitivity to cytokines [27], viral diversity before initiation of ART [28], and parameters of humoral immunity such as anti-p24 antibody titres [8]. Interestingly, neither the quantity nor the quality of HIV-specific

CD8 T-cell responses has previously been found to be predictive for the ability to control HIV replication after STI [23, 29].

In conclusion, we show that the presence of HLA-Bw4 significantly impacts on the control of viral load after STI during chronic HIV infection. Whether the increased capacity to suppress HIV-1 replication associated with HLA-Bw4 warrants reappraisal of STI as a treatment option in selected patient populations depends on the findings of future studies.

Acknowledgements

We thank the patients for participating in the SHCS and in these treatment interruption trials, the nurses and physicians at the various SHCS centres for excellent patient care, the SHCS data centre in Lausanne for data management and Marie Christine Francioli for administrative assistance.

This study was financed in the framework of the Swiss HIV Cohort Study, supported by the Swiss National Science Foundation (SNF grant #33CS30-134277). M.S and C.H. were supported by the Swiss National Science Foundation (grants # PP00P3_128461/1 and 31003A_135677, respectively).

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PROJECT 2.

Modulation of the natural killer cell KIR repertoire by cytomegalovirus infection

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Journal: Eur J Immunol. 2013 Feb;43(2):480-7.

My contribution to work:

Sample preparation, participation in experimental design and performance of experiments, participation in data analysis and interpretation, participation in manuscript writing.

Abstract

Patients carrying activating killer cell immunoglobulin-like receptor (KIR) genes are significantly protected from cytomegalovirus (CMV)-associated complications after solid organ or hematopoietic stem cell transplantation. Whether previous infection with CMV affects natural killer (NK)-cell function in healthy donors is unknown.

We studied the KIR repertoire and alterations of KIR expression after in vitro exposure to CMV in 54 healthy donors. The expression of neither activating nor inhibitory KIRs was different at baseline between 23 seropositive and 31 seronegative donors. However, after co-culture of NK-cells with CMV-infected fibroblast cells, expression of the inhibitory receptors KIR2DL1 and KIR2DL3 and the activating receptor KIR3DS1 significantly increased in CMV-seropositive donors. In CMV-seronegative donors, changes were subtle and restricted to the subset of NK-cells expressing NKG2C. Expansion of inhibitory KIRs occurred exclusively in donors carrying the cognate HLA class I ligands, whereas the presence of the putative ligand HLA-Bw4 was not necessary for the expansion of KIR3DS1-expressing NK-cells.

Our data show that previous infection with CMV does not alter the resting NK-cell receptor repertoire, but appears to modify how NK-cells respond to re-exposure to CMV in vitro.

Introduction

NK cells are an important component of the immune system in the control of viral infection [1]. Unlike B and T cells, NK cells do not display rearranged receptors but instead are regulated by the integration of signaling from germline encoded activating and inhibitory receptors. One important and incompletely characterized family of receptors are the killer cell immunoglobulin-like receptors (KIRs) [2]. KIRs are almost exclusively expressed on NK cells and encoded by 15 different gene loci, nine inhibitory iKIRs, and six activating aKIRs. The KIR genes cluster in chromosome 19, forming haplotypes composed of 7–11 individual KIR genes. The most common haplotype in Caucasians contains mostly iKIRs accompanied by a single or no aKIR gene and is called “A” haplotype [3]. Haplotypes containing more than one aKIR gene are collectively termed “B” haplotypes. Most iKIRs recognize HLA class I ligands and function as important receptors in the maintenance of NK-cell self-tolerance. In contrast, neither the ligands nor the function of most aKIRs have been established [4].

We have recently shown in patients undergoing solid organ transplantation a protective effect of B haplotype genes regarding posttransplant CMV infection and reactivation [5, 6]. Similar studies have shown congruent results for donor activating KIR genotype in recipients of hematopoietic stem cell transplantation [7, 8]. These data suggest that NK cells might recognize CMV-infected cells via activating KIR receptors.

Primary CMV infection most frequently occurs subclinically, and no studies have so far studied NK cells during primary CMV infection. However, recent evidence suggests that murine NK cells may display immunological memory comparable to that of B and T lymphocytes [9, 10]. In mice infected with murine CMV, the repertoire of Ly49 (the murine homologue of KIR) on NK cells stays permanently altered [11]. The potential for CMV to modulate NK-cell surface receptors is underlined by the fact that in humans, latent CMV infection has been shown to induce permanent up-regulation of the activating NK-cell receptor natural killer cell group antigen 2C (NKG2C) [12–14]. Collectively, these data suggest that latent CMV infection might lead to changes in the KIR repertoire of NK cells or might alter the NK-cell response to CMV in vitro.

We therefore assessed in a cohort of healthy donors the expression of inhibitory and activating KIR receptors. KIR repertoire was assessed both in freshly collected NK cells as well as after co-culture with a CMV-infected fibroblast cell line.

Materials and methods

Healthy donor buffy coats and sera were collected under an ethical committee approved protocol after written informed consent from all study participants. PBMCs were extracted by using Ficoll. IgG antibodies as a sign of previous infection with CMV were detected using a commercially available assay (Architect CMV IgG, Abbott). DNA was extracted from an aliquot of cells by NucleoSpin DNA Extraction Kit (Macherey-Nagel, Düren, Germany), and stored at −20°C until use. The remaining mononuclear cells were cryopreserved until use as described below.

Antibodies and flow cytometry

mAbs used to stain cell-surface and intracellular Ags were: CD3 (OKT3, eBioscience), CD56 (HCD56, BioLegend), KIR2DL1 (143211, R&D), KIR3DL1 (DX9, Miltenyi), KIR2DL3 (180701, R&D), KIR2DL1/DS1 (HP-MA4, BioLegend), KIR3DL1/S1 (Z27.3.7, Beckman Coulter), NKG2A (Z199.1, Beckman Coulter), NKG2C (134591, R&D Systems), KIR2DS4 (JJC11.6, Miltenyi), KIR2DL5 (UP-R, BioLegend), KIR2DL2/S2/L3 (DX27, Miltenyi), Ki-67 (20Raj1, eBioscience), CD107a (H4A3, BD- Pharmingen), and IFN-γ (B27, BD Pharmingen). Samples were acquired on a DAKO CyAn ADP nine-color flow cytometer (Beckman Coulter). For all analyses of NK-cell subsets, we gated on the CD56+/CD3- subset. FACS plots were analyzed with FlowJo software version 9.2. Propidium iodide (BD Pharmingen) was used to exclude dead cells from the analysis.

CMV co-culture

Healthy donor PBMCs (0.2×10^6) were cultured in the presence of 5000 MRC-5 fetal human lung fibroblast cells (kindly provided by H. Hirsch, Basel) on 96-well

plates in 200 μL of DMEM plus L-glutamine, 1 mg/mL D-glucose and pyruvate (GIBCO), 10% FCS (Sigma-Aldrich), and 1000 U penicillin/streptomycin (GIBCO). Cells were cultured at 37°C for 14–21 days, and half of the co- culture medium was replaced weekly. At indicated days, cells were harvested and analyzed by FACS for analysis of KIR and NKG2A expression. The MRC-5 cell line was infected with a WT strain of CMV (kindly provided by H. H. Hirsch, Basel) the day before culture and also weekly during the changing of culture medium. Co-culture with uninfected MRC-5 was used as a negative control. Successful infection of MRC-5 cells by CMV was assessed in control cultures demonstrating cytopathic effects.

Genotyping and quantitative PCR

KIR genotype was assessed using sequence-specific primer PCR [25]. Known ligands for inhibitory KIR were typed by PCR-sequence-specific primer (HLA-C1 group versus HLA-C2 group), and by using a real-time PCR method that detects HLA-Bw4 ligands [26, 27]. For quantitative PCR, NK cells were purified from PBMCs using the NK-Cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). RNA was extracted from purified NK cells (NucleoSpin RNAII, Macherey-Nagel) and reverse transcribed by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol. The real-time PCRs were performed by Applied Biosystems 7500 Real-Time PCR System in 10 μL reaction mixture volumes containing 1× Power SYBR Green I Master Mix (Applied Biosystems, Warrington, UK), 0.3 μM of KIR-specific primers [25] or 0.3 μM housekeeping gene (GAPDH) (Forward: 5'-GAC CCC TTC ATT GAC CTC AAC TAC A-3') Reverse: 5'-CTA AGC AGT TGG TGG TGC AGG-3') and 1 μL of postreverse-transcription mixture. PCR cycling conditions were set to 2 min at 50°C and 10 min at 95°C followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. The melting curve stage was added to the program in order to control samples' quality.

Statistics

Resting KIR repertoire expression was compared between CMV-seropositive and -

seronegative donors by unpaired *t*-test. KIR expression after CMV co-culture was compared by paired *t*-test in samples exposed to CMV versus cells from the same donor cultured in the absence of CMV. All *p*-values presented are two-sided and were considered significant if < 0.05.

Results

NK-cell inhibitory and activating KIR repertoire is not altered by CMV infection

Fifty-four healthy donors were genotyped for the non-framework genes 2DL1, 2DL2, 2DL3, 2DL5, 3DL1, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, and 3DS1. KIR gene frequencies were comparable in 23 CMV-seropositive and 31 seronegative donors and within the range of published prevalences for Caucasian donors (data not shown). The expression of cell surface inhibitory (2DL1/CD158a, 2DL2/3/CD158b, 2DL5/CD158f, 3DL1/CD158e1) and activating (2DS1/CD158h, 2DS4/CD158i, 3DS1/CD158e2) KIRs by flow cytometry was equally comparable between CMV-seronegative and CMV-seropositive patients (Supporting Information Fig. 1A–E, H and J). No antibodies are available against KIR2DS3 and KIR2DS5, and all antibodies that detect KIR2DS2 cross-react with the inhibitory isoform KIR2DL2. We therefore used quantitative PCR to compare the expression of these receptors in purified NK cells from CMV-seropositive and -seronegative donors. Again, no significant differences were detected between CMV-seropositive and CMV-seronegative donors for KIR2DS2, KIR2DS3, or KIR2DS5 (Supporting Information Fig. 1F, G and I).

Previous data demonstrated the expansion of NK cells expressing the activating receptor NKG2C in CMV-seropositive donors [13]. To assess whether NK cells expressing activating KIR receptors are enriched within the NKG2C subset, we analyzed co-expression of KIR2DS1 and KIR3DS1 with NKG2C on NK cells. In agreement with previous studies, we found higher expression of NKG2C in seropositive donors. However, co-expression of NKG2C with activating KIR2DS1 and KIR3DS1 was not different in CMV-seropositive or -seronegative donors (data not shown). Collectively, these data show that the resting NK-cell KIR repertoire is not modulated by previous CMV infection.

NK cells expressing KIR2DL1, 2DL3, and 3DS1 expand after CMV co-culture in CMV-seropositive donors

We next assessed how NK-cell subsets respond to in vitro exposure to CMV using a co-culture model using the fibroblast line MRC-5 (which supports CMV replication in vitro and carries all relevant ligands to inhibitory KIRs, that is, HLA groups C1, C2, and Bw4) in the presence or absence of CMV. In both CMV-seropositive and CMV-seronegative donors, the frequency of NK cells within the PBMC population increased during CMV co-culture (day 0: 8 and 6%, day 21: 17 and 20%, respectively, for seropositive and seronegative donors). Compared with non-infected MRC-5, co-culture with CMV-infected MRC-5 induced specific changes in the KIR repertoire (Fig. 1). KIR repertoire changes on the total NK-cell population were exclusively detected in CMV-seropositive donors. The frequency of NK cells expressing the inhibitory receptors KIR2DL1, KIR2DL2/3, and natural killer cell group antigen 2A (NKG2A) increased significantly in PBMCs co-cultured with CMV-infected MRC-5 cells (Fig. 1A, B, and D), if NK cells were derived from a donor carrying anti-CMV-IgG antibodies. No expansion of KIR3DL1 was observed (Fig. 1C). Strikingly, no expansion of KIR2DL1 and KIR2DL2/3 expressing NK cells occurred in CMV-seronegative donors upon co-culture on CMV-infected MRC-5. Of the activating receptors studied, we found no significant change in the expression of KIR2DS1 (Fig. 1E), whereas the frequency of KIR3DS1-expressing NK cells increased significantly after co-culture with CMV-infected MRC-5 (Fig. 1F). This was exclusively observed if the donor had previously undergone CMV infection. Importantly, both in CMV-seropositive and CMV-seronegative donors, NK cells were polyclonal after co-culture, as evidenced by a variegated pattern of KIR and NKG2A expression. In CMV-seronegative donors, the only alteration induced by CMV infection was an increase in the expression of NKG2A by day 21.

As NKG2C expression has previously been shown to be up-regulated in patients during and after CMV replication [13,15,16], we assessed total NKG2C expression and KIR expression on NKG2C+ cells before and after 14-day culture, as a more sensitive assay directly investigating putative CMV-specific NK cells. NKG2C expression was nonsignificantly elevated in CMV-seropositive donors compared with that in seronegative donors at baseline. After culture, we detected

a significant increase in NK-cell NKG2C expression, if PBMCs were cultured on infected compared with culture on non-infected MRC-5, which was restricted to CMV-seropositive patients (Fig. 2A). Confirming results obtained on total NK cells, expression of KIR2DL1 but not of KIR3DL1 increased on NKG2C+ cells (Fig. 2C and D). Interestingly, a small but statistically significant increase in KIR2DL1 on NKG2C+ was detected also in CMV-seronegative donors; however, this increase was much smaller than that seen in CMV-seropositive donors (Fig. 2C).

To discriminate between expression of KIR2DL2/S2 and KIR2DL3, we next cultured PBMCs from donors carrying the genes for all three receptors. Co-staining of a KIR2DL3 specific Ab with an Ab recognizing KIR2DL2/S2/L3 allowed us to distinguish between expression of KIR2DL2/S2 and KIR2DL3 (Fig. 3A). In five CMV-seropositive donors, strong expansion of KIR2DL3-expressing NK cells was documented, while co-culture with CMV-infected fibroblasts had no impact on the expression of KIR2DL2/S2 (Fig. 3B and C).

To address whether the increased expression of KIR-expressing cells represents true expansion, we determined cell number weekly during the 21-day co-culture with MRC-5 in the presence or absence of CMV. The NK-cell number contracted during the first week, followed by an expansion of NK cells exclusively in seropositive donors in the presence of CMV (Supporting Information Fig. 2). Staining for the proliferation marker Ki-67 corroborated these results: infection of MRC-5 with CMV led to a massive up-regulation of Ki-67 on NK cells if these stemmed from CMV-seropositive donors (Fig. 2B). Interestingly, when the KIR repertoire was assessed on Ki-67+ cells, we noted expansion of KIR2DL1/Ki-67 double positive but not of KIR3DL1/Ki-67 double positive cells after co-culture with CMV-infected MRC-5 (Fig. 2E and F).

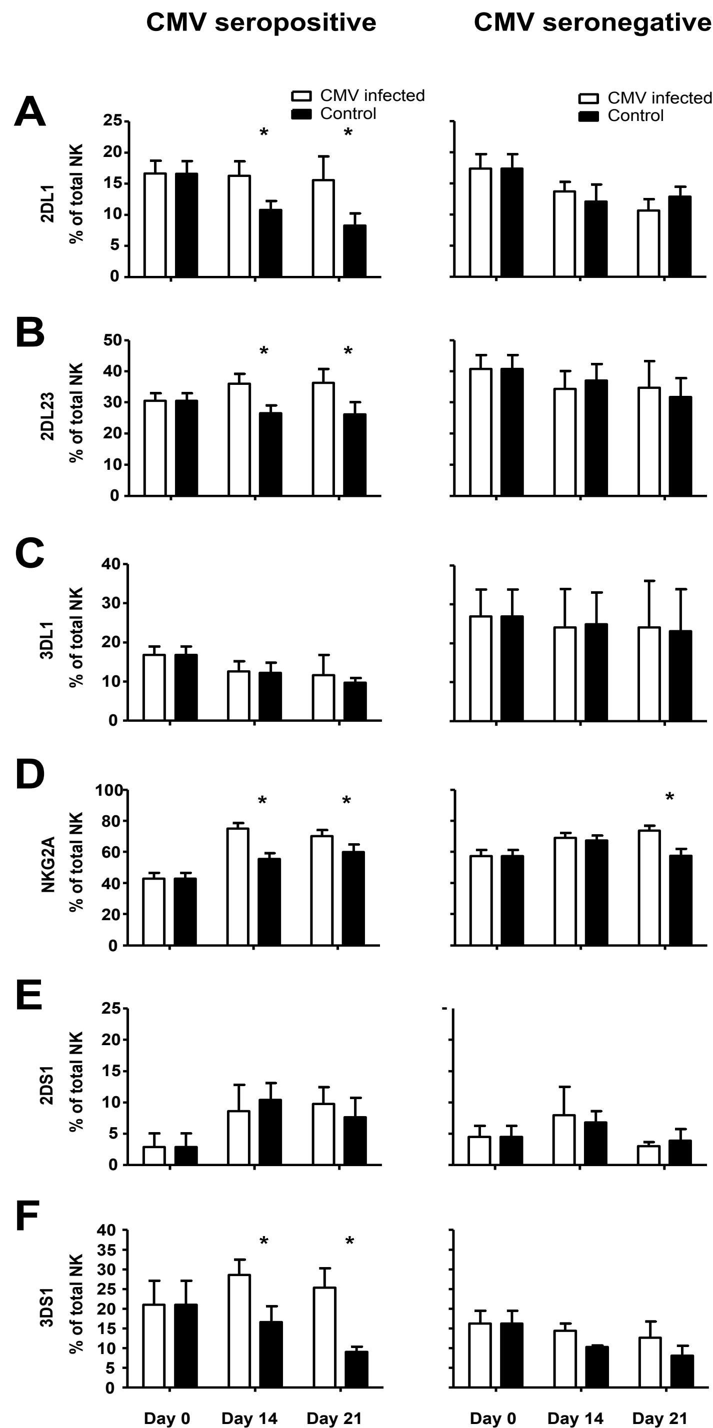


Figure 1. Changes to the NK-cell KIR repertoire after co-culture with CMV-infected fibroblasts. PBMCs from CMV-seropositive or negative donors were co-cultured for 21 days with CMV-infected fibroblasts and the percentage of (A) KIR2DL1-, (B) KIR2DL2/3-, (C) KIR3DL1-, (D) NKG2A-, (E) KIR2DS1- and (F) KIR3DS1-expressing cells within the total NK-cell population were determined by gating on $CD56^{+}/CD3^{-}$ cells and then for individual KIRs. Data are shown as mean + SEM of 24 donors pooled from 6 experiments. Comparison between groups was made by Student's T-test, * $p < 0.05$.

Expansion of inhibitory KIR-expressing NK cells is dependent on the presence of KIR ligands

We next aimed to characterize factors influencing the expansion of KIR-expressing NK cells. HLA-C1 group Ags are the ligand for KIR2DL2/S2/L3, while HLA-C2 group Ags are the ligands to KIR2DL1 [17]. If CMV-seropositive donors were stratified according to their KIR ligand status, an expansion of KIR2D-expressing NK cells occurred only in the presence of the cognate KIR ligand: KIR2DL1 expanded only in donors carrying a C2 ligand (Fig. 4A and B), whereas KIR2DL2/S2/L3 NK cells expanded exclusively in the presence of the cognate group C1 ligand (Fig. 4C and D).

While no ligand has been identified for the activating KIR receptor KIR3DS1 [18], genetic association studies have suggested an epistatic interaction of KIR3DS1 with HLA-Bw4 in HIV infection [19]. Analysis of Bw4-status in conjunction with KIR3DS1 expression in our population showed that expansion of KIR3DS1 occurred irrespective of the presence of Bw4 (day 21 KIRDS1 expression in CMV-exposed versus CMV non-exposed cells in seropositive donors: mean 23 versus 8% in Bw4-negative, and 31 versus 11% in Bw4-positive donors, $p < 0.05$ for both comparisons).

Increase in inhibitory KIR expression is due to expansion of KIR+ cells

To address the question, whether the increase in KIR expression induced by exposure to CMV was due to expansion of NK cells expressing KIRs at the initiation of co-culture or due to induction of KIRs in initially KIR- NK cells, we depleted PBMCs of NK cells expressing either KIR2DL1 or KIR2DL3. After co-culture with CMV-infected MRC-5, NK cells remained negative for KIR2DL1 and KIR2DL3, demonstrating that the increase in expression of the respective KIR was most likely due to expansion of KIR+ NK cells rather than induction of KIR expression in KIR- NK cells (data not shown). As KIR3DS1 expression is detectable only barely above background staining on primary NK cells [20], flow cytometric sorting of KIR3DS1+ from KIR3DS1- cells was not possible, and formal proof that the increase in KIR3DS1 detected after exposure to CMV is still lacking.

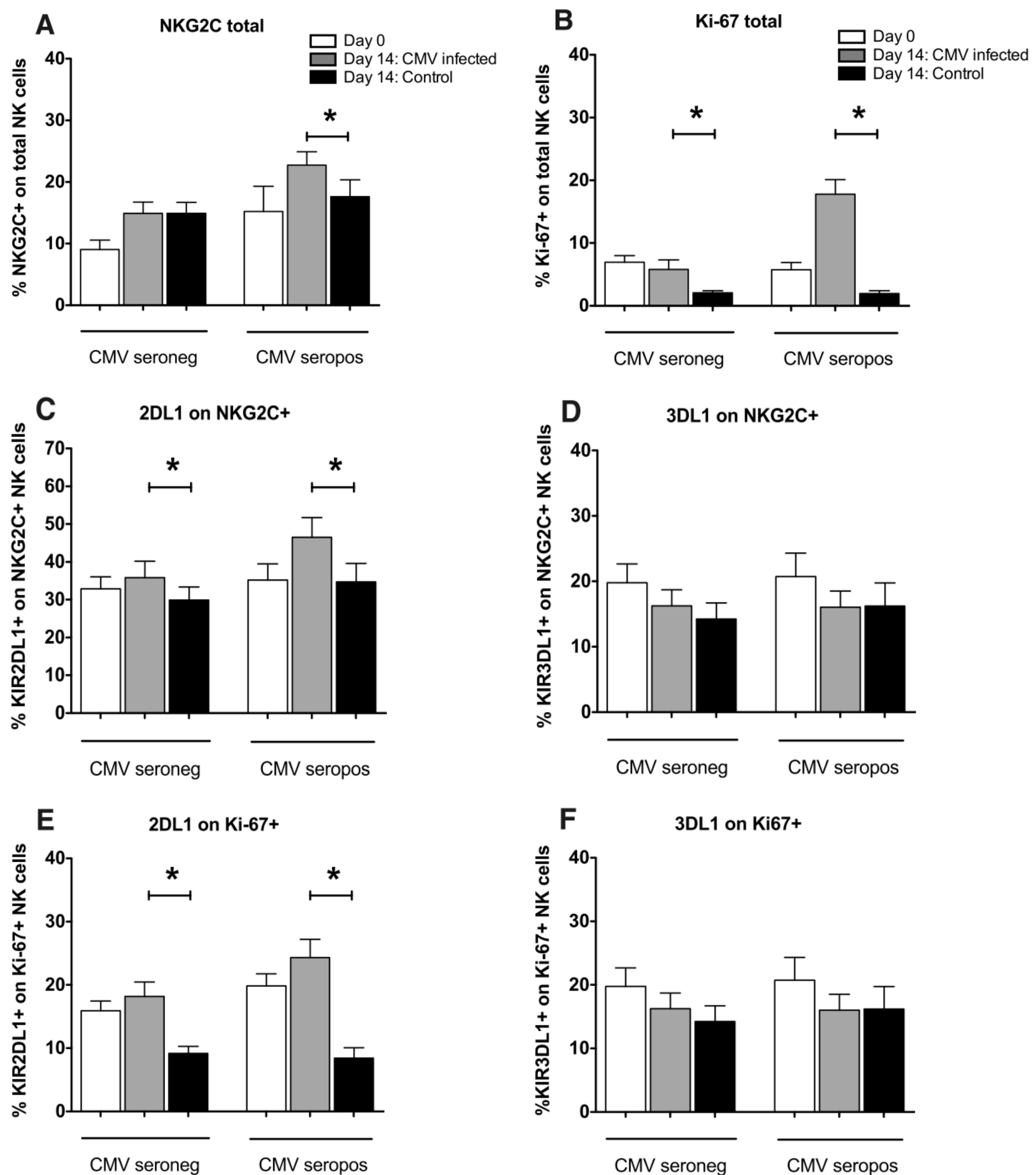


Figure 2. Changes in NKG2C and Ki-67 expression after NK-cell co-culture with CMV-infected fibroblasts. PBMCs from CMV-seropositive or seronegative donors were co-cultured for fourteen days with CMV-infected fibroblasts and the frequency of (A) NKG2C expression, and (B) Ki-67 expression was measured on NK-cells after gating on $CD56^{+}/CD3^{-}$ cells. (C-F) Similarly, (C, E) KIR2DL1 and (D, F) KIR3DL1 expression was assessed within $NKG2C^{+}$ and Ki-67^{+} cells respectively. Data are shown as mean + SEM of 20 donors pooled from 2 experiments. Comparison between groups was made by Student's T-test, * $p < 0.05$.

Changes in KIR repertoire do not depend on the presence of B- and T-lymphocytes

To exclude the possibility that changes in KIR repertoire were induced by the presence of B- and T lymphocytes, we cultured FACS-sorted NK cells from CMV-

seropositive donors in the presence of MRC-5 with and without CMV. Changes in the KIR repertoire were closely recapitulated by those found if PBMCs were co-cultured from the same donors, showing that the specific expansion could not be ascribed to the presence of lymphocytes other than NK cells in the co-culture assay (Supporting Information Fig. 3).

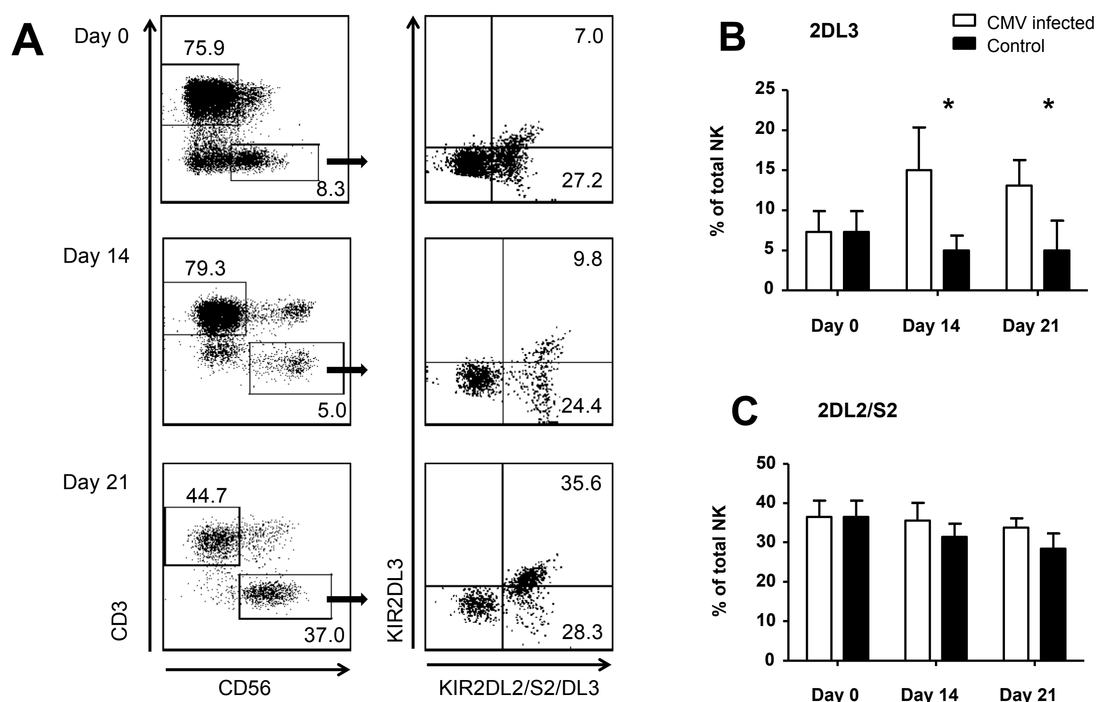


Figure 3. Expansion of KIR2DL3-expressing NK-cells after PBMC co-culture with infected fibroblasts. PBMCs from CMV-seropositive donors were co-cultured for 21 days with CMV-infected fibroblasts. (A) A representative flow cytometry plot of the gating strategy for $CD56^{+}/CD3^{-}$ cells is shown. (B, C) The percentage of (B) KIR2DL3- and (C) KIR2DL2/S2-expressing NK-cells was determined by flow cytometry. Data are shown as mean + SEM of 5 donors pooled from two experiments. Comparison between groups was made by Student's T-test, * $p < 0.05$.

CMV infection leads to degranulation and cytokine production by NK cells

In order to assess how NK cells respond functionally to exposure to CMV infected target cells, we assessed CD107a expression as a marker of degranulation and IFN-γ production by intracellular cytokine staining. After two and 3 weeks of culture, all NK-cell subsets of CMV-seropositive and -seronegative donors exposed to CMV in vitro degranulated and produced IFN-γ at the level of positive controls (PMA), suggesting nonspecific activation (data not shown). When analyzed earlier, we detected a significant increase in degranulation and IFN-γ production in CMV-exposed NK cells already at 3 days of co-culture. Extending previous results, degranulation and cytokine production were stronger in CMV-

seropositive than in CMV-seronegative donors, and were significantly higher for the HLA-C binding KIR2DL1 than for the HLA-B binding KIR3DL1 (Fig. 5).

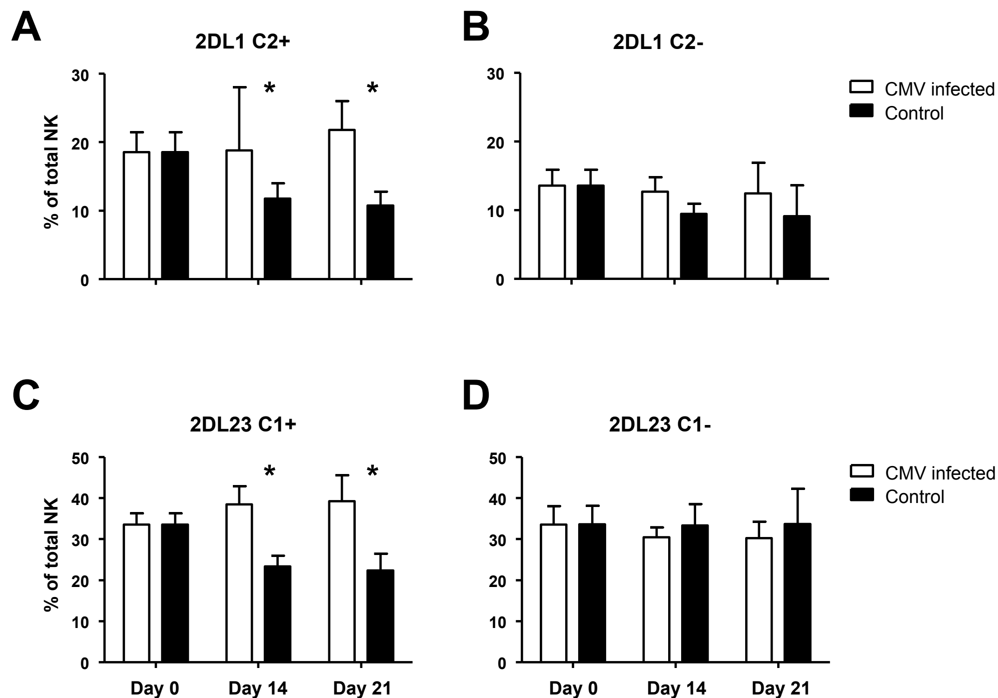


Figure 4. Expansion of KIR2DL1 and KIR2DL2/3-positive NK-cells in relationship to the presence of HLA KIR ligands. PBMCs from CMV-seropositive or -seronegative donors were co-cultured for 21 days with CMV-infected fibroblasts and the percentage of (A, B) KIR2DL1- and (C, D) KIR2DL2/3-positive NK-cells was determined in patients, which did (A, C) or did not (B, D) carry the KIR ligands HLA-C1 and HLA-C2 respectively. Data are shown as mean + SEM of 5-11 donors pooled from 2 experiments. Comparison between groups was made by Student's T-test, * $p < 0.05$.

Discussion

This analysis of the impact of previous infection with CMV on the KIR repertoire of NK cells was prompted by the observation that transplant recipients are relatively protected from CMV replication if they carried B-haplotype associated activating KIR genes [5–8]. In our most recent analysis, protective effects were most evident in carriers of activating KIR genes located in the telomeric part of the KIR haplotype [6]. This part of the KIR gene cluster contains the activating receptors KIR2DS1, KIR3DS1, and KIR2DS5. The strong linkage disequilibrium between these genes makes it unlikely that population-based genetic association studies will be helpful in further identifying the resistance locus [21]. We there- fore aimed in this study to analyze if previous infection with CMV alters the repertoire of KIR expression both in freshly isolated cells as well as after exposure to CMV in an in vitro co-culture model.

In agreement with previous data investigating NK-cell receptor repertoire of CMV-seropositive and -seronegative donors, we find no evidence that the KIR repertoire of resting NK cells is altered by previous infection with CMV [12, 14]. In contrast, significant alterations in the KIR repertoire occurred after exposure of NK cells to CMV in vitro. We observed a specific expansion of NK cells expressing the inhibitory receptors KIR2DL1, KIR2DL3, and NKG2A, as well as of NK cells expressing the activating receptor KIR3DS1. Expansion of KIR2DL1 and KIR2DL3 occurred only in the presence of the cognate HLA-C ligands, whereas KIR3DS1+ NK cells expanded independently from the presence of the putative ligand HLA-Bw4.

Our results are intriguing in several ways: regarding the aKIR-mediated protection, we show that of the aKIR receptors to which antibodies exist, KIR3DS1 is the only one to expand in response to stimulation with CMV. This is in agreement with our population-based studies, which localized the locus of resistance to the telomeric part of the KIR haplotype, which contains — among other KIR receptor genes — the gene coding for KIR3DS1 [6]. Interestingly, expansion of KIR3DS1-expressing cells is irrespective of the presence of the putative KIR3DS1-ligand HLA-Bw4, suggesting that KIR3DS1 might bind a ligand outside of the context of HLA. Potential candidate ligands, which will need to be investigated in the future, may include UL18, a CMV-encoded HLA-like decoy protein, which has previously been shown to bind the inhibitory receptor LIR-1 [22].

Strikingly, NK cells expressing the inhibitory receptors KIR2DL1, KIR2DL3, and NKG2A were also found to expand in response to in vitro exposure to CMV. KIR2DL1 and KIR2DL3 bind mutually exclusive subsets of HLA-C Ags, whereas HLA-E is the ligand for NKG2A. The notion that a receptor conveying an inhibitory signal leads to expansion of cells expressing the receptor might appear unintuitive. However, recent studies have revealed that the inhibitory KIR/HLA interaction may be disrupted by peptides antagonizing the binding of KIRs to cognate HLA [23]. Whether such "peptide antagonism" is indeed responsible for the expansion of NK cells carrying inhibitory receptors will need to be addressed in future experiments.

Finally, the changes of NK-cell receptor repertoire in response to exposure to CMV occurred almost exclusively in patients with previous exposure to CMV, as measured by CMV IgG seropositivity. Only in a sensitive analysis gating first on

NKG2C+ cells, were we able to also document an up-regulation of HLA-C binding KIRs in CMV-seronegative donors. While NK cells are traditionally seen as innate immune cells without the capacity for memory formation, recent studies in mice have suggested that NK cells share many features with effector cells of adaptive immunity, including the capacity to elicit memory responses [10, 24]. Whether the fact that changes in the NK-cell repertoire after in vitro expo- sure to CMV truly represent a function of NK-cell memory to human CMV cannot be concluded with certainty, as CMV persists latently in infected patients, and might chronically stimulate NK cells.

In conclusion, we show that receptor repertoire of circulating NK cells is not altered by previous infection with CMV. After exposure to CMV in vitro, however, an HLA class I ligand dependent expansion of KIR2DL1+ and KIR2DL3+ cells occurs, along with expansion of cells expressing NKG2A and KIR3DS1. Changes to the NK-cell receptor repertoire were confined to CMV-IgG positive patients.

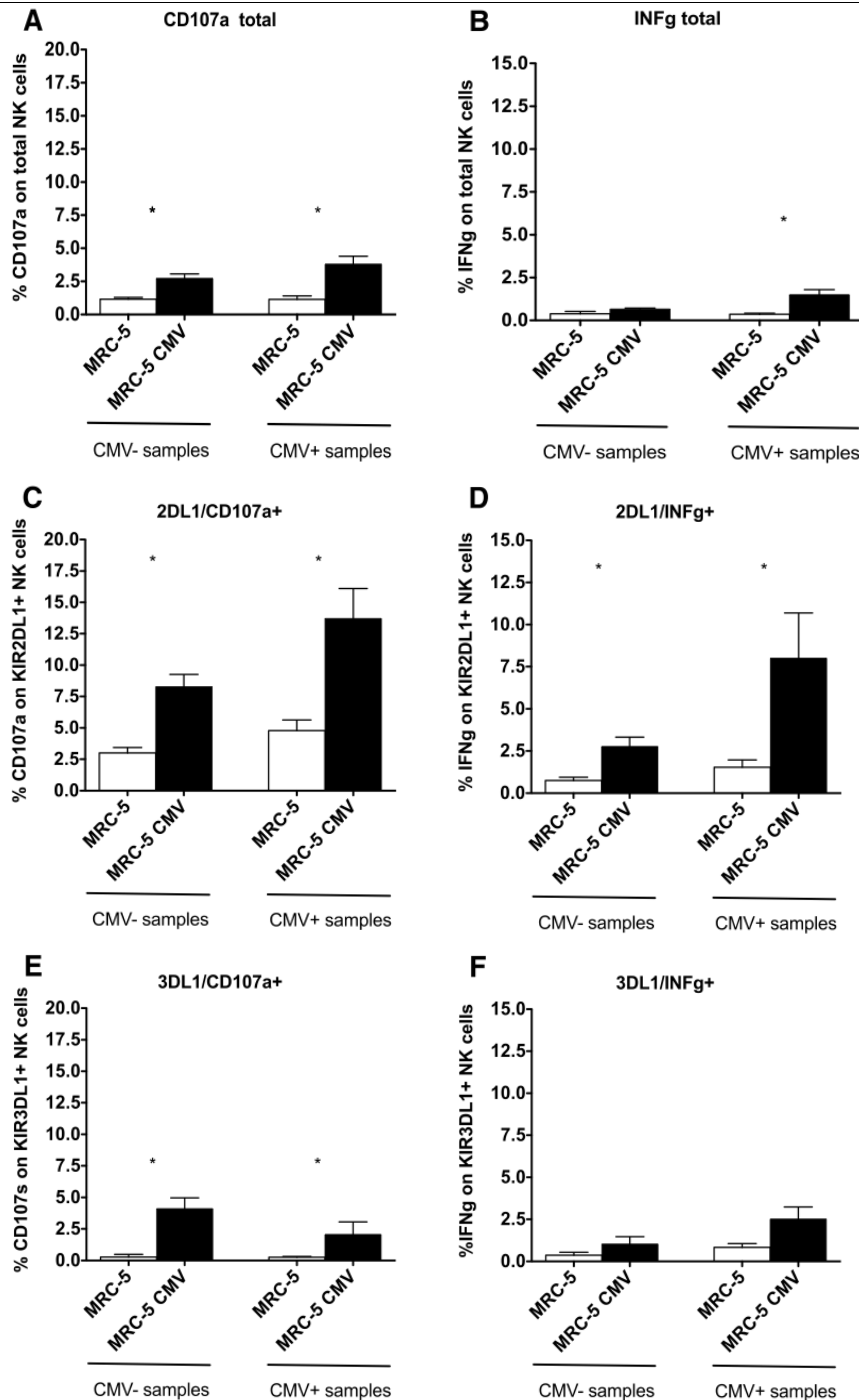
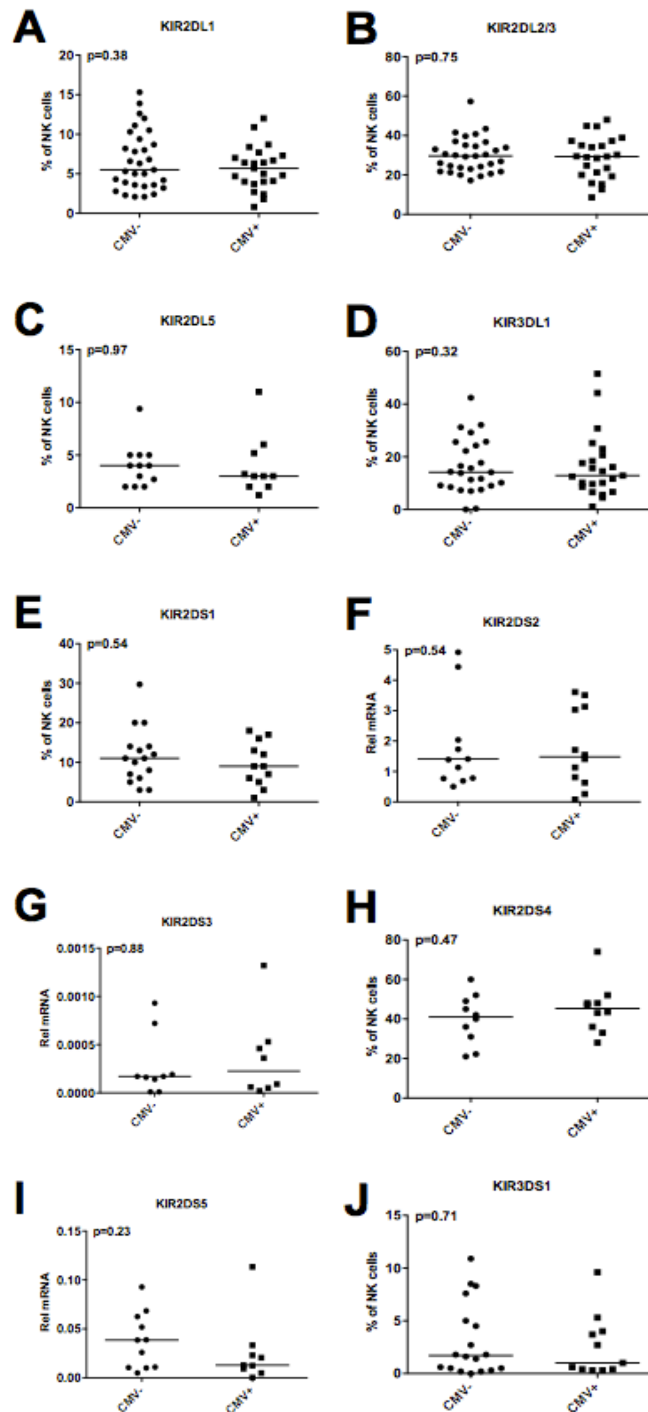


Figure 5. Functional analysis of NK-cells in PBMCs cultured for three days in the presence of MRC-5 with or without CMV. PBMCs from CMV-seropositive or -seronegative donors were co-cultured for 3 days with fibroblasts infected or not with CMV. (A, C, E) Surface CD107a expression and (B, D, F) intracellular expression of IFN-gamma was assessed by gating on (A, B) $CD56^{+}/CD3^{-}$ NK-cells and within NK-cells expressing (C, D) KIR2DL1 and (E, F) KIR3DL1. Data are shown as mean + SEM of 5 donors pooled from 2 experiments. Comparison between groups was made by Student’s T-test, * $p < 0.05$.

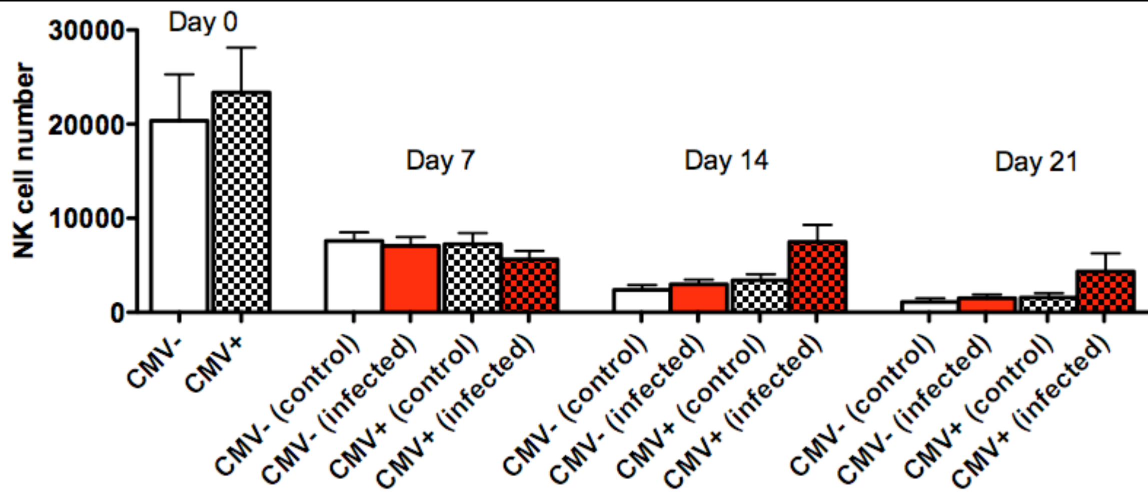
Acknowledgments

This study was supported by grants from the Swiss National Science Foundation (grant PPOOP3 128461 / 1 to M.S.) and from the “Stiftung Forschung Infektionskrankheiten”. The authors would like to express their gratitude to Beatrice Hess (Institute of Microbiology, Basel University) for help in setting up the CMV co-culture.

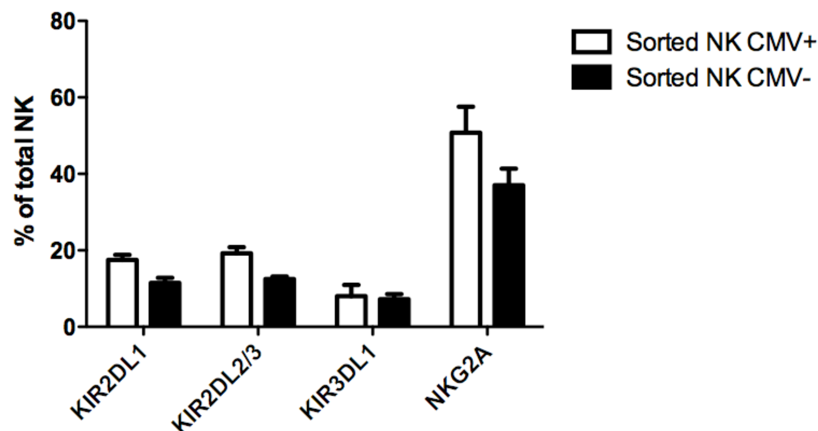
Supporting information



Supporting Information Figure 1. NK cell KIR and NKG2A expression in CMV- seropositive and seronegative donors. PBMCs from CMV-seropositive (CMV+) or CMV-seronegative (CMV-) donors were stained for cell surface expression of the inhibitory receptors (A) KIR2DL1, (B) KIR2DL2/3, (C) KIR2DL5, (D) KIR3DL1 and of the activating receptors (E) KIR2DS1, (H) KIR2DS4, and (J) KIR3DS1 after gating on $CD56^{+}/CD3^{-}$ NK cells. mRNA quantity was compared for the activating receptors KIR2DS2, KIR2DS3, and KIR2DS5 in immunomagnetically sorted NK cells by qRT-PCR. Data represent 6 experiments performed in 54 donors. Expression of each KIR is shown only in donors that carry the respective KIR gene. Horizontal lines represent means. Comparison between groups was made by Student’s T-test.



Supporting Information Figure 2. Expansion of NK cells after co-culture with CMV- infected fibroblasts. Total number of NK cells derived from CMV-seropositive (CMV+) and CMV seronegative donors (CMV-) was assessed during three-week culture in the presence of the fibroblast cells infected or not (control) with CMV by a combination of trypan blue cell counting and FACS phenotyping. Data are pooled from 2 experiments involving a total of 10 donors. Bars represent means and whiskers the standard error of the mean. Comparison between groups was made by Student's T-test.



Supporting Information Figure 3. Expression of KIR and NKG2A in FACS-sorted NK cells co-cultured with CMV-infected fibroblasts. FACS-sorted NK cells from CMV-seropositive donors were co-cultured for 21 days with fibroblasts in the presence or absence of CMV and the expression of inhibitory KIR- and NKG2A receptors was compared by flowcytometry in cultured samples. Data are pooled from 2 experiments involving a total of 5 donors. Bars represent means and whiskers the standard error of the mean.

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PROJECT 3.

A comprehensive analysis of the binding of anti-KIR antibodies to activating KIRs

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Journal: Genes and Immunity 2013 Jan;15(1):33-7.

My contribution to work:

Providing idea, sample preparation, experimental design, performance of experiments, data analysis and interpretation and participation in manuscript writing.

Abstract

Analysis of killer cell immunoglobulin-like receptor (KIR) expression has been notoriously difficult because of the cross-reactivity of available antibodies, in particular between activating and inhibitory isoforms. We undertook a comprehensive study of available anti-KIR antibodies binding to activating KIRs (a-KIRs). Using cell lines stably transfected with a-KIRs (KIR2DS1–S5 and KIR3DS1), we confirmed documented binding specificities. In addition, we show that clones HPMA4 and 143211—previously assumed to be specific for KIR2DS1/L1 and KIR2DL1, respectively—bind KIR2DS5 and KIR2DS3 (HPMA4), and KIR2DS5 (143211). Other antibodies with previously undocumented binding were JJC11.6 (recognizing KIR2DS3) and 5.133 (recognizing all a-KIRs except KIR2DS1 and KIR2DS3). The novel KIR2DS5 reactivities were confirmed by blocking with soluble KIR-Fc fusion proteins, and by reverse transcriptase-PCR analysis of sorted primary natural killer cells. In conclusion, we show formerly undocumented binding properties of anti-KIR antibodies. These cross-reactivities should be taken into account when analyzing KIR expression.

Introduction

The expression of killer immunoglobulin-like receptors (KIRs) on the surface of natural killer (NK) cells presents considerable interindividual variability [1]. Variation in KIR gene content [2], KIR allelic polymorphism [3, 4], clonal variegation of the NK-cell repertoire [5], and other factors such as subclinical infection [6], all account for phenotypical diversity of NK-cell repertoires, which differ even among individuals with identical KIR genotype. Together with the extensive polymorphism of human leukocyte antigen alleles, many of which encode KIR ligands, this variability shapes host immune responses that depend on the interaction between KIRs and the human leukocyte antigen in a highly personalized manner [7]. An accurate phenotypic picture of the NK-cell KIR repertoire can thus be a valuable tool in forecasting individual susceptibility to auto- immune disease or viral infection, or the graft versus leukemia effect observed in some types of hematopoietic stem cell transplantation [8].

Unfortunately, the specificity of antibodies to detect KIR surface expression on NK cells is not absolute, owing to the high ectodomain homology among different KIR proteins. In particular, the cross-reactivity of antibodies recognizing both the inhibitory and activating members of the KIR family has hampered a complete and precise phenotypical analysis of the KIR repertoire [9]. In addition, the reactivity of some anti-KIR antibodies currently used in flow cytometry remains incompletely characterized, due in part to the redefinition of KIR alleles and antibody specificities [10, 11]. We undertook this study to systematically assess the reactivity of commercially available anti-KIR antibodies with activating KIR (a-KIR) molecules using transfected NK cell lines.

Materials and Methods

NK cell transfectants

Complementary DNA for all the known human a-KIR genes were obtained by reverse transcriptase-PCR from peripheral blood mononuclear cell of donors genotyped by single specific primer-PCR [20]. The entire coding region, including

the leader peptide, of the most prevalent allele of each a-KIR gene (KIR2DS1*002, KIR2DS2*001, KIR2DS3*001, KIR2DS4*001, KIR2DS5*002 and KIR3DS1*013), was cloned and inserted into the retroviral vector pMX-Puro (a generous gift from Professor L. Lanier, UCSF, USA). Cloned plasmids were sequenced bidirectionally and aligned to KIR sequences using the current IPD-KIR database (http://www.ebi.ac.uk/ipd/kir/). Each KIR–pMX construct was transfected into packaging Phoenix A cells (gift from L. Lanier) and viral supernatants used to transduce NKL cells, an NK cell line lacking a-KIR expression (gift of Dr. AM Garcia-Lora, University Hospital Virgen de las Nieves, Granada, Spain) and transduced NKL cells were selected in 0.5 mg ml^{-1} puromycin. NK cells expressing a-KIR were grown in RPMI (Invitrogen, Carlsbad, CA, USA) supplemented with 10% human AB serum and 100 U ml^{-1} rhIL-2 (Proleukin, Novartis, Basel, Switzerland). Owing to the low expression of 2DS3 and 3DS1, additional transfections were performed with these KIRs in the NK cell line YT.

Flow cytometry

To test the reactivity of the panel of antibodies shown in Supplementary Table 1 against the different a-KIRs, between $5x10^4$ and 10^5 transfected NK cells were incubated with each antibody in FACS buffer (2% FCS in phosphate buffered-saline), washed, stained with a viability dye (Sytox blue, Invitrogen) and analyzed on a Cyan ADP Beckman-Coulter flow cytometer (Beckman Coulter, Brea, CA, USA). The anti-KIR antibodies were purchased commercially from the vendors listed in Supplementary Table 1, used at saturating concentrations. Other antibodies used were anti-CD3 (clone SK7), CD14 (clone 61D3) and CD19 (clone HIB19, all from eBioscience, San Diego, CA, USA) and CD56 (clone HCD56, Biolegend, San Diego, CA, USA). Antibody isotope controls were used in parallel, and non-viable cells were excluded from the analysis. Intracellular staining was done similarly after a short fixation step (15 min) and using permeabilization buffer (Biolegend) instead of FACS buffer, following the manufacturer's protocol.

Production of recombinant KIR-Fc fusion proteins

Baculoviral constructs encoding the ectodomains of 2DS1, 2DS2, 2DS3, 2DS4

and 2DS5 in frame with the CH2 and CH3 domains of human IgG1 (Fc region) were kindly provided by P. Parham (Stanford University, USA). As revealed by sequencing, these a-KIR alleles were the same as those used in the cell transfectants. The constructs were subcloned into pFL [21] and transfected by electroporation into packaging DH10EMBacYFP cells to obtain bacmid DNA. After confirming integrity and frame by sequencing, 2 mg of bacmid DNA was transfected with Cellfectin (Invitrogen) into Sf21 cells to obtain baculovirus supernatant V0. Two rounds of amplification followed to ensure high titer virus supernatants (V2), which were then used for recombinant protein production in a serum-free culture medium (SF-4 Baculo Express, Bioconcept, Allschwil, Switzerland) at 27°C. KIR-fusion proteins were purified by protein A-sepharose affinity chromatography and analyzed by polyacrylamide gel electrophoresis. For blocking experiments, staining of KIR transfectants with a test antibody was performed either in the absence or after pre-incubation of the antibody with each of the KIR-Fc fusion proteins at 100 mg ml^{-1} for 30 min at room temperature.

NK-cell sorting and reverse transcriptase-PCR for KIRs

Peripheral blood mononuclear cells were stained sequentially with the HPMA4 antibody for 30 min (1:50 dilution), followed by an overnight incubation with the EB6b antibody (1:50 dilution) or with 143211 exclusively in FACS buffer and an antibody cocktail consisting of anti-CD3, CD14, CD19 and CD56 antibodies for NK-cell gating. The different cell subpopulations obtained in donors with either KIR2DS5+ or KIR2DS5- genotype were sorted by FACS with an Aria cell sorter (BD Biosciences, San Jose, CA). Each sorted subset was immediately processed for RNA extraction with the Reliaprep kit (Promega, Madison, WI, USA) and reverse transcription (High-Capacity cDNA Reverse Transcription kit, Applied Biosystems-Life Technologies, Carlsbad, CA, USA). Intron-spanning primers used in previous studies [20, 22] were used to amplify KIR2DL1 (F: 5’-GCA G CA CCA TGT CGC TCT T-3’; R: 5’-CCT GCC AGG TCT TGC G-3’), KIR2DS1 (F: 5’-TCT CCA TCA GTC GCA TGA A-3’; R: 5’-TTA TGC GTA TGA CAC CTC CTG ATG-3’) and KIR2DS5 (F: 5’-AGA GAG GGG ACG TTT AAC C-3’; R: 5’-CAA GCA GTG GGT CAC TTG A-3’) by quantitative PCR using a conventional master mix (Promega). PCR products were run by agarose electrophoresis to confirm

amplicon size.

Results and discussion

Published results have shown that the NKL cell line is devoid of surface KIR expression except KIR2DL4 [12]. In agreement with these data, none of the anti-KIR antibodies tested stained parental NKL cells, with the exception of the KIR2DL4 antibody Mab33, showing weak reactivity. NKL cells transfected with KIR2DS1, KIR2DS2, KIR2DS4 and KIR3DS1 were stained with monoclonal antibodies known to have affinity for these KIRs (clones EB6b, DX27, FES172 and Z27, respectively). As no specific antibodies for KIR2DS3 and KIR2DS5 are commercially available, cells transfected with these KIRs were positively identified with a pan-KIR2D MAb (clone NKVFS1). Flowcytometric characterization of the transfected NK cell lines is summarized in Figure 1a. Of note, expression of KIR2DS3 was low and mostly intracellular, in agreement with previous reports showing that this KIR is retained in the cytosol in transfected cell lines [13]. Surface expression of KIR3DS1 was weak in comparison with the other a-KIR genes, but comparable to the expression of this KIR gene on primary NK cells, or in transfected BaF3 cells [14]. The intracellular and low expression, respectively, of KIR2DS3 and KIR3DS1 was similar in the YT cell line.

Each anti-KIR antibody stained the corresponding a-KIR transfectant and not the others as predicted, but some unexpected stainings were observed. Most remarkably, antibodies with nominal specificity for KIR2DL1/2DS1 (clone HPMA4) and anti-KIR2DL1 (clone 143211) consistently stained cells transfected with KIR2DS5. In contrast, another antibody against KIR2DL1/2DS1 (clone EB6b) did not show any reactivity against KIR2DS5 (Figure 1b). The HPMA4 antibody also stained the KIR2DS3 transfectants (Figure 1b) after intracellular staining, although the low expression of this KIR led to stainings of lower intensity, in both NKL and YT transfectants. Other anti-KIR antibodies showing unexpected cross-reactivities were clone JJC11.6 (anti-KIR2DS4), against KIR2DS3 in intracellular staining experiments, and clone 5.133 (anti-KIR3DL1/3DL2), which cross-reacted with all transfected cell lines except those expressing KIR2DS1 and KIR2DS3 (Figures 1c and d).

To confirm the reactivity of the HPMA4 and 143211 antibodies with

KIR2DS5, and to more conveniently assess the cross-reactivity of these antibodies against KIR2DS3, we used recombinant a-KIR-Fc fusion proteins in blocking experiments. Each KIR-Fc protein blocked the staining of the corresponding transfectant with its identifying antibody, indicating correct folding of the Fc fusion proteins. For example, the KIR2DS1-Fc fusion abrogated staining of KIR2DS1 transfectants by the HPMA4 antibody, and while the KIR2DS2-Fc and KIR2DS4-Fc did not affect staining by this antibody, pre-incubation with the KIR2DS3- or the KIR2DS5-Fc fusion proteins completely blocked it (Figure 1e). A similar experiment was conducted using KIR2DS5 transfectants and showed identical results, confirming that the D1 and D2 domains of KIR2DS3 and KIR2DS5 are recognized by the HPMA4 antibody (Figure 1e). Additionally, the reactivity of KIR2DS5 transfectants against the 143211 antibody could be totally reversed by pre-incubation with KIR2DS5-Fc fusion proteins, also confirming the interaction of this antibody with KIR2DS5. In contrast, the very weak staining of 2DS3 transfectants by 143211 (Figure 1b) could not be confirmed in this assay using blocking by KIR2DS3-Fc (Figure 1e).

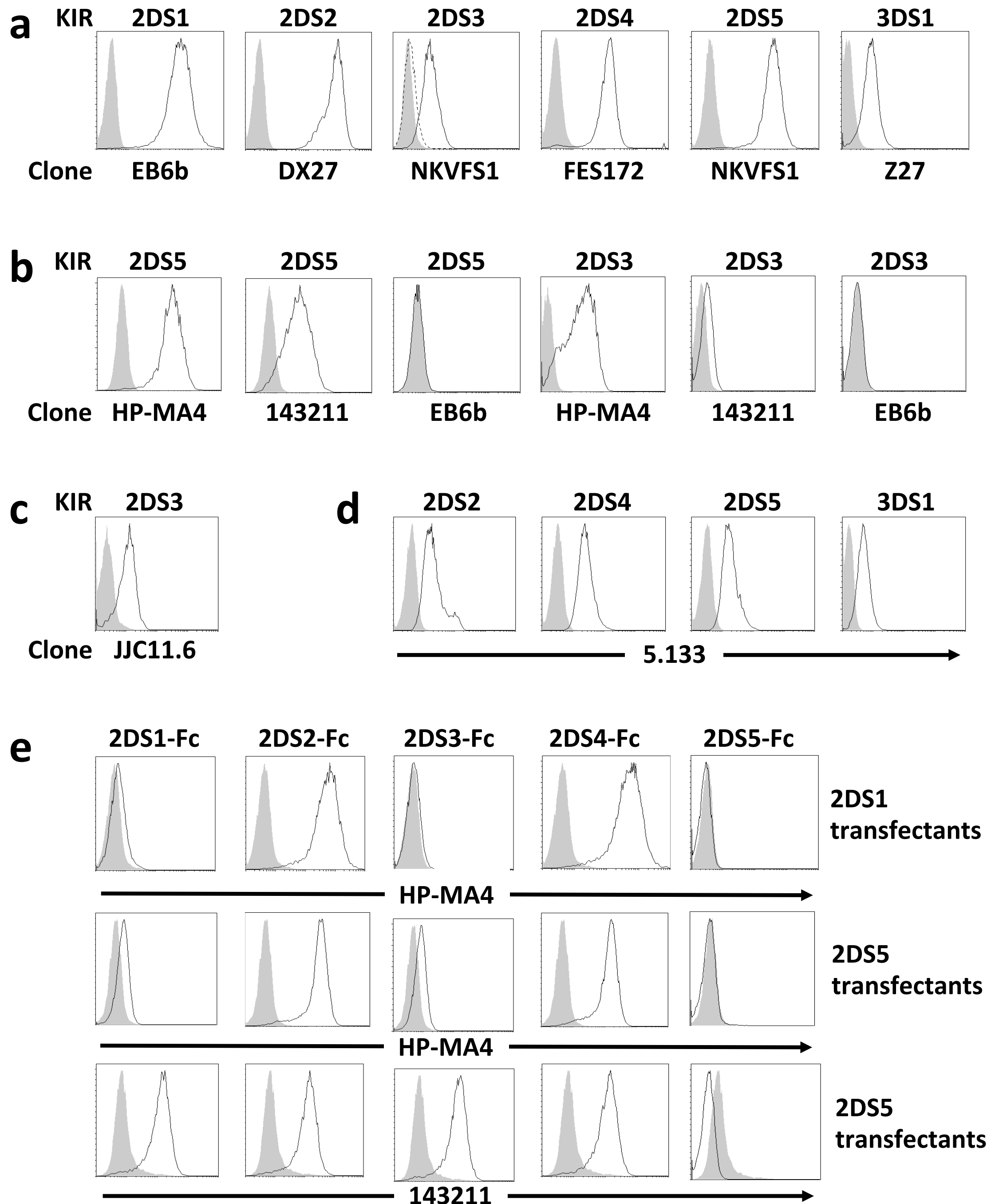

Figure 1. (a) NK cells transfected with a-KIRs (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5 and KIR3DS1). Histograms show staining of the a-KIR-transfected NK cells (solid line) stained each with the corresponding anti-a-KIR antibody (see text for details), along with isotype antibody controls, represented by filled gray histograms. Staining for KIR2DS3 is shown with (solid line) and without (dashed line) cell permeabilization. (b) The monoclonal antibody HPMA4 stains NKL cells transfected with KIR2DS3 (permeabilized) and KIR2DS5; 143211 stains KIR2DS5 and very weakly stains KIR2DS3 (permeabilized); whereas EB6b does not stain either KIR2DS3 or KIR2DS5. (c) The KIR2DS4 antibody JJC11.6 stains KIR2DS3 transfectants (permeabilized). (d) The KIR3DL1/3DL2 5.133 Mab stains KIR2DS2, KIR2DS4, KIR2DS5 and KIR3DS1 transfectants. (e) Staining of NKL2DS1 or NKL2DS5 transfectants with different antibodies in the presence of different KIR-Fc conjugates. Staining of KIR2DS1-transfected NK cells with the KIR2DL1/2DS1 antibody HPMA4 is blocked after pre-incubation of the antibody with KIR2DS1-Fc-, KIR2DS3-Fc- or KIR2DS5-Fc-chimeras (100 mg ml^{-1}), but not with similar concentrations of KIR2DS2-Fc- or KIR2DS4-Fc-chimeras (upper row). Similar results were observed with HPMA4 antibody staining of KIR2DS5 transfectants (middle row). Staining of KIR2DS5 transfectants with the 143211 antibody is blocked by pre-incubation with KIR2DS5-Fc-chimeras only (lower row).

We additionally investigated whether the differential staining properties of the HPMA4 and EB6b antibodies could be exploited to identify surface expression of KIR2DS5 in peripheral blood NK cells. A group of peripheral blood mononuclear cell samples from donors genotyped as positive (n=8) and negative (n=7) for KIR2DS5 were double-stained sequentially with these antibodies. Using this strategy, a cell subpopulation of NK cells compatible with KIR2DS5 surface expression was observed (Figure 2a). In all KIR2DS5+ donors, a discrete subset of HPMA4+ EB6b- cells was found, amounting to 2–10% of the total NK cells, in contrast with KIR2DS5- donors, in whom it was absent. This subset represents KIR2DL1- KIR2DS1- KIR2DS5+ cells, but other NK cells may express KIR2DS5 in combination with KIR2DL1 and KIR2DS1. This possibility was searched for by reverse transcriptase-PCR in sorted cells. As seen in Figure 2b, HPMA4+ EB6b- cells expressed the KIR2DS5 transcripts and none of the other two KIRs, but KIR2DS5 transcripts were also found in cells expressing KIR2DL1 and KIR2DS1. Notably, the staining intensity of KIR2DS1 containing subsets by HPMA4 was relatively weak because of sequential staining with HPMA4 followed by EB6b, in which the latter antibody competed for binding of HPMA4 (confirmed by sequential staining of KIR2DS1 transfectants, data not shown).

Additionally, in two donors negative for KIR2DL1 and positive for KIR2DS5 by genotype, we were able to evaluate the total percentage of KIR2DS5 on peripheral blood NK cells by staining with the 143211 antibody. Expression of KIR2DS5 was detected in 2% and 4% of NK cells, respectively (Figure 2c). The identity of this population was confirmed as previously by sorting and reverse transcriptase-PCR for KIR2DS5 (Figure 2d).

In another two donors with a KIR2DS3+ KIR2DS5- genotype, we failed to detect intracellular staining by HPMA4 of presumed intracellular KIR2DS3. Whether this is due to low expression of KIR2DS3 in primary NK cells, or to the specific allele (KIR2DS3*002) in KIR2DS5- individuals [15], (which was different from the more frequent KIR2DS3*001 allele used in transfected cell lines) remains unresolved.

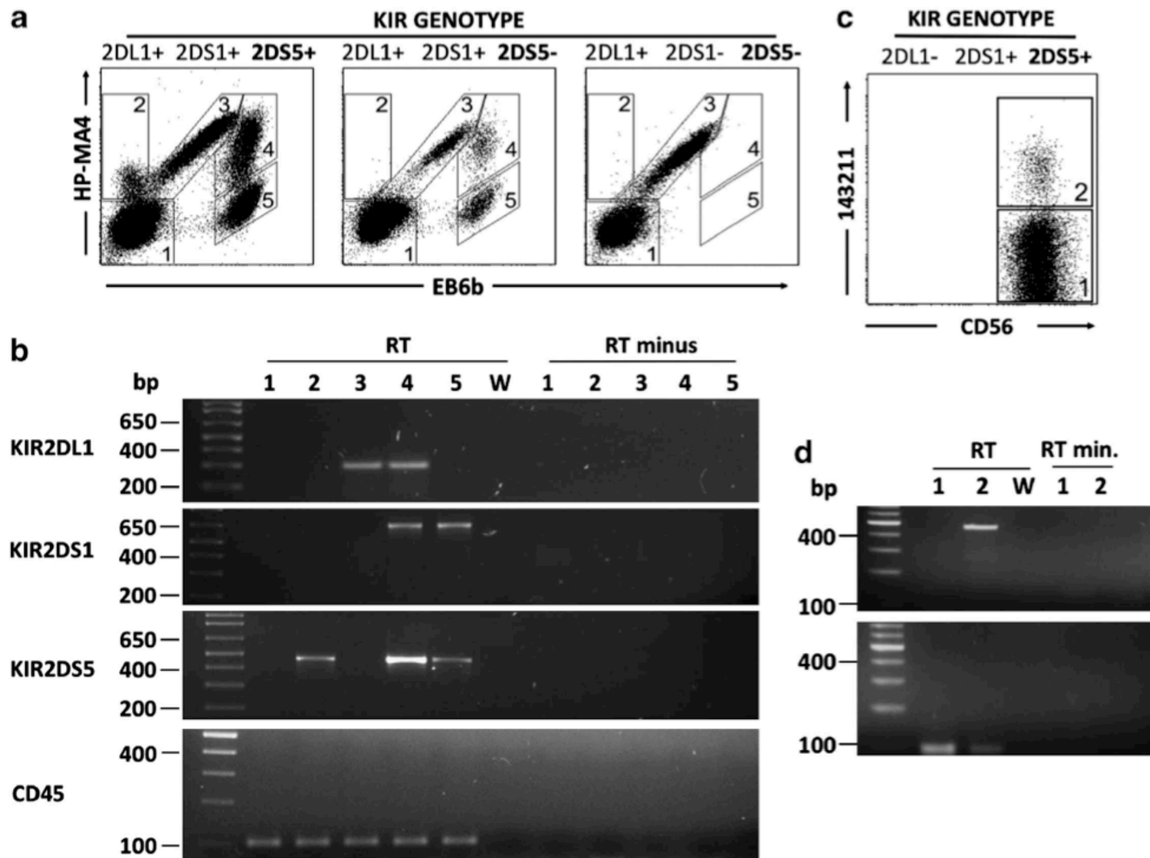

Figure 2. (a) Representative dot plot showing gated live peripheral blood NK cells double-stained sequentially with the HPMA4 (PE) and the EB6b (APC) antibodies in cells from donors with the denoted genotype. Only in donors genotyped positive for KIR2DS5 there is a subpopulation of HPMA4+ EB6b- stained cells (subpopulation 2). (b) RT-PCR of KIR2DL1, KIR2DS1, KIR2DS5 and CD45 (control) of sorted cells corresponding to the cell subsets indicated in (a) and a water control (W). Subset 1 does not contain any of the analyzed KIRs, subset 2 contains only KIR2DS5 transcripts, subset 3 contains only KIR2DL1 transcripts, subset 4 contains a mixture of all the three transcripts and subset 5 contains only KIR2DS1 and KIR2DS5. On the right panel, the RT-minus control is shown, for example, the same samples were subjected to the reverse transcription protocol in the absence of enzyme. (c) Representative dot plot showing gated live peripheral blood NK cells stained with the 143211 antibody in cells from a donor with the denoted genotype. In the absence of KIR2DL1, the 143211- positive subpopulation 2 includes all the KIR2DS5-positive NK cells. (d) RT-PCR of KIR2DS5 and CD45 (control) of sorted cells corresponding to the cell subsets indicated in (c) and a water control (W). KIR2DS5 transcripts are only found in population 2. On the right panel, the RT-minus control is shown.

Only recently has it been shown that KIR2DS5 is expressed on NK-cell surfaces by using a non-commercial antibody [16, 17]. The function of KIR2DS5 in NK cells is uncertain at present, as the ligands interacting with it remain unknown, although it could potentially modulate the reactivity of NK-cell subsets in certain circumstances. The lack of specific antibodies have so far limited the study of KIR2DS5, but now its presence and relevance in health and disease can be assessed with commercially available antibodies. Likewise, the study of the clonal architecture of the NK-cell repertoire can be further refined. The shown cross-reactivity with KIR2DS5 could have led in the past to inaccurate estimations of

subset frequencies or affected the purity of sorted cells based on the reactivity with these antibodies [18]. Importantly, the inheritance of the KIR2DS5 gene is strongly linked to that of KIR2DS1 [19].

In conclusion, the analysis presented here defines the reactivity of available antibodies against the most prevalent a-KIRs. As a major novel finding, clone HPMA4 cross-reacts with KIR2DS5 and KIR2DS3. Co-staining of HPMA4 with antibodies specific for KIR2DS1/L1 allows detection of surface KIR2DS5

Acknowledgments

We thank Professor P. Parham, Stanford, Professor L. Lanier, UCSF, and Dr. AM Garcia-Lora, Granada, Spain, for reagents and cells, and Professor Kurt Ballmer-Hofer, PSI for resources for protein expression. This study was supported by grants from the Swiss National Science Foundation (grant PP00P3_128461/1) and by Krebsforschung Schweiz (grant KFS-3030-08-2012).

Supplementary Table.

Clone	Nominal specificity	Isotype	Additional specif. found	Vendor
HPMA4	2DL1, 2DS1	IgG2b	2DS3, 2DS5	Biolegend
HP3E4	2DL1, 2DS1, 2DS4	IgM	-	BD
EB6b	2DL1, 2DS1	IgG1	-	Beckman
11PB6	2DL1, 2DS1	IgG1	-	Miltenyi
143211	2DL1	IgG1	2DS5	R & D
DX27	2DL2, 2DL3, 2DS2	IgG2a	-	Biolegend
CH-L	2DL2, 2DL3, 2DS2	IgG2b	-	BD
GL183	2DL2, 2DL3, 2DS2	IgG1	-	Beckman
180701	2DL3	IgG2a	-	R & D
mAb 33	2DL4	IgG1	-	Biolegend
UP-R1	2DL5	IgG1	-	Biolegend
FES172	2DS4	IgG2a	-	Beckman
JJC11.6	2DS4	IgG1	2DS3	Miltenyi
NKVFS1	pan KIR2D	IgG1	-	Miltenyi
Z27	3DL1, 3DS1	IgG1	-	Beckman
5.133	3DL1, 3DL2	IgG1	2DS2, 2DS4, 2DS5, 3DS1	Miltenyi
DX9	3DL1	IgG1	-	BD

KIR antibodies used in this study. Their established specificities and additional specificities found are indicated.

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PROJECT 4.

Characterization of novel binding specificities of activating KIR2DS3 and KIR2DS5 to leukemic cell lines

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Manuscript

My contribution to work:

Experimental design, performance of experiments, data analysis and interpretation, manuscript writing.

Abstract

Numerous literature data suggest a general participation of B haplotype donor activating killer cell immunoglobulin-like receptors (KIR) in the elimination of residual leukemia after hematopoietic stem cell transplantation (HSCT). However, there is no direct evidence for binding of activating KIR to leukemia cells and these conclusions are drawn mainly from associations between donor KIR genotype and transplant outcome. High linkage disequilibrium between activating KIR genes makes it unlikely that further gene association studies will identify KIR genes responsible for the beneficial effects.

We therefore biosynthesized soluble forms of activating KIRs as KIR-Fc conjugates and checked their binding to a panel of leukemic cell lines. We also measured their binding affinities. Considering the binding to HLA class I molecules of inhibitory KIR, we evaluated if activating KIR share the specificity. Moreover, we undertook a trial to confirm the function of activating KIRs by expressing them on NK cell line – NKL, which were destined for functional assays. Finally, we checked functional consequences of binding using assays with primary NK cells.

We detected binding of KIR2DS3-Fc and KIR2DS5-Fc conjugate to an unknown ligand on the surface of HLA class I-deficient K562 cell line as well as KIR2DS3-Fc and KIR2DS5-Fc to HLA class I ligands on the surface of HEL and Namalwa cells. Binding was independent of HLA-C subgroups, and relatively weak when compared to inhibitory KIR receptor. The functional assays of KIR2DS5 positive subpopulation on primary NK cells against K562 cell line demonstrated a moderate input of that receptor in target cell killing.

Introduction

The function of the NK cells is regulated by surface receptors, among which the killer cell immunoglobulin-like r eceptor (KIR) family is one of most important [1- 3]. This group consists of inhibitory and activating members, and therefore activation of NK cells is a result of a balance of signals derived from receptors with opposing function.

Inhibitory KIRs recognize ligands among HLA class I molecules. For example, KIR2DL1 recognizes HLA-C molecules defined as subgroup C2. In contrast, KIR2DL2 and KIR2DL3 bind in general HLA-C (C1) epitopes [4-7]. Based on the high structural similarity, it could be expected that their activating counterparts exhibit similar binding preferences. This is the case for the KIR2DL1/DS1 receptor pair, in which the HLA-binding residues are conserved, enabling KIR2DS1 to bind HLA-C (C2) [8]. Binding of KIR2DS1 to HLA-C2 was confirmed functionally using cellular experiments [9, 10]. However, for the other activating KIRs - except for KIR2DS2, possibly binding to HLA-C (C1 subgroup) [11] - only for KIR2DS4 binding to some HLA-A and HLA-C subtypes was demonstrated using HLA-coated beads [12, 13]. Specificities of remaining activating KIRs are unknown. Despite this, HLA molecules are plausible candidates as ligands to the remaining activating KIR. Arguing in favor of that theory is the simultaneous evolutionary emergence of HLA-C accompanied by lineage III KIR expansion in primates [14, 15]. Also, in cases where the interaction is proven for both inhibitory and activating KIRs, the domains responsible for contact with HLA class I molecules are highly similar [6, 8]. The lack of a detectable binding of most activating KIR in previously reported experiments in the published literature may be due to allelic polymorphism of KIR genes or may be due to modulation of binding properties by peptides presented on HLA. The latter was extensively demonstrated for the interactions between KIR2DL1 and HLA-Cw4 and also between KIR2DL2 and HLA-Cw3, which are modified by substitution of amino acids in position 7 and 8 (P7 and P8) of presented peptides [16, 17]. A similar example comes from studies on the influence of Epstein-Barr Virus (EBV) infection on KIR2DL1/DS1 binding, which shifts binding affinity from KIR2DL1 to KIR2DS1 [18]. Alternatively, it was also shown that KIR2DS4 binds an unknown

ligand on melanoma cell lines devoid of HLA expression; and that KIR3DL2 binds CpG oligodeoxynucleotides causing the internalization of the complex into Toll-like Receptor 9 (TLR9) containing endosomes [19, 20]. Hence, KIRs are also potentially able to bind non-HLA ligands.

The KIR genes segregate as haplotypes containing combinations of inhibitory and activating KIRs. These haplotypes are divided according to presence of one or more activating KIRs into haplotypes A or B, respectively. Hence, the haplotype A contains mostly inhibitory KIRs with one (KIR2DS4) or no activating KIR, whereas B haplotypes carry additional (mostly activating) KIR genes [21, 22]. Retrospective studies have demonstrated that patients suffering from acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia and plasma cell myeloma (PCM) receiving hematopoietic stem cell transplantation from donors carrying B haplotypes exhibit reduced rates of disease relapse [23-28].

In this study we produced activating KIR-Fc conjugates in order to screen panels of malignant cell lines in a search for their ligands and check the interaction strength. Antibody blocking assessed whether ligands belong to group of HLA class I molecules. The function of receptors was evaluated by using primary NK cells.

Materials and Methods

Production of recombinant KIR-Fc fusion proteins

Baculoviral constructs encoding the ectodomains of 2DS1, 2DS2, 2DS3, 2DS4, and 2DS5 in frame with the CH2 and CH3 domains of human IgG1 (Fc region) were kindly provided by P. Parham (Stanford University, USA). As revealed by sequencing, these activating KIR alleles were the most prevalent (KIR2DS1*002, KIR2DS2*001, KIR2DS3*001, KIR2DS4*001, KIR2DS5*002 and KIR3DS1*013). The KIR-Fc proteins were produced according the same scheme as previously described [29]. Briefly, the constructs were subcloned into pFL [30] and transfected by electroporation into packaging DH10EMBacYFP cells to obtain bacmid DNA. After confirming integrity and frame by sequencing, 2 μg of bacmid DNA was transfected with Cellfectin II (Invitrogen, Carlsbad, CA, USA) into Sf21

cells to obtain baculovirus supernatant V0. Two rounds of amplification followed to ensure high titer virus supernatants (V2), which were then used for recombinant protein production in protein-free culture medium (Insect-XPRESS protein-free insect cell medium with L-glutamine, Lonza, Verviers, Belgium) at 27°C. KIR-fusion proteins were purified by protein A-sepharose affinity chromatography and analyzed by polyacrylamide gel electrophoresis. Their specificity was confirmed by blocking experiments as previously shown [29]. The concentration was assessed basing on absorbance at 280 nm by Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Rockford, IL, USA) and after its correction by molar extinction coefficient.

Antigen coated bead screening of KIR-Fc fusions binding specificity

The screening of KIR-Fc conjugates binding to a broad panel of HLA antigens was performed by the FlowPRA Specific Antibody Detection Test (One Lambda, Canoga Park, CA, USA) on the Luminex 200 (Luminex, Austin, TX, USA) platform as previously described [31, 32]. In short, the beads coated by HLA antigens derived from EBV-transformed human B-cell lines were incubated with KIR-Fc conjugates in concentrations of 10 μg/ml, 50 μg/ml or 200 μg/ml for 30 min. at room temperature. Beads were washed and incubated FITC-conjugated anti-human IgG, washed again and destined for measurement [33].

Generation of activating KIR expressing NKL cell lines

The NKL cell line was transduced by a retroviral transduction system and maintained in culture as described in material and methods section in Project 3. The test of functionality of the expressed receptors is described in functional assay section of that manuscript.

Cell lines and PBMCs

All human leukemic cell lines were a kind gift from A. Wodnar-Filipowicz (Department of Biomedicine, University Hospital Basel, Basel, Switzerland) and are summarized in Table.1. Their hematopoietic origin and viral infection status

information comes from the CABRI database (www.cabri.org). The lymphoblastoid cell lines BM21 and SPL were purchased from EMBL-EBI, IPD-IMGT/HLA cell bank and considering their HLA class I phenotype were used as KIR2DS1-Fc binding controls. All the cell lines were cultured with RPMI 1640 medium (Gibco, Paisley, UK) enriched with 10% fetal bovine serum (Sigma-Aldrich, Steinheim, Germany) and Penicillin (1000 U/ml)/Streptomycin (1000 µg/ml) (Gibco). Healthy donor buffy coats were collected in under an ethical committee approved protocol. Peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep (Axis Shield PoC AS, Oslo, Norway) gradient and frozen.

Genotyping

KIR genotype was assessed using sequence-specific primer PCR [34]. Known ligands for activating KIR2DS1 were typed by PCR-sequence-specific primer (HLA-C1 group versus HLA-C2 group) [35]. Four digit HLA genotyping was performed by Jean Villard (Immunology and Transplantation Unit, University Hospital Geneva, Geneva, Switzerland).

Flow cytometry

As previously mentioned the anti-KIR antibodies were used to evaluate of KIR-Fc conjugate integrity (existence of epitopes recognized by their corresponding antibodies). These KIR-Fc conjugates were incubated with the following antibodies prior to adding them to KIR expressing NKL cell line: anti-KIR2DL1/DS1/DS3/DS5 (clone HP-MA4, Biolegend), anti-KIR2DL1/DS3/DS5 (clone 143211, R&D Systems, Minneapolis, MN, USA), anti-KIR2DL2/DS2/DL3 (clone DX27, Biolegend, San Diego, CA, USA), anti-KIR2DS4 (clone FES172, Beckman Coulter, Brea, CA, USA), and anti-KIR2D (clone NKVFS1, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), were all PE-labeled and used in dilution 1:50. Depletion of antibodies by KIR-Fc conjugates and lack of their binding to KIR expressing NKL the same receptor as applied soluble form confirmed the quality.

In order to test binding of activating KIR-Fc conjugates to lymphoblastoid or leukemic cell lines, KIR-Fc at a concentration of 50 µg/ml were pre-incubated for 20 min. at 4°C in the dark with the PE-conjugated goat anti-human IgG (One

Lambda) at 1:100 dilution. Meanwhile, cells (10^5 per single staining) were washed twice with FACS buffer (2% FCS in PBS) in 96 U-bottom well Falcon plates (Becton Dickinson, Franklin Lakes, NJ, USA). Afterwards, the cells were incubated for 30 min. at 4°C in FACS buffer in the dark with labeled KIR-Fc conjugates, washed twice, and stained with a viability dye (Sytox blue, Invitrogen, Carlsbad, CA) in dilution of 1:20000. Additional staining with the PE-conjugated goat anti-human IgG (without KIR-Fc) was performed as control of unspecific binding of the secondary antibody. Since the KIRs are highly homologous, they constituted isotype controls to each other and differences in their binding guarantee the interaction via KIR domains, rather than via IgG-Fc. For additional confirmation of KIR-Fc binding specificity, KIR-Fc conjugates were biotinylated by Lightning-Link Type A Biotin Antibody Labeling Kit (Novus Biological, Cambridge Science Park, Cambridge, UK) according to the manufacturer's manual, added to target cell lines, which were pre-incubated with KIR-Fc blocking reagent in dilution 1:20 for 20 min. in 4°C, and detected by conjugate of streptavidin-PE (Southernbiotech, Birmingham, UK) in 1:50 dilution.

For primary NK cells functional assays the flow cytometric sequential staining according materials and methods section of Project 3. was performed. As degranulation marker anti-CD107a-V450 antibody (BD Biosciences) was applied.

The HLA class I expression on cell lines was evaluated by PE-labeled anti-HLA class I antibody (W6/32, Biolegend). In blocking assays, the incubation with unlabeled anti-HLA class I antibody (W6/32, Biolegend) at a concentration 50 µg/ml for 30 min. at 4°C and two washes with FACS buffer prior to incubation with KIR-Fc conjugated, as well as anti-HLA-C antibody (DT9) at the same concentration received as a kind gift from R. Apps (Institute of Pharmacology and Physiology, University of Bristol, Bristol, UK) were applied. All analyzes were performed on a Cyan ADP Beckman-Coulter flow cytometer (Beckman Coulter). FACS plots were prepared with FlowJo software v. 9.4.

Functional assays

The functionality of activating KIRs in NKL cell line was determined by redirected cytotoxicity chromium release assay using as target cells the mouse mastocytoma cell line expressing FcγR receptor as earlier mentioned [13]. Two million target

cells were first incubated for 1 hour in 37°C with 200 μCi of radioactive chromium (^{51}Cr isotope, Hartmann Analytic GmbH, Braunschweig, Germany) in 400 ul of RPMI medium. Afterwards, these cells were washed and incubated with unlabeled anti-panKIR2D antibody (NKVFS1, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) at a concentration of 1 mg/ml for 30 min. in 4°C, mixed with respective effector cells in effector:target (E/T) ratios 40:1, 20:1, 10:1 and 5:1, and incubated for 4 hours in 37°C in U-bottom 96-well plate. Total reaction volume was 200 ul. All samples were analyzed in triplicates. The efficacy of target cell lysis by effector cells was proportional to released radioactive chromium in 50 ul of medium containing released radioactive chromium, transferred to LumaPlate-96 (PerkinElmer, Waltham, MA, USA), dried overnight and measured by Top Count NXT (PerkinElmer) microplate scintillation and luminescence counter. Total lysis was calculated in percentage using the formula: (specific release - spontaneous release)/(total release - spontaneous release) × 100.

Further functional assays used the 721.221, 721.221C1 and 721.221C2 cell lines as target cell with additional application of KIR-blocking antibodies anti-KIR2DL1/DS1 (EB6b, Beckman Coulter, Brea, CA, USA) and anti-panKIR2D antibodies. Due to use of primary NK cells in functional assays checking functional consequences of activating KIR binding to a target cell, degranulation was evaluated by assessment of CD107a as previously recommended [36]. E/T ratio was low (40:1) in order to avoid maximal cytotoxicity effect and exhaustion of effector cells. The assay was carried out for 6 hours after mixing the cells, from which after 1 hour brefeldin A (Biolegend) in ratio 1:1000 was added.

Cell line	Origin or leukemia type	EBV status
697	Acute lymphoblastic leukemia (cALL) (B-cells)	neg
721.221	B-cell lymphoma	pos
721.221C2	B-cell lymphoma	pos
AML-193	Acute monocytic leukemia AML (FAB M5)	neg
BM21	Lymphoblastoid cell line (B-cells)	pos
BV-173	Chronic myeloid leukemia (Pre-B CML)	neg
Daudi	Burkitt lymphoma (B-cells)	pos
DOHH2	Non-Hodgkin Lymphoma (B-cell)	pos
HEL	Erythroid leukemia (monocyte-macrophage AML M6)	neg
HL60	Acute myeloid leukemia (FAB M2)	neg
Jiyoye	Burkitt lymphoma (B-cells)	pos
Jurkat	Acute lymphoblastic leukemia ALL (T-lymphocytes)	neg
K562	Chronic (erythrocytic-megakariocytic) leukemia CML	neg
Kasumi-1	Acute myeloid leukemia AML (FAB M2)	neg
MOLM13	Acute myeloid leukemia AML (FAB-M5a)	neg
Namalwa	Burkitt lymphoma (B-cells)	pos
NB-4	Acute promyelocytic leukemia APL (FAB M3)	neg
Raji	Burkitt lymphoma (B-cells)	pos
REH	Acute lymphoblastic leukemia (pre-B ALL)	neg
SD-1	Acute lymphoblastic leukemia (pre-B ALL)	pos
SUP-B15	Acute lymphoblastic leukemia (pre-B ALL)	neg
SPL	Lymphoblastoid cell line (B-cells)	pos
THP-1	Acute monocytic leukemia AML (monocytes)	neg
U937	Hystiocytic leukemia (monocytes)	neg

Table 1. Lymphoblastoid or leukemic cell lines used for KIR-Fc binding screening.

Results

Control assays confirm the KIR2DS1, but neither KIR2DS2 nor KIR2DS4 specificity

After production of activating KIR-Fc conjugates, their specificity was checked using different methods. Using the previously described depletion assay, binding of anti-KIR antibodies to KIRs expressed on cell lines was abolished by pre-incubation with saturating amounts of KIR-Fc conjugates [29]. All conjugates showed the expected specificity (data not shown).

Since there exists literature data concerning ligands for KIR2DS1, KIR2DS2 [9-11, 18, 37, 38] and KIR2DS4 (HLA-A*1102, -C*0501, -C*1601 and -C*0202) [13], we next compared the binding of those activating KIR-Fc to HLA-bearing beads with existing published data [31, 32]. The working concentration of conjugates was optimized and set up to 50 µg/ml. The screening was carried out with a set of beads coated with a broad panel of HLA class I allotypes allowing to dissect precisely the binding specificity towards HLA-A, -B, and -C molecules. As expected, the binding of KIR2DS1-Fc to molecules from HLA-C2 subgroup was observed (Figure 1), but in contrast to previous data [11, 13], no binding of KIR2DS4 to HLA-A*1102, HLA-C*0202, HLA-C*0501 and HLA-C*1601 was detected. In general, no activating KIR conjugates exhibited binding to HLA-A or HLA-B molecules (data not shown).

Next, we tested KIR-Fc conjugate specificity by assessing binding to cell lines and primary cells. First, EBV-positive lymphoblastoid cell lines of known HLA configuration were used. Based on previous results, the KIR2DS1-Fc conjugate was tested in the binding assay with cell lines expressing a KIR2DS1 ligand (BM21, C2/C2) or devoid of a ligand (SPL, C1/C1). As shown in Figure 2A, strong binding of KIR2DS1-Fc conjugate to the BM21 cell line was noted, whereas no interaction with the SPL cell line was seen. The same result was obtained applying the HLA negative 721.221 cell line transfected to express single HLA-C1 and HLA-C2 subgroup antigens (data not shown). Finally, weak binding was also detected to healthy donor peripheral mononuclear cells, if cells were derived from a donor carrying the HLA-C2 specificity (Figure 2B).

Further screening reveals new binding capabilities of activating KIRs

Based on literature data indicating an advantage in leukemia-free survival in AML patients receiving allogeneic hematopoietic stem cell transplantation from a donor carrying activating KIRs [23], we next screened twenty leukemia cell lines with KIR-Fc conjugates. The cell lines included in our studies were diverse in terms of cell type, their development stage, type of leukemia, as well as expression of HLA molecules on their surface and EBV-infection status (Table 1). The screening revealed binding of both KIR2DS3-Fc and KIR2DS5-Fc conjugates to K562, HEL and Namalwa cell lines, but not the others (Figure 2C). The selective binding of only some, but not all KIR-Fc suggests specificity. To confirm that, KIR-Fc conjugates were biotinylated and added to target cells, which were earlier incubated with FcR blocking reagent. The binding detection was made by streptavidin-PE conjugates (Figure 3).

The KIR-Fc conjugates exhibit rather weak binding affinity

We next assessed binding affinity of activating KIR-Fc conjugates. Inhibitory KIRs demonstrate a broad range of affinity with K_d values between 10 nM to 1 µM [39], while the K_d of the KIR2DS1 to HLA-Cw4 was estimated to be 30 µM [9]. That is in accordance to regulatory mechanisms of NK cells, where affinity of activating KIRs should be equal or lower than that of inhibitory counterparts in physiologic conditions. Therefore, the mentioned values were a reference for our measurements. We performed flowcytometric binding assays of KIR-Fc conjugates to target cells in serial dilutions. Based on median fluorescence intensity (MFI), with highest values corresponding to saturation, we estimated the following K_d values: 0.8 µM, 5.0 µM and 7.0 µM at room temperature for KIR2DS1-Fc (BM21 cells), KIR2DS3-Fc and KIR2DS5-Fc (HEL and Namalwa cells) respectively taking into account their 102 kDa molecular mass (Figure 4). The K_d value of KIR2DS3-Fc and KIR2DS5-Fc conjugates binding to K562 cell line was estimated to approximate 10 µM for both of them (data not shown).

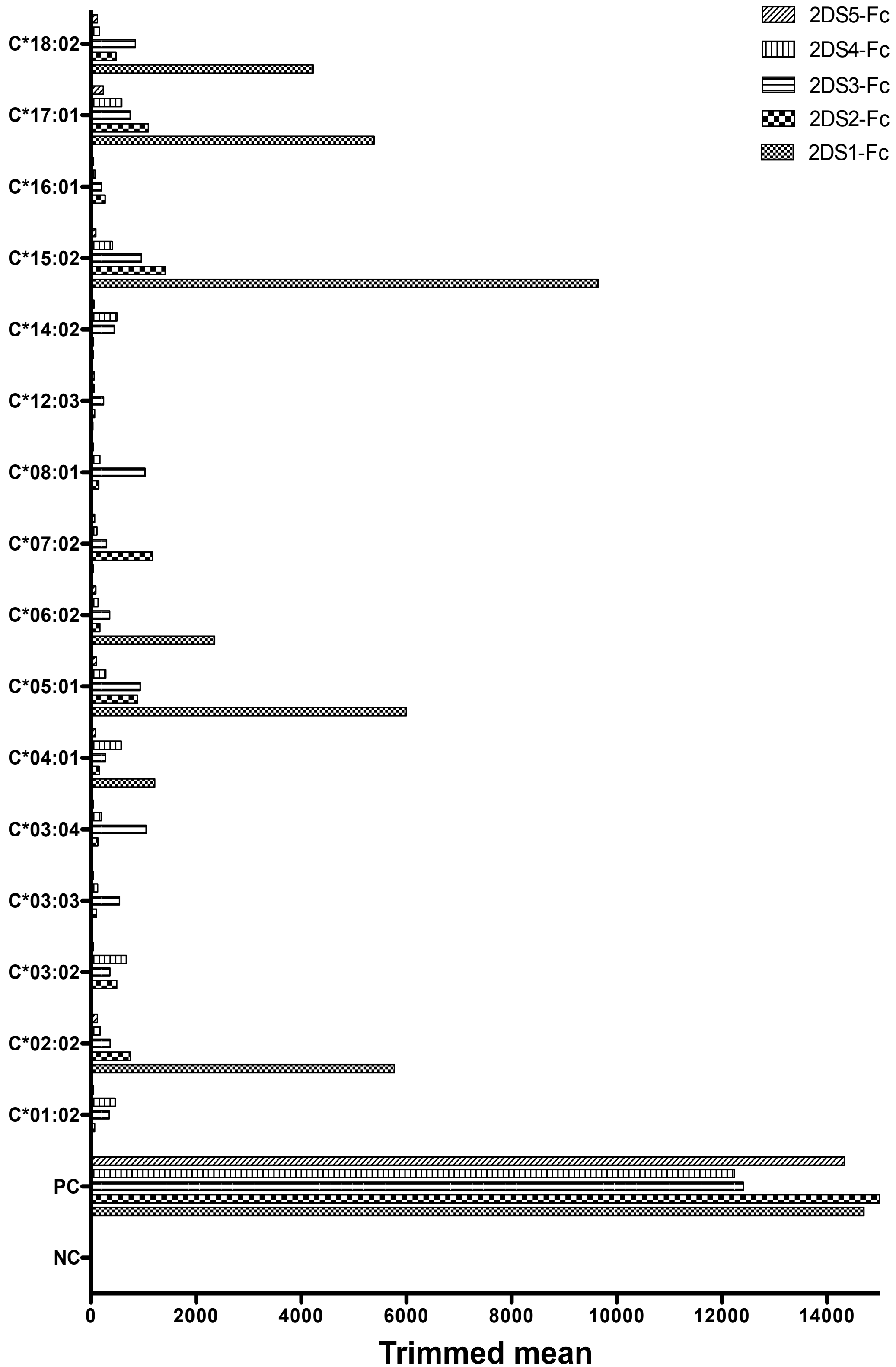

Figure 1. Binding of human KIR2DS-Fc conjugates (KIR2DS1-Fc, KIR2DS2-Fc, KIR2DS3-Fc, KIR2DS4-Fc and KIR2DS5-Fc) to HLA-C (C1 and C2) coated beads presented as trimmed mean. NC - negative control, PC – positive control.

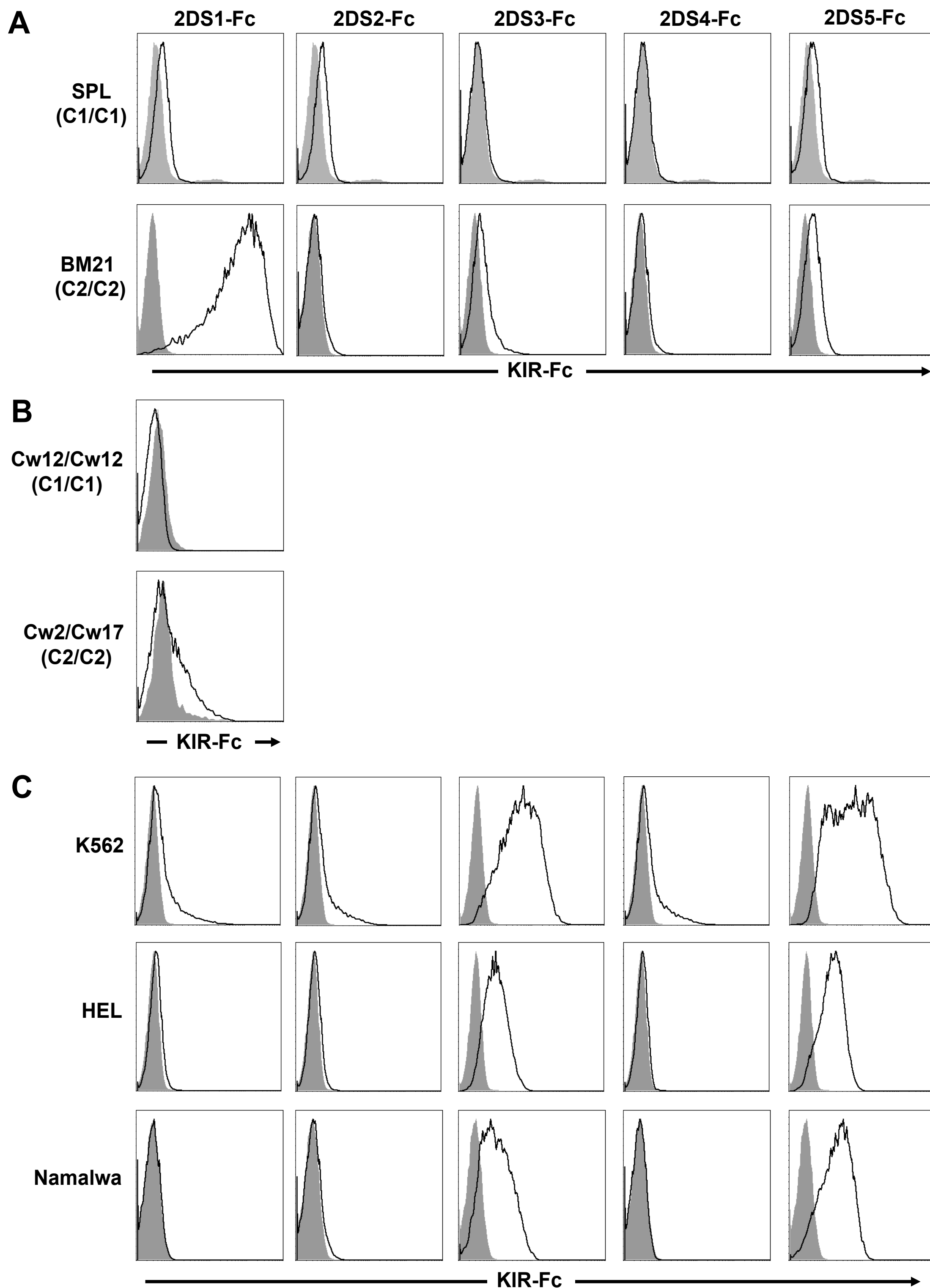

Figure 2. A) Binding of KIR2DS-Fc conjugates (KIR2DS1-Fc, KIR2DS2-Fc, KIR2DS3-Fc, KIR2DS4-Fc and KIR2DS5-Fc) to EBV-positive lymphoblastoid SPL (C1/C1) and BM21 (C2/C2) cell lines. **B)** Binding of KIR2DS1-Fc to PBMC from donors being either C1/C1 homozygote (upper panel) or C2/C2 homozygote (lower panel). **C)** Binding of KIR2DS-Fc conjugates (KIR2DS1-Fc, KIR2DS2-Fc, KIR2DS3-Fc, KIR2DS4-Fc and KIR2DS5-Fc) to leukemic cell lines (K562, HEL, Namalwa). In all cases the KIR2DS-Fc binding is represented by black solid line and secondary antibody alone binding is presented by gray filled histograms.

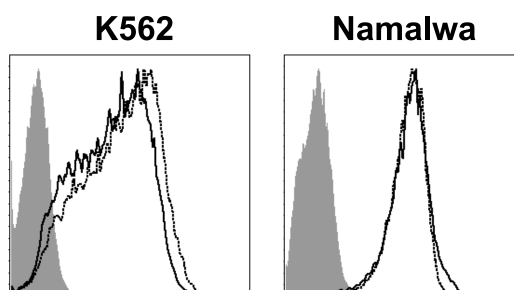

Figure 3. Binding specificity test with biotinylated KIR2DS5-Fc to K562 (left panel) and Namalwa (right panel) cells. The continuous line shows the binding of only KIR2DS5-Fc conjugate, dashed line depicts the binding of KIR2DS5-Fc conjugate after application of FcR blocking reagent and gray filled histograms illustrates the binding of secondary goat anti-human IgG antibody only.

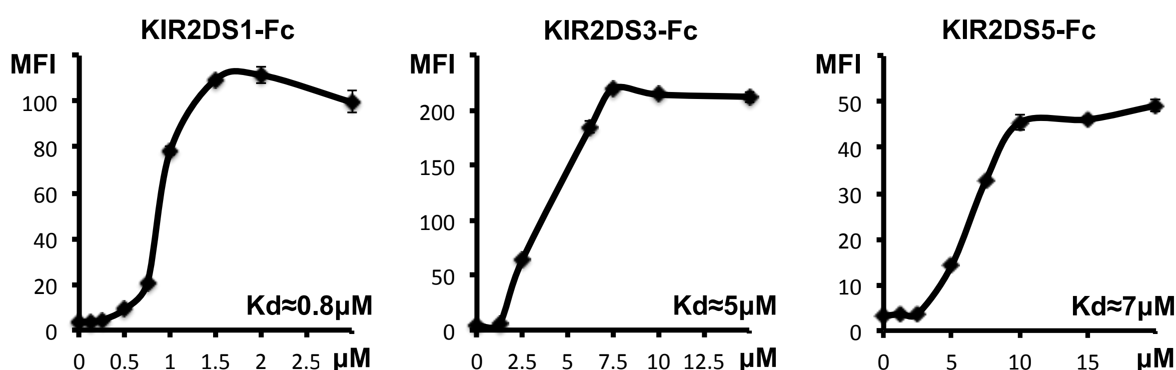

Figure 4. Binding strength of KIR2DS1-Fc (left panel), KIR2DS3-Fc (middle panel) and KIR2DS5-Fc (right panel) conjugates assessed measuring the median fluorescence intensity (MFI) of respective KIR-Fc conjugates by flow cytometry in series of dilution (different molar concentrations).

W6/32 antibody blocks the binding of KIR2DS3-Fc and KIR2DS5-Fc to HEL and Namalwa cell lines

The interaction of inhibitory KIRs with HLA class I molecules and the high structural similarity of KIRs suggests that some HLA class I molecules alone or as complexes with peptides could be potential ligands for activating KIRs. Therefore, inhibition of KIR-Fc conjugate binding by pre-incubation with anti-HLA-A,-B,-C (clone W6/32) and anti-HLA-C (clone DT9) antibodies was carried out. Binding of the KIR2DS1-Fc conjugate to HLA-C2 on the BM21 cell line was only slightly reduced by W6/32 antibody (Figure 5A, upper panel). Similarly, the DT9 antibody did not block the interaction of both KIR2DS1-Fc conjugates (Figure 5A, lower panel). In contrast, the W6/32 antibody totally abolished the binding of KIR2DS3-Fc (data not shown) and KIR2DS5-Fc to the HEL and Namalwa cell lines (Figure 5B). The DT9 antibody did not interfere with the interaction of the KIR2DS3-Fc

(data not shown) KIR2DS5-Fc conjugate with both leukemic cell lines (Figure 5B).

Killing of KIR2DS5-binding and KIR2DS5 non-binding cell lines by KIR2DS5 positive primary NK cells

Functional consequences of a cell ligand binding to KIR2DS5-Fc may be an increased capacity for NK cell activation and elimination of leukemic cells by corresponding receptor. Therefore, we performed a functional assay applying the cross-reactivity staining method (described in materials and methods section of Project 3). This would allow us to evaluate differences in degranulation in KIR2DS5+ primary NK cells against K562 (KIR2DS5-staining) or 721.221 (non-staining) cell lines. Both cell lines are devoid of HLA expression on their surface, so no blocking effect from interaction with inhibitory KIRs is likely. We compared relative degranulation of KIR2DL1-/S1-/S5-, KIR2DL1+, KIR2DS1+/DS5+ and KIR2DS5+ NK cell subpopulation in donors homozygous for HLA-C2. As expected due to licensing, the KIR2DL1-/S1-/S5- NK cell subpopulation degranulated less than KIR2DL1+ NK cells (Figure 6A) but more strongly than KIR2DS1+S5+ NK cells (Figure 6B). In turn, KIR2DS5+ NK cell subpopulation degranulated at a similar level as KIR2DL1-/S1-/S5- NK cell subpopulation against K562 cells, but significantly less against 721.221 cells (Figure 6C). That may suggest an input of KIR2DS5 receptor in NK cell activity and its functionality.

Functionality test of single activating KIR expressing NKL cell lines

Single activating KIR-transduced NKL cells would be a useful tool to study the function of aKIRs. Activating KIRs were therefore expressed on the surface of NKL cells by retroviral transduction. The functionality of expressed receptors was tested by a redirected cytotoxicity assay against mouse P815 cells. As shown in Figure 7A (left panel), the NKL cells do not lyse P815. NKL transfected with KIR2DS1 lysed P815 in the presence of a redirecting antibody (Figure 7A, right panel). Similar data were obtained for the other single activating KIR expressing NKL cells, except for NKL2DS3, which showed rather weak and intracellular expression of the receptor (data not shown). This confirmed the presence and functionality of all cellular elements needed for signaling and degranulation.

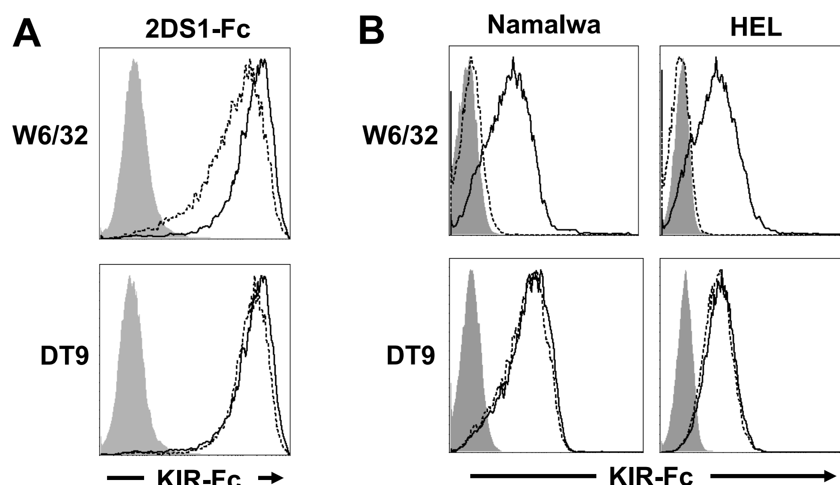

Figure 5. Histograms show influence of anti-HLA-A,B,C (W6/32) antibody (upper row) and anti-HLA-C (DT9) antibody (lower row) on binding: **A)** of KIR2DS1-Fc (left side) to a ligand on the surface of the BM21cell line and **B)** of KIR2DS5-Fc to a ligand on the surfaces of Namalwa (left side) and HEL (right side) cell lines. In all panels continuous line shows the binding of only KIR2DS-Fc conjugate, dashed line depicts the binding of KIR2DS-Fc conjugate after antibody blocking and gray histograms illustrates the binding of secondary goat anti-human IgG antibody only.

The use of single activating KIR expressing NKL cells against P815 cell line is, however, a very artificial system. Confirmation in another assay is necessary. Therefore killing assays of NKL2DS1 and NKL2DS2 effector cells against 721.221 cell lines expressing HLA-C1 or HLA-C2 ligands with and without KIR2L1/DS1 blocking antibody were performed (Figure 7B). In every case difference decrease of lysis was observed in the presence of antibody. Similar results were obtained against K562 cells and no difference was visible, when anti-panKIR antibody was applied (data not shown). This suggests that reduced lysis was rather due to depletion of effector cells than due to blocking of an activating interaction. In summary, this system did not demonstrate functionality when used against physiological ligands and was therefore not used for confirmation of function of activating KIRs.

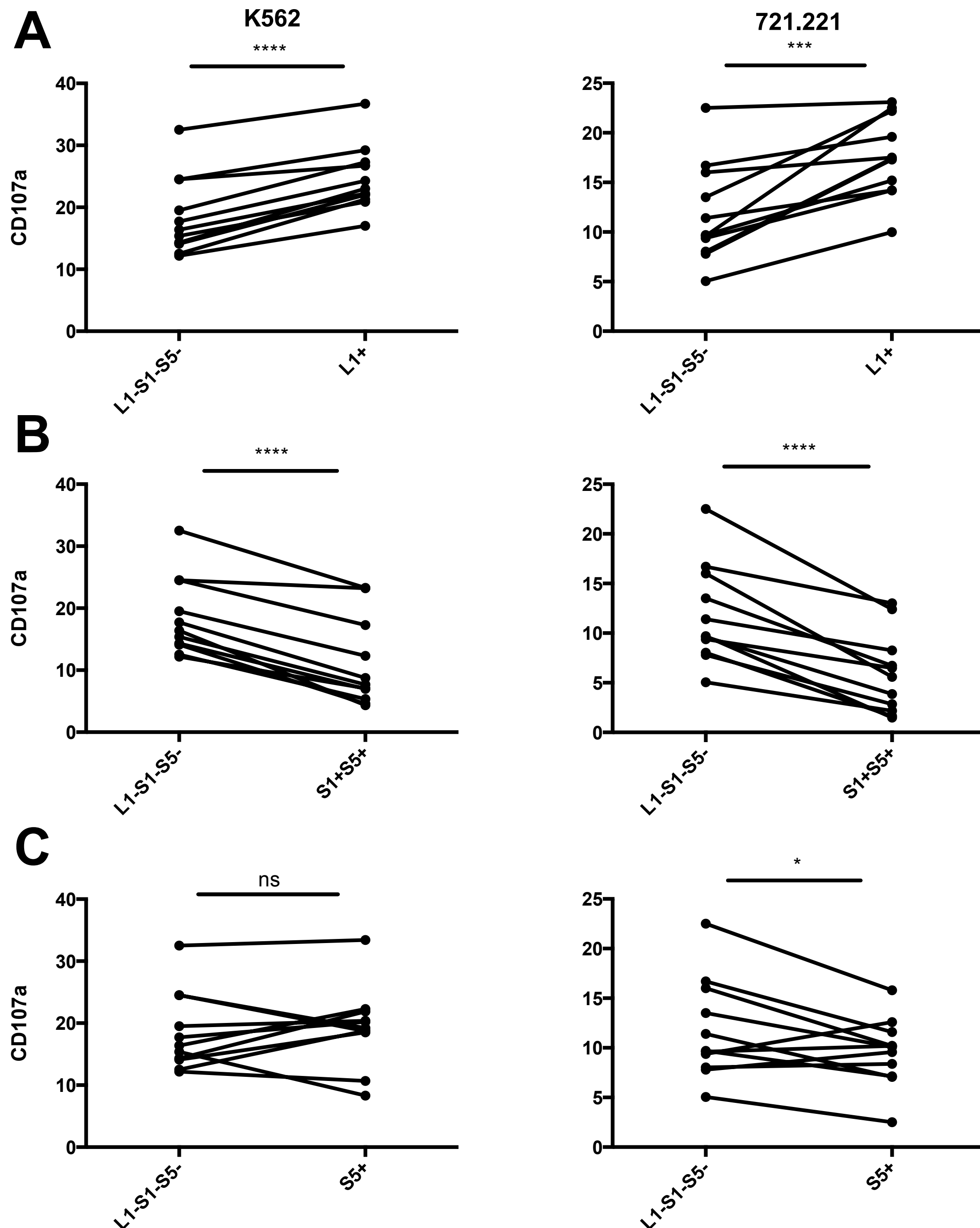

Figure 6. Plots comparing degranulation of different populations of KIR2DL1+, KIR2DS1+ and KIR2DS5+ donors against K562 and 721.221 cell lines **A)** KIR2DL1-DS1-DS5- subpopulation to KIR2DL1+ in K562 ($p<0.0001$) and 721.221 ($p=0.0006$) cells **B)** KIR2DL1-DS1-DS5- subpopulation to KIR2DS1+/S5+ in K562 ($p<0.0001$) and 721.221 ($p<0.0001$) cells **C)** KIR2DL1-DS1-DS5- subpopulation to KIR2DS5+ in K562 ($p<0.6182$) and 721.221 ($p=0.0476$) cells. Statistical calculations were computed by paired t-Student test in 11 samples (n=11).

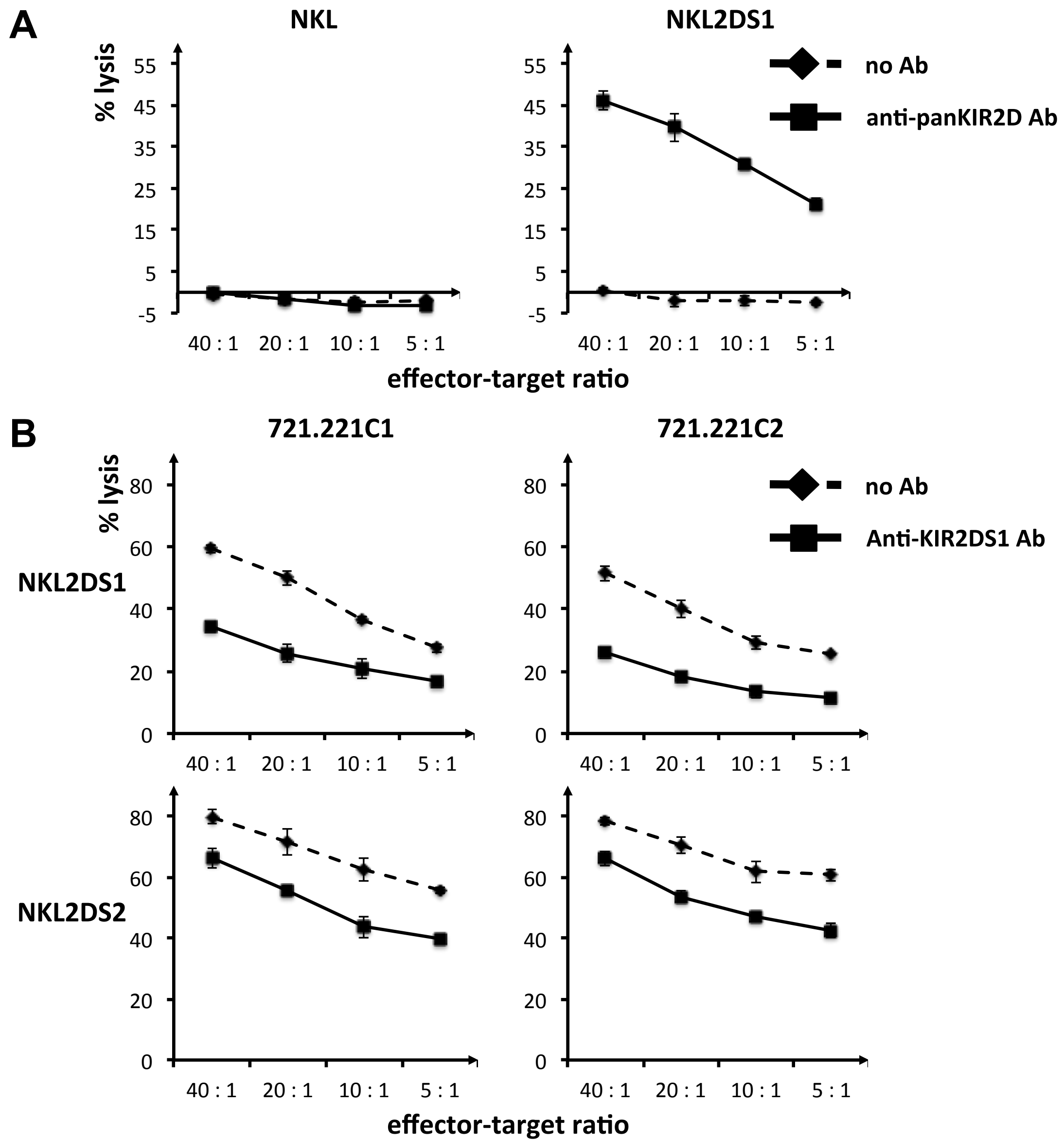

Figure 7. Plots comparing killing efficacy (percentage of lysis) of untransfected and activating KIR transfected NKL cell lines. Mean vales from triplicated wells are taken to evaluate target cells lysis in every E/T ratio (40:1, 20:1, 10:1, 5:1 in presence or absence of respective blocking antibodies **A)** NKL (left panel) and NKL2DS1 (right panel) effector cells against P815 cell lines. **B)** NKL2DS1 killing assay was performed against 721.221C1 (upper left panel) and 721.221C2 (upper right panel). NKL2DS2 killing assay was performed against 721.221C1 (lower left panel) and 721.221C2 (lower right panel).

Discussion

The literature data indicate statistically clear advantages in terms of antiviral protection or antileukemic response in individuals carrying activating KIRs. The more precise explanation of mechanisms driving these beneficial effects could provide valuable information allowing for the prediction of antiviral reaction as well as optimal donor selection for bone marrow transplantation. For this reason we performed the search for ligands of activating KIRs aiming to evaluate more precisely their function.

Similar to previous studies [13, 31] for KIR receptor binding screening we applied KIR-Fc conjugates of human origin and produced in insect cells in order to increase the yield. The binding of KIR2DS1-Fc to HLA-C (C2 subgroup) was confirmed, but no binding of KIR2DS2-Fc by use of HLA-coated beads was visible [40]. This is in contrast to recent data, where the recognition of HLA-C (C1 subgroup) by that receptor was demonstrated on primary cells [11]. That inconsistency may be explained by differences in experimental systems: the interaction between HLA class I molecules and soluble forms of KIRs may not be equivalent to the situation when the molecules are placed on cellular membrane. Cellular assays might be better in visualization of low avidity interactions. In case of the KIR2DS4-Fc conjugate, the lack of binding shown previously [13] probably results from use of another HLA-beads kit, which may contain a different repertoire of peptides in HLA context, which would confirm the dependence of the interaction on a peptide [16]. Unfortunately, no exact source of those HLA molecules is given from manufacturer. Similarly, we could confirm the KIR2DS1-Fc binding on C2/C2 lymphoblastoid cell line, but not the binding of KIR2DS2-Fc to SPL (C1/C1) cell line. The weak binding strength of KIR2DS1-Fc to C2/C2 primary cell line mirrors the physiological state where it has to be easily counterbalanced by inhibitory KIR, preventing the NK cell activation.

In the following binding screening a new specificity of KIR2DS3-Fc and KIRDS5-Fc conjugates was discovered. Considering the intracellular physiological expression of the first [41], for its activity an additional factor causing externalization on the cellular membrane may be necessary. Taking into account the parameters characterizing these three binding cell lines like their origin, cell

type, and viral status (Table 1 and 2), all mentioned features could be excluded as binding influencers. HEL cells represent monocyte-macrophage AML and the binding confirms the role of activating KIRs against the disease in general [23, 26], but no binding was seen in case of the others AML cell lines. In case of the Namalwa cell line, there is no literature proof for interaction of KIR2DS5 receptor with Burkitt's lymphoma blasts. Surprisingly, no binding of KIR2DS1-Fc was seen in any leukemic cell line. That may be caused by low expression of HLA by leukemic cells [42, 43] or peptide repertoire change in malignant transformed cells. KIR2DS3-Fc and KIR2DS5-Fc binding to HLA class I negative K562 cell line suggests a ligand, which is structurally similar to HLA class I molecules. The potential candidates could be searched among HLA-related stress-induced molecules MICA and MICB or in ULBP protein family. That hypothesis requires further investigation.

The studies of binding affinities of KIR-Fc conjugates indicated a micromolar (μM) range of K_d value, which is weaker than for inhibitory KIRs (range from 10 nM to 1,0 μM) [6, 18, 39] This may be expected as stronger binding of inhibitory receptors to a HLA molecule on a "healthy" cell possibly prevents interaction of activating receptors and triggering the killing by NK cells. That affinity may however depend on different factors – polymorphisms of KIR itself as well as HLA-presented peptide, if the potential ligand is an HLA member, and be shifted towards activating KIRs.

Cell line	A1	A2	B1	B2	C1	C2
HEL	03:01	32:01	35:01	35:08	04:01	04:01
Namalwa	03:01	68:02	07:02	49:01	07:01	07:02

Table 2. HLA genotyping results of KIR2DS3-Fc and KIR2DS5-Fc binding cell lines.

The blocking of interaction by W6/32 antibody abolishes only in a small part the interaction between KIR2DS1 to HLA. The crystal structure of the KIR-HLA interaction, however, clearly shows the KIR binding to HLA-C2 molecules and is dependent on positions 80 in the α1 groove from the upper side involving also contact with amino acids P7 and P8 of the peptide [16, 17, 21]. It is very probable that W6/32 does not constitute a steric hindrance for KIR2DS1-Fc binding. In contrast, the W6/32 antibody totally abolishes KIR2DS3-Fc and KIR2DS5-Fc binding to HEL and Namalwa cell lines, which confirms that HLA class I molecules

are their ligands [16, 18, 44]. The genotyping results and the difference in blocking effect by W6/32 and DT9 antibodies rather exclude binding of KIR2DS3-Fc and KIR2DS5-Fc to ligands from HLA-C group. The HEL and Namalwa cell lines share the same subtype of HLA-A (*03:01) molecule, but no binding to another cell line carrying it – WT47 (data not shown) excludes it as ligand itself. More probably, a combination of HLA-A or HLA-B molecules in complex with presented peptides creates their binding domains, as demonstrated by Fadda et al. [45]. The DT9 antibody seems to bind to a location, which does not interfere with KIR binding epitopes.

The use of the HLA class I negative K562 cell line [46] binding KIR2DS5-Fc on its surface makes it possible to evaluate the potential killing effect of KIR2DS5 receptor in primary NK cells. The HLA-negative cell line 721.221 was used as a negative control. We detected a modest difference in reactivity of KIR2DS5-expressing NK cells against K562 and 721.221: degranulation of KIR2DS5+ cells was lower against 721.221 but not against K562, when compared to KIR2DL1-/DS1/DS5- cells. This may be indirect evidence for an activating interaction between KIR2DS5 and a ligand expressed on K562, but will need further study.

Experiments using activating KIR transfectants in order to evaluate more precisely the function of receptors were not successful despite their high activity in redirected cytotoxicity assay. The reason for that may be relative low affinity of KIRs and overcoming of their function by receptors from other groups present on NKL cell line (e.g. 2B4, NKG2C, NKG2D etc.) blocking possible interactions.

Collectively our data show new potential binding possibilities for activating KIRs within or outside of the HLA class I context. Additionally we demonstrate moderate modulation of NK cell activity by KIR2DS5 receptor. Further investigations including immunoprecipitation and mass-spectrometry in order to identify these ligands as well as further functional assay are necessary for prediction of possible KIR2DS5 receptor diagnostic or immunotherapeutic application.

Acknowledgments

We kindly thank:

Jean Villard (Immunology and Transplant Unit, Geneva University Hospital, Switzerland) for HLA genotyping of cell lines,

Kurt Ballmer-Hofer (Laboratory of Biomolecular Research, Department of Biology and Chemistry, Paul Scherrer Institute, Villigen, Switzerland) for help in developing of KIR-Fc conjugates protocol and production,

Richard Apps (Institute of Pharmacology and Physiology, University of Bristol, Bristol, UK) for DT9 antibody,

Stephan Grzesiek, Irena Burmann, Marco Rogowski (Biozentrum, University of Basel, Basel, Switzerland) for help in production of KIR-Fc conjugates and

Aleksandra Wodnar-Filipowicz (Department of Biomedicine, University Hospital Basel, Basel, Switzerland) for providing the leukemic cell lines.

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75-80.

FINAL DISCUSSION AND PERSPECTIVES

In the last years, the significant contribution of KIR to the immune response of NK cells against viral infections and tumors became clear. The possibility to better predict outcome of malignant and infectious diseases by assessing KIR genotype and phenotype, and the promising immunotherapeutic potential of NK cells encourages deeper studies.

Strategies to predict therapeutic response in order to improve treatment efficacy and decrease costs are used more and more frequently. For example, the search for markers predicting immunologic responses in HIV infected patients undergoing HAART identified a subgroup of patients carrying CX3CR1-249I or CX3CR1-280M alleles with superior response [130]. Similarly, we investigated the possibility to screen for factors associated with progression to AIDS like: KIR, their HLA class I ligands and SNPs as potential predictors of control of viral replication after STI. We found a modest association of HLA-Bw4 with low viral loads after STI. This strategy might be expanded in the future, e.g. by including HLA-B alleles, and particular SNPs (HLA-C-35, HCP5, ZNRD1, CCR5 Δ32 and CCR2 V64I), CCR5 haplotypes and CCL3L1 copy number variation which have previously been used to distinguish long-term non-progressors, from progressors, or even rapid progressors [131]. If such an expanded panel would be useful in the setting of STI remains unresolved at this time.

Our further studies concerning an antiviral role of NK cells were directed towards their interaction with CMV. CMV was chosen as target pathogen, as previous studies have indicated that CMV infection influences NK cell phenotype [132]. Moreover, recent studies showed protection from CMV reactivation after kidney transplantation and after stem cell transplantation [102] in patients carrying activating KIRs [105]. In contrast to our study, which detected an influence of CMV infection on KIR repertoire only after in vitro challenge with the virus but not in resting NK cells, a similar study investigating a larger cohort identified changes also in resting NK cells between CMV seropositive and –seronegative donors [133]. The expansion of functional NK cell subsets expressing KIR2DL1 and KIR2DL3 in the presence of their cognate ligands triggered by co-cultivation with CMV-infected fibroblasts is possibly caused by dynamic changes in MHC class I

expression and substitution of presented peptide repertoire [134]. Presentation of viral peptides has been shown to decrease interaction affinity and NK cell inhibition provoking a “missing-self” effect. Similarly to the studies of Beziat et al. [133] we demonstrate increased NKG2C expression in response to CMV, suggesting possible synergy of NK cell surface molecules within an immunological synapse. The role of each component requires, however, further investigation. Our results confirm the potential role of KIR3DS1 receptor, belonging to the telomeric part of the KIR haplotype, in the response to CMV infection. In contrast to the protective role of KIR3DS1 in HIV, which occurs only in the presence of HLA-Bw4, we found no such association in our studies on CMV.

In order to better understand possible roles of activating KIRs, we created soluble forms of activating KIRs, and single activating KIR transduced cell lines. This allowed us to screen commercially available anti-KIR antibodies for cellular specificities. We found new specificities including KIR2DS3 and KIR2DS5, which we applied for a new method of flow cytometric analysis.

The binding of KIR2DS3-Fc and KIR2DS5-Fc conjugates to HEL and Namalwa cell lines, is inhibited by an anti-HLA class I antibody and HLA genotyping results strongly suggest HLA-A or HLA-B in complex with peptides as ligands. The important role of peptides in the KIR-HLA interaction was previously demonstrated for KIR2DL3 and HLA-Cw3 [135, 136]. Identification of peptides allowing KIR2DS3 and KIR2DS5 binding is currently ongoing.

The KIR2DS5-Fc binding to the HLA deficient cell line K562 is similar to the previously reported binding of KIR2DS4 to melanoma cell lines [137]. Consequences of binding *in vivo* are difficult to predict. Our functional assay applying sequential antibody staining showed a tendency of higher response of KIR2DS5+ NK cells against K562 (KIR2DS5-Fc staining, blastic phase of chronic myeloid leukemia) than against 721.221 (KIR2DS5-Fc non-staining). Interestingly, a small retrospective study found a protective role of donor KIR2DS5 in reducing relapse after allogeneic HSCT for chronic myeloid leukemia [138].

In summary, our data suggest a high diagnostic potential of activating KIRs. The identification of their ligands could not only help in developing a more detailed understanding of their function, but also improve their predictive application. Similarly, the effect of KIRs in hematopoietic stem cell transplantation, in terms of

leukemia elimination and viral reactivation, as an immunotherapeutic tool is worthy of further investigation.

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