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Pro-Inflammatory Cytokines

Unleash Natural Killer Cell Potential

For Tumor Therapy

– NK Cells Want To Break Free –

Referees:

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DECLARATION

I hereby declare that the thesis at hand has been written by myself, independently and with no other sources and aids than quoted. The work described in this thesis was performed at the German Cancer Research Center (DKFZ), Heidelberg, Germany in the research group 'Innate Immunity' under the supervision of PD Dr. Adelheid Cerwenka. This thesis has not been submitted to another university for dissertation.

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Für meine Eltern.

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1. ZUSAMMENFASSUNG

Natürliche Killer (NK)-Zellen bilden als Teil des angeborenen Immunsystems die erste Abwehr gegen Virusinfektionen und maligne Transformationen und stellen daher einen vielversprechenden Anwendungsbereich für die Immuntherapie gegen Krebs dar. Die vollständige Aktivierung der NK-Zellen wird jedoch durch inhibitorische Signale eingeschränkt, die durch die Interaktion von inhibitorischen Killer-Immunglobulin-ähnlichen Rezeptoren (KIRs) auf den NK-Zellen mit körpereigenem HLA-I auf autologen Tumor Zellen vermittelt werden. Es ist daher überaus wichtig für die Immuntherapie gegen Krebs, die KIR-vermittelte Hemmung der NK-Zellen zu umgehen, um das volle Potenzial von autologen NK-Zellen ausschöpfen zu können. Obwohl die molekularen Mechanismen der *de novo* Genexpression von KIR-Rezeptoren während der Entwicklungsphase von NK-Zellen ausreichend ergründet sind, ist wenig über eine mögliche Regulation auf reifen NK-Zellen bekannt.

In dieser Studie präsentieren wir eine neue Strategie, wie NK-Zellen vorübergehend von KIR-vermittelter Hemmung befreit werden können, um den autologen NK-Zell–Transfer in der Therapie von HLA-I positiven Tumoren zu verbessern. Stimulation von reifen NK-Zellen, isoliert aus dem menschlichen Blut, mit einer Kombination der Zytokine Interleukin (IL)-12, IL-15 und IL-18 führte zu einer verminderten Oberflächenexpression der bedeutenden inhibitorischen KIR2DL2/L3, KIR2DL1 und KIR3DL1 Moleküle. Im Gegensatz dazu zeigten andere Rezeptoren keine Veränderung oder erfuhren sogar eine Hochregulierung wie z.B. die alpha-Kette des IL-2 Rezeptors (CD25). Die ausgeprägteste Herabregulation der KIR-Rezeptoren war zwei Tage nach Zytokinstimulation sichtbar und konnte auf erniedrigte KIR-mRNA-Spiegel zurückgeführt werden. Die Verringerung der KIR Expression war transient und sowohl die mRNA als auch die Oberflächenexpression der KIR-Rezeptoren konnte durch Kultivierung in IL-2 oder IL-15 erneut induziert und wiederhergestellt werden.

Die verringerte Expression der KIR2DL2/L3 Rezeptoren auf IL-12/15/18–aktivierten NK-Zellen war mit einer geringeren KIR-vermittelten Hemmung und mit einer erhöhten CD16-abhängigen Zytotoxizität verbunden. Vor allem aber ermöglichte die erniedrigte

Expression der KIR2DL2/L3 Rezeptoren eine deutlich verbesserte Zytotoxizität der IL-12/15/18–aktivierten NK-Zellen gegenüber Tumorzellen, die den passenden HLA-I Liganden trugen. Zusätzlich konnten wir eine Herabregulierung von inhibitorischen KIR-Molekülen auf NK-Zellen in einem Infektionsmodell des humanen Zytomegalie Virus (HCMV) beobachten, welches ein physiologisches System mit hohen Konzentrationen an pro-inflammatorischen Zytokinen (wie z.B. IL-12) darstellt.

Zusammenfassend beschreibt unsere Studie einen neuen Mechanismus der Herunterregulation von inhibitorischen KIR-Rezeptoren durch pro-inflammatorische Zytokine auf reifen NK-Zellen. Die herabgesenkte Expression der KIR-Rezeptoren auf IL-12/15/18–aktivierten NK-Zellen führte zu einer verminderten Hemmung durch entsprechende HLA-I Liganden, wodurch potente Effektorzellen für die Behandlung HLA-I positiver Tumore generiert werden konnten. Diese Ergebnisse implizieren, dass die hier aufgezeigte vorübergehende Resistenz gegen KIR-vermittelte Inhibition die Behandlungsmöglichkeiten der Immuntherapie deutlich verbessern könnte, insbesondere bezüglich der Infusion von autologen NK-Zellen.

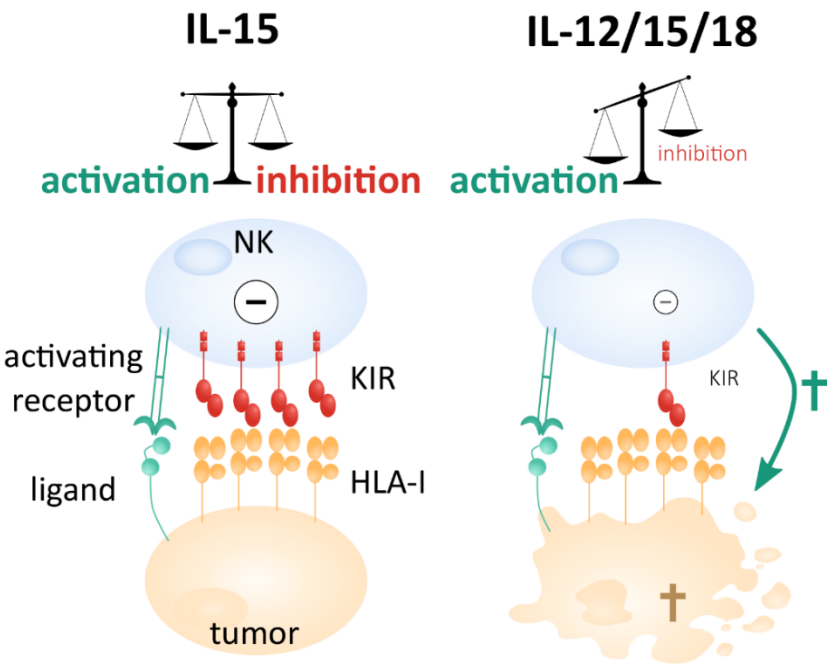
2. SUMMARY

Natural killer (NK) cells form the first line of defense against viral infections and malignant transformation and therefore represent promising targets for cancer immunotherapy. However, NK cell anti-tumor efficacy in the patient is often impaired through inhibitory signals mediated by the interaction of inhibitory killer immunoglobulin-like receptors (KIRs) with self–HLA-I expressed on autologous tumor cells. Therefore, it is crucial to circumvent KIR-mediated self-inhibition in immunotherapy to unleash autologous NK cell potency. Although the molecular mechanisms of *de novo* KIR expression during NK development are well established, little is known about regulation of their expression on mature NK cells.

We here present a new strategy of transiently and safely releasing NK cells from KIR-mediated inhibition to improve autologous NK cells transfer against HLA-I–expressing tumors. Stimulation of mature peripheral blood NK cells with a combination of the cytokines interleukin (IL)-12, IL-15 and IL-18 resulted in decreased surface expression of the major inhibitory KIR2DL2/L3, KIR2DL1 and KIR3DL1 molecules. In contrast, other NK receptors remained unchanged or even got upregulated such as the IL-2 receptor alpha-chain (CD25). Most pronounced KIR downregulation was observed two days after cytokine exposure and was attributed to decreased KIR mRNA levels. Downregulation of KIR expression was transient and KIR mRNA and surface expression could be re-induced upon culture in IL-2 or IL-15. Reduced KIR2DL2/L3 expression on IL-12/15/18–activated NK cells was associated with less KIR-mediated inhibition and increased CD16-dependent cytotoxicity in redirected lysis assays. Importantly, downregulation of KIR2DL2/L3 expression enabled improved cytotoxicity of IL-12/15/18–stimulated NK cells against cognate HLA-I–expressing tumor targets. Additionally, we observed downregulation of inhibitory KIR molecules on NK cells after 3 days in a human cytomegalovirus (HCMV) infection model, representing a physiological setting with high concentrations of pro-inflammatory cytokines such as IL-12.

Taken together, our study reports a novel mechanism of KIR downregulation on mature peripheral blood NK cells by pro-inflammatory cytokines. Decreased KIR expression of

IL-12/15/18–activated NK cells translated into reduced inhibition by self–HLA-I, generating potent effectors cells for the treatment of HLA-I–expressing tumors. These results imply that the transient resistance to self-inhibition might greatly improve immunotherapy protocols especially for autologous NK cell infusions.



IL-12/15/18–induced downregulation of inhibitory killer immunoglobulin-like receptors (KIRs) on NK cells translated into reduced KIR-mediated inhibition and enhanced cytotoxicity against cognate HLA-I–expressing tumor cells, indicating high potency in autologous infusions. Adapted from the graphical abstract from Ewen et al. 2018 Eur. J. Immunol. [710].

3. INTRODUCTION

3.1. The immune system

Every species is constantly exposed to diverse pathogens such as bacteria, viruses, parasites or fungi and has developed strategies to protect the organism from pathogenic threat. Vertebrates have developed a complex system consisting of specialized organs, tissues, effector cells and soluble molecules to fight foreign pathogen and to protect against malignant transformation of the own body. Therefore, the immune system can detect and discriminate healthy from non-self or altered-self cells but is also carefully regulated to not attack healthy tissue. The importance of a functional immune system is illustrated through the link of immune dysfunctions or immunodeficiencies to severe disorders such as autoimmune diseases and an increased risk of lethal infections and of developing cancer [1]. Two major branches of the immune system are distinguished in vertebrates according to their mechanism and specificity of antigen recognition, kinetics of the immune response and the capability of memory formation: The evolutionary conserved innate immunity and the adaptive immunity, which has evolved in vertebrates including rodents and humans [2].

3.1.1. The innate immune system

The innate immune system is characterized by its rapid immune response and based on a germline encoded receptor repertoire recognizing a variety of different pathogens. It is conserved among plants, invertebrates and vertebrates and virtually every species has developed basic defense mechanisms against pathogens. The first defenses of the body are anatomical as well as chemical barriers such as the skin or the acidic milieu in the stomach. An intact epithelial barrier can prevent pathogens to enter the body and mucosal surfaces additionally produce antimicrobial peptides such as β-defensins. Innate immunity is further mediated by humoral (e.g. the complement system) as well as cellular components which include phagocytic cells (macrophages, monocytes, dendritic cells and neutrophils), granulocytes (eosinophils and basophils), mast cells and innate lymphoid cells (ILCs and NK cells) [2].

Cells of the innate immune system express germline-encoded receptors that recognize molecules expressed on stressed and malignantly transformed cells, or molecular microbial structures such as pathogen-associated molecular patterns (PAMPs). These PAMPs are evolutionary conserved structures common to many pathogens and include complex carbohydrates found on bacterial cell walls (peptidoglycans, lipopolysaccharides) and nucleic acids typical for viruses or bacteria (unmethylated CpG DNA, double-stranded RNA). Similar to PAMPs, innate receptors can also detect endogenous ligands associated with cellular stress, so called damage-associated molecular patterns (DAMPs), such as the chromatin-associated protein high-mobility group box 1 (HMGB1), heat shock proteins or chromatin DNA [3].The best characterized pathogen recognition receptors (PRRs) are the Toll-like receptors (TLRs) expressed on phagocytic cells like monocytes, macrophages, dendritic cells or neutrophils [3]. These sensory cells can directly induce effector functions via phagocytosis and degradation of the pathogen, but also orchestrate both innate and adaptive immunity through recruiting and activation of other immune cells. The production of cytokines helps to shape and amplify a specific immune response while chemokines act as chemo-attractants attracting cells from the blood circulation into lymphoid organs or tissues. Phagocytic uptake of pathogens by dendritic cells (DCs), also named professional antigen-presenting cells (APCs), leads to their maturation and migration to the lymph node where they present antigens to adaptive immune cells. Presentation of processed antigens by APCs on their major histocompatibility complexes (MHC) class II is a major mechanism to activate antigen-specific immune cells. In general, many mechanisms of the innate immune system not only eliminate pathogens, but also function to prime and enhance the adaptive immune response [2,4–6].

Another important part of the cellular innate immunity comprises the family of innate lymphoid cells (ILCs), including natural killer (NK) cells, which will be described in detail in section 3.2.

3.1.2. The adaptive immune system

The hallmarks of the adaptive immune system are the delayed, but yet strong and antigen-specific immune response and the capability to generate immunological memory, providing long-lasting immunity. Adaptive lymphocytes develop in the bone marrow from a common lymphoid progenitor (CLP) and comprise B cells, that mature in the bone

marrow, and T cells that further differentiate in the thymus. Each B and T lymphocytes express a unique surface receptor with a single antigen-specificity. The high diversity of the antigen-specific receptors is formed by somatic recombination of various gene segments encoding the B cell (BCR) and T cell receptor (TCR). The random rearrangement of V(D)J gene segments requires the recombinases RAG-1/2 and can result in 10^{14} - 10^{18} receptors with different specificities enabling the adaptive immune system to respond against a vast variety of antigens [7,8]. To ensure tolerance, T cell clones with self-reactive or non-functional TCRs are subsequently deleted or inactivated in the thymus through positive and negative selection [2].

Upon encounter of their cognate antigen, activation of antigen-specific naïve T and B cells leads to clonal expansion in secondary lymphoid organs and differentiation into effector lymphocytes highly efficient against the specific pathogen. The clearance of the infection is followed by a contraction phase and formation of a small pool of long-lived memory cells, which are capable of mounting a faster and stronger recall response after secondary challenge with the same antigen. Long-lasting immunity through the formation of immunological memory marks the basis of successful vaccination. Immunological memory has mostly been accounted to be a feature of adaptive immune cells. However, a growing body of experimental evidence suggests that also innate immune cells can possess adaptive features such as longevity and superior recall responses, blurring the lines of innate and adaptive immunity [9–11]. This aspect will be discussed in more detail in section 3.6.

The B cell receptor (BCR) is a membrane-bound immunoglobulin (Ig) and directly binds native antigens without the need of presentation and can therefore detect extracellular pathogens. In addition, B cells can also function as APCs and present processed antigens on MHC class II. Upon activation and maturation into plasma cells, B cells secrete a soluble form of the BCR, termed antibodies, which constitute the humoral arm of the adaptive immunity and possess the same antigen specificity as the BCR. Antibodies are Y-shaped molecules composed of two identical heavy and two light chains. Depending on the heavy chain, five different antibody or Ig isoforms exist (IgA, IgD, IgE, IgG and IgM) with different functions and localizations in the body. Antibodies further consist of a constant Fc region and two highly variable regions created through somatic recombination, enabling recognition of a vast variety of antigens. Antibodies can either directly neutralize antigens or exert different functional outcome via their Fc part such as opsonization of pathogens

for phagocytes, antibody-dependent cytotoxicity (ADCC) or activation of the complement system [2,12].

The T cell receptor (TCR, CD3) is composed of two chains, the α- and β-chain, that undergo somatic rearrangement and determine the highly variable antigen specificity. The TCR can only recognize antigens that are processed and presented on MHC molecules. $CD4^+$ T helper cells recognize peptides in the context of MHC class II molecules on APCs, while $CD8^+$ T cells interact with MHC class I that is expressed on any nucleated cell. Upon activation, naïve $CD8^+$ T cells differentiate into cytotoxic lymphocyte effector cells (CTLs), which are potent producers of interferon-gamma (IFN-γ) and mediate their cytotoxicity via secretion of perforin and granzymes [2].

In addition to the antigen-specific TCR engagement, T cells require co-stimulatory signals provided by APCs in order to become fully activated. This includes interaction of the B7 family ligands with co-stimulatory CD28 on T cells or with inhibitory CTLA-4, which functions as checkpoint to inhibit excessive T cell activation. In addition, cytokines produced by adjacent immune cells can polarize T cell differentiation into effector cells with different properties and functions. After activation, $CD4^+$ T cells can differentiate into different subtypes of cytokine producing helper cells, depending on the co-stimulatory cytokine milieu. The main lineages comprise regulatory T cells (T_{regs}) and T_H1, T_H2 and T_H17 helper cells, which are defined by their characteristic cytokine expression profile and function [13]. T_H1 cells characteristically produce IFN-γ to activate macrophages, DCs and B cells and to fight intracellular pathogens. T_H1 subsets are differentiated in the presence of interleukin (IL-) 12 and IL-18, whereas IL-4 triggers the polarization of T_H2 cells. Key cytokines of T_H2 cells are IL-4, IL-5 and IL-13 to assist B cells, mast cell and basophils in the control of extracellular parasite infections such as helminths. T_H17 cells produce IL-17 and IL-22 and play a major role in clearance of extracellular bacteria and fungi at mucosal surfaces. Transforming growth factor beta (TGF-β) can induce regulatory T cells (T_{regs}) expressing the transcription factor FoxP3 (forkhead box protein P3) and the IL-2 receptor α-chain (CD25). Regulatory T cells can inhibit other effector cells through secretion of immunosuppressive cytokines e.g. TGF-β and IL-10, limiting an ongoing immune response but also preventing autoimmunity [2].

In addition to T cells with a TCR composed of α- and β-chains, also γδ-T cells exist, which have a limited TCR diversity but can recognize antigens in a non-MHC restricted manner [14].

γδ-T cells exhibit features of both innate and adaptive immunity and are enriched at epithelial surfaces. Another cell type bridging innate and adaptive immunity are natural killer T (NKT) cells, which co-express an αβ-TCR (CD3) and surface markers typically associated with natural killer cells e.g. NK1.1. NKT cells recognize lipids or glycolipids presented by CD1d, are activated by DC-derived IL-12 and subsequently stimulate activate T_H1 cells and CTL mediated immunity [15].

3.2. Natural killer cells

Natural killer (NK) cells are large granular immune cells that constitute about 5 – 10 % of lymphocytes in the peripheral blood. They have been first described in 1975 as a subset of lymphocytes distinct to T or B cells and have been named according to their function of spontaneous or 'natural' cytotoxicity without the need of prior sensitization [16–18]. Observations that these natural killers are specifically cytotoxic against malignant cells lacking expression of self-MHC class I proposed the 'missing self' hypothesis (see section 3.3) of NK specificity [19]. NK cells have traditionally been classified as classical innate immune cells because, unlike B and T cells of the adaptive immune system, they express germline encoded receptors that do not require RAG recombinase-mediated rearrangement [20,21]. However, a recent study has reported a role for the RAG recombinase in NK cell development and cellular fitness [22] and accumulating evidence suggests adaptive features of NK cells, blurring the borders between innate and adaptive immunity [10,23–25].

Natural killer cells act as first line of defense against various pathogens, viral infections and malignant cells [26,27]. Besides their ability to directly eliminate and kill infected and transformed cells, NK cells function through production of cytokines and chemokines that shape the innate and adaptive immune response [28,29]. They provide protection against viral infection and metastatic dissemination and play a central role in pregnancy and graft rejection [30–34].

3.2.1. Definition

NK cells represent a unique subset of lymphocytes, distinct from T and B cells, which recognize their targets in an inherent and MHC–unrestricted manner based on a germline-

encoded receptor repertoire [20]. NK cells belong to a larger family of innate lymphoid cells (ILCs), which are involved for instance in tissue homeostasis and immunity in mucosal tissues [35,36]. Three subsets of ILCs have been described to date based on their functionality and transcription factors, with NK cells classified as part of the subgroup of ILC1 [37–39].

In flow cytometry, human NK cells are usually phenotypically defined as $CD3^{-}CD56^{+}$ lymphocytes. The T cell marker CD3 needs to be excluded, since CD56 is also expressed on $CD3^{+}$ NKT cells [15,40]. In addition to CD56, NKp46 has been proposed as general NK marker due to its expression on mouse NK cells [41–43]. Both markers have their disadvantages since CD56 negative NK cells have been detected in certain diseases [44]. At the same time, expression of NKp46 is not restricted exclusively to NK cells and might be a subject of regulation in certain conditions such as infections or in NK cells with adaptive features [38,43,45]. In order to discriminate NK cells from other ILC1, the transcription factor Eomes, the presence of cytolytic granules and expression of NKp80 are currently suggested as NK cell specific markers [39,46,47].

3.2.2. NK cell development:

Compared to murine NK cell development, most conclusions about NK precursors in humans are derived from *in vitro* differentiation data or from monitoring NK cell reconstitution after hematopoietic stem cell transplantation (HSCT). However, it is wildly accepted in the field that NK cell and ILC development takes place in the bone marrow from $CD34^{+}$ hematopoietic stem cells (HSC) into a common lymphoid progenitor (CLP) that can also give rise to other lymphocytes as B and T cells [28,48,49]. But the bone marrow might not be the only site of NK cell development, as precursors have been found in several other tissues as the thymus, lymph nodes, liver, gut and uterus [50–54]. Extramedullary compartments might represent important sites of NK development and terminal differentiation, shaping NK diversification and functional heterogeneity by the unique milieus of different tissues. *De novo* acquisition of CD122, the IL-15Rβ chain, marks the commitment to the NK cell precursor (NKP) [48–50,55] and IL-15 has been shown to be essential for further differentiation, maturation and the survival of NK cells in the periphery [56–58]. In addition, a complex interplay of multiple transcription factors drives commitment to the NK cell lineage such as ID2, PU.1, Ets-1, TOX, NFIL3 as well as Tbet and Eomes at later stages of NK cell development [59–63]. Immature NK cells (iNK) differentiate

further into mature NK cells (mNK) in the periphery where they gain their effector functions, i.e. the capability of IFN-γ production and cytotoxicity, and sequentially acquire functional receptors such as CD56, CD94, NKG2A, NKp46, NKG2D and eventually CD16 and killer immunoglobulin-like receptors (KIRs) [52,53,64–67].

3.2.3. NK cell subsets

In humans, $CD56^{+}CD3^{-}$ NK cells can be further divided into two subsets on the basis of their CD56 expression levels, into $CD56^{bright}$ and $CD56^{dim}$ NK cells [26,68–70]. The $CD56^{bright}$ subset comprises approximately 10 % of NK cells in peripheral blood but is enriched in secondary lymphoid organs [54,66,70,71]. They are attributed to have a predominantly immunomodulatory role due to their high potential of cytokine production upon monokine (IL-12 or IL-18) activation but contain rather low levels of perforin and granzyme [71–74]. $CD56^{dim}$ NK cells constitute the prevalent subset of human peripheral (pb)NK cells and display a high capacity of target cell-induced cytotoxicity and cytokine production [68,70,73–75]. In addition, $CD56^{dim}$ NK cells express the FcγRIII (CD16), allowing them to exert antibody-dependent cytotoxicity (ADCC) [12]. $CD56^{dim}$ NK cells are less efficient effectors upon cytokine activation in comparison to the $CD56^{bright}$ subset, but are potently stimulated upon activating receptor engagement [71–74]. Increasing evidence suggests that $CD56^{bright}$ cells are precursors of $CD56^{dim}$ NK cells, as the latter possesses lower proliferative capacity associated with shorter telomers [64,66,76]. $CD56^{bright}$ NK cells predominate neonatal tissues and umbilical cord blood and are the first population to be detected after engraftment following HSCT in humanized mouse models and patients [64,77–80]. Several reports suggest linear maturation from $CD56^{bright}$ to terminally differentiated $CD56^{dim}$ NK cells characterized by coordinated changes of surface receptors associated with maturation, activation and homing [64,65,77]. In contrast to $CD56^{dim}$ NK cells, the $CD56^{bright}$ subset is uniformly high for CD94/NKG2A but lacks the expression of CD16 and KIRs [68]. The continuous differentiation from $CD56^{bright}$ ($CD94/NKG2A^{hi}CD16^{-}KIR^{-}CD57^{-}$) to $CD56^{dim}$ ($CD94/NKG2A^{-}CD16^{+}KIR^{+}CD57^{+}$) NK cells is marked by a progressive decrease in NKG2A expression and an inverse correlation of sequential KIR and CD57 acquisition via phenotypical and functional intermediates ($CD56^{dim}CD94/NKG2A^{+}CD16^{+}KIR^{+/-}CD57^{-}$) [64,65,77,81,82].

3.2.4. NK cell effector functions

Natural killer cells serve as the first line of defense in the elimination of transformed or virally infected cells. Their effector function is mainly characterized by granzyme/perforin-mediated cytotoxicity and by the production of cytokines, but can also involve immune-modulatory functions [27]. The integration of all signals delivered by activating and inhibitory receptors decides whether or not the NK cell becomes effectively activated and determines the kind of effector function [83–85]. The final response is further influenced by cytokines, chemokines and additional immune cells in the local microenvironment of the NK cell.

Cytotoxicity

NK cells have several modes of inducing death of a target cells, either via the secretion of perforins and granzymes or via membrane-bound death receptor-related pathways.

Death receptors include the ligand-receptor pairs FasL-Fas (CD95L-CD95), TNF-TNFR and TRAIL-TRAILR of the TNF superfamily [86–88]. The interaction between NK cells expressing death ligands and death receptors on target cells induces the recruitment of various intracellular adaptors leading to the formation of the death-inducing signaling complex (DISC) [89]. The DISC recruits and proteolytically activates the pro-caspases 8 and 10 and thereby initiates the enzymatic caspase cascade, resulting in apoptosis of the target cell [86,90].

The second mechanism of NK cell-mediated target cell killing involves the release of lytic granules containing pre-formed perforin and granzyme [91]. Upon recognition of a target cell, an immunological synapse is formed between both cells, inducing the exocytosis of cytotoxic granules in close proximity to the target cells [92,93]. Perforin molecules form pore-like structures in the membrane of the target cell, thereby disrupting the membrane integrity and allowing the entry of granzyme molecules in the cytoplasm [94]. The serine proteases granzyme A and B induce apoptosis through initiation of the caspase cascade or via mitochondrial depolarization and DNA fragmentation mediated by proteolytic cleavage [95].

Antibody-dependent cellular cytotoxicity (ADCC) also functions via the perforin/granzyme pathway [96,97]. Antibody-coated target cells can be cross-linked by FcγRIII (CD16), the low-affinity receptor for IgG, triggering a strong NK cell cytotoxic response that does not require additional co-activation [12,98,99]. The capacity of strong NK cell effector and anti-cancer

functionality upon ADCC is currently exploited in the clinical application of therapeutic antibodies [100,101].

Cytokine production

NK cells present an important source of various cytokines and chemokines such as IFN-γ, TNF-α, GM-CSF and IL-6 [75]. IFN-γ is considered to be the signature cytokine of NK cells and has been demonstrated to have anti-proliferative and pro-apoptotic effects on cancer cells via upregulation of caspases, FasL and TRAIL [102]. The combined secretion of TNF-α and IFN-γ has been shown to induce senescence in cancer cells [103]. In addition, IFN-γ stimulates antigen presentation on MHC class I and II molecules and promotes macrophage functionality [27,104]. Cytokine secretion and the release of cytotoxic granules are regulated differentially, enabling specific NK cell responses through combinatory mechanisms or by either mechanism independently [105,106]. Furthermore, cytokine production by NK cells not only contributes to target cell death but also influences and shapes the adaptive immune response of T and B cells [5,6,107,108]. In addition, activation of NK cells by cytokines such as IL-15, IL-12 or IL-18 can further augment NK cell effector functions [109].

3.3. NK cell receptors and their ligands

NK cells express a variety of adhesion molecules and receptors for cellular ligands as well as for cytokines and chemokines. The following chapter will outline a small selection of the most important receptors relevant for the context of this thesis. Activation of NK cells is tightly regulated by a delicate balance of signals delivered via activating and inhibitory receptors [75,83,106,110]. Activating receptors primarily recognize stress-induced ligands on virally infected or transformed cells thereby enabling NK cells to detect and eliminate potentially harmful cells. Inhibitory receptors mostly bind conventional or non-conventional self–MHC class I molecules and can thereby distinguish between self and non-self thus protecting healthy cells and sensing cells that lack self-MHC-I [32,111–114]. The integration of all signals delivered by activating and inhibitory receptors decides whether or not the NK cell becomes effectively activated and determines the kind of effector function [83–85,115]. The final response is further influenced by cytokines, chemokines and additional immune cells in the local microenvironment of the NK cell. In steady state conditions, inhibitory signals dominate through the interaction of inhibitory receptors

recognizing self–MHC class I on healthy cells. A seminal study by Klas Kärre and colleagues revealed that NK cell cytotoxicity is triggered by tumor cells that have lost self–MHC-I expression on their surface [19]. MHC class I molecules are expressed on all healthy nucleated cells thereby preventing NK cells from being activated during normal conditions. In contrast, MHC-I is often downregulated on virus-infected or transformed cells to evade detection by $CD8^+$ T cells [116], thus becoming susceptible to NK cell mediated killing ('missing-self' recognition) [19,117–119]. Deficiency of MHC-I is not sufficient to trigger full NK cell activation but in addition requires co-expression of ligands for activating receptors [84,112,120,121]. These ligands can be pathogen-derived or induced by cellular stress upon viral infection or malignant transformation [32,113,122–124]. This 'induced-self' model explains how NK cells can overcome the inhibitory signals if MHC-I expression is preserved, as well as their inability to attack healthy cells with no or low MHC-I levels (e.g.

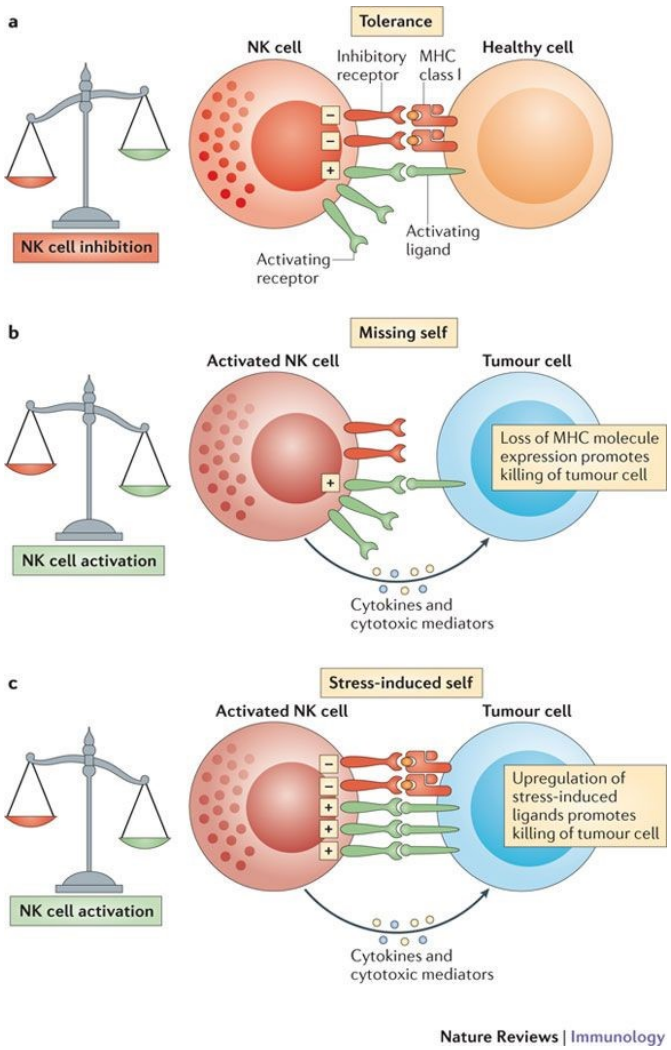


Figure 3.1: NK cell activation by missing self or induced self. NK cell tolerance (a) to healthy cells expressing cognate self–MHC class I molecules is mediated by dominant inhibitory receptors counteracting activating signals. (b) Down-regulation of MHC-I molecules on tumor cells results in loss of NK cell self-inhibition ('missing self') shifting the balance towards NK cell activation. (c) Stress-induced upregulation of activating ligands on tumor cells can overcome inhibitory signals of self–MHC-I leading to 'stress-induced self' triggered activation of NK cells. Adapted from Vivier et al. Nat Rev Immunol 2012 [610].

erythrocytes or neurons). Downregulation of activating NK ligands or expression of decoy MHC-I molecules hence display one strategy of immune escape of viruses and cancer [125–127].

3.3.1. Activating NK receptors

In contrast to B or T cells, NK cells express a wide array of germline encoded activating receptors, which can act in synergy to potently stimulate NK cell effector functions [84,98,111]. Most activating receptors signal via immunoreceptor tyrosine-based activation motifs (ITAMs) leading to recruitment and activation of the tyrosine kinases Syk and Zap70 [106,115]. ITAM signaling leads to NK activation by inducing signaling pathways of phosphatidyl-inositol-3-OH kinase (PI3K), phospholipase C and Vav [83,128]. Activating NK receptors can be classified according to their function and structure or their association with adapter molecules such as DAP10 or DAP12.

Natural Cytotoxicity Receptors

One major family of activating NK receptors is composed of the natural cytotoxicity receptors (NCRs) which include NKp30, NKp44, NKp46 and NKp80 [41,47,129–131]. They belong to the immunoglobulin superfamily and associate with different intracellular adaptor molecules bearing ITAMs such as CD3ζ, FcεRIγ or DAP12 [132–135]. NKp30, NK46 and NKp80 are constitutively expressed on mature human NK cells and NKp46 is also a marker for mouse NK cells [41,47,130,132]. Expression of NKp44 is not detected on resting NK cells but is induced by IL-2 or IL-15 activation [129,133].

A variety of pathogen-encoded ligands have been characterized for the NCRs, such as viral hemagglutinins or PfEMP1 of Plasmodium falciparum for binding and activation of NKp46 and NKp30 [131,136,137]. Human cytomegalovirus (HCMV) protein pp65 has been described as a viral ligand of NKp30 [138] and binding of heparan sulfate proteoglycans to NKp30, NKp44 and NKp46 has been reported [139–141]. Recently, interaction of NKp30 with the fungal cell wall component β-1,3-glucan has been demonstrated [142] and complement factor P has been identified as a new ligand for NKp46 [143]. Although cellular or tumor-derived ligands for some NCRs remain elusive, they have been shown to play a major role in NK-mediated tumor immunosurveillance [131,144–146]. For instance, a recent publication could reveal a new function of NKp46-mediated IFN-γ release in controlling tumor architecture and metastatic

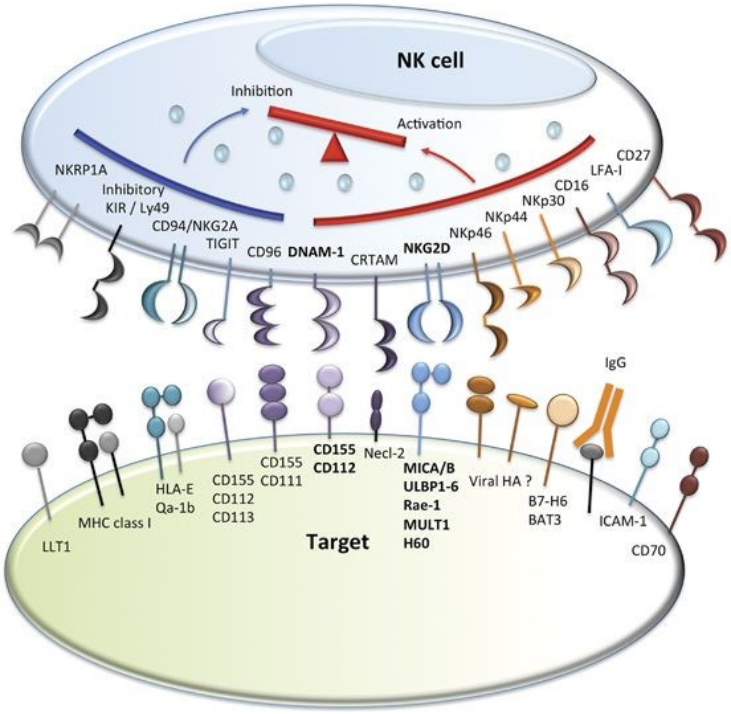


Figure 3.2: NK cell receptors and their ligands determine the balance of activation and inhibition. A selection of important activating and inhibitory receptors and their cognate ligands are depicted. Adapted from Chan et al. Cell Death Differ 2014 [751]

dissemination [147]. For NKp30 two bona fide cellular ligands have been identified: B7-H6 [148], expressed on the surface of tumor cells ,and BAT-3 (BAG-6) [149], which is released in exosomes. Shedding or down-regulation of B7-H6 has been elucidated as an immune-escape mechanism of tumor cells [150,151]. A recent publication has described the platelet-derived growth factor DD (PDGF-DD) as a ligand for NKp44, involved in cell cycle arrest and tumor control *in vivo* [152]. Furthermore, NKp44 has been shown to bind the mixed-lineage leukemia-5 protein (MLL-5) [153] and the proliferating cell nuclear antigen (PCNA), an inhibitory cellular ligand assisting in tumor immune-evasion via blocking the receptor [154]. NKp80 is expressed on all NK cells in human peripheral blood and has been recently suggested as a marker for discrimination of conventional NK cells and other members of the ILC family [46,47]. NKp80 has been shown to recognize the activation-induced C-type lectin (AICL) [155], which is upregulated on myeloid cells after TLR stimulation and also on NK cells after activation with IL-12/15/18 [156].

Natural Killer Group 2 Receptors

The class of natural killer group 2 (NKG2) receptors belong to the C-type lectin-like family and comprises activating as well as inhibitory receptors [157–159]. NKG2C and NKG2D are activating members, whereas NKG2A represents an inhibitory receptor and will be discussed in more detail in section 3.3.3.

The **NKG2C** receptor is expressed as a disulfide-linked heterodimer with CD94 on human and mouse NK cells and on subsets of human memory T cells [134,160]. NKG2C/CD94 couples to the ITAM-bearing adaptor molecule DAP12, thereby transmitting activating signals [161]. CD94/NKG2C recognizes the non-classical MHC class I molecule HLA-E as a ligand in humans and Qa-1b in mice [162,163]. Furthermore, the expansion of a NKG2C expressing subset upon HCMV infection has been linked to the generation of memory-like NK cells with adaptive features (see section 3.6.2) [164–166].

NKG2D is a homodimeric receptor and does not associate with CD94. It is expressed on the surface of all human and mouse NK cells and a fraction of $CD8^+$ and γδ-T cells [167,168]. Upon receptor ligation, NKG2D signals via the adaptor molecule DAP10 in humans and DAP10 and DAP12 in mice resulting in Vav-1 phosphorylation and activation of phosphatidylinositol-3 kinase (PI3K) [169–172]. NKG2D detects stress-induced MHC class I homologue ligands, which are upregulated upon viral or malignant transformation and genotoxic stress [113,173,174]. They include MHC class I chain-related gene (MIC)-A, MIC-B and the UL16 binding proteins (ULBP) 1 - 6 [167,175,176]. In mice, the respective NKG2D ligands are the retinoic acid early transcript-1 molecules (Rae-1), murine UL16-binding-like transcript-1 (MULT-1) and histocompatibility antigen 60 (H-60) [122,123,177,178]. These ligands are expressed below threshold on healthy tissues but have been shown to be induced upon viral or malignant transformation and genotoxic stress e.g. due to DNA damage and heat shock response pathways [173,174,179–182]. NKG2D is often targeted in immune evasion strategies for instance via shedding of ligands from tumor cells or TGF-β mediated receptor downregulation [183–186]. A recent publication emphasizes the impact of NKG2D-mediated anti-cancer immunity by blocking the shedding of NKG2D ligands from the surface of tumor cells [187]. The importance of NKG2D in tumor immunosurveillance has been demonstrated for several cancer entities *in vivo* [173,188–190].

CD16 (FcγRIIIA)

Complete activation of resting human NK cells requires combined signals of stimulation via two or more receptors [98]. A potent exception is CD16, the low affinity FcγRIIIA, which is expressed on most mouse NK cells and on the human $CD56^{dim}$ subset. It associates with the ITAM-containing FcεRIγ chain and CD3ζ [191,192]. CD16 binds the Fc part of IgG class antibodies and therefore enables NK cells to exert antibody-dependent cell-mediated cytotoxicity (ADCC) against antibody coated targets [12,193,194]. Recently, our group has

demonstrated the importance of CD16-mediated ADCC in anti-cancer immunity and innate memory formation [99].

3.3.2. Co-stimulatory receptors

DNAX accessory molecule-1 (DNAM-I, CD226)

The co-stimulatory receptor DNAX accessory molecule-1 (DNAM-I, CD226) is a member of the immunoglobulin superfamily and signals via ITAM motifs and PKC recruitment [195,196]. It is constitutively expressed on most human NK cells, on approximately 50% of murine NK cells and on subsets of T cells and myeloid cells [197–199]. CD112 (Nectin-2) and CD155 (polio virus receptor, PVR), two members of the nectin family, have been identified as ligands for DNAM-I and are broadly distributed on hematopoietic, endothelial and epithelial cells [200–203]. These ligands are upregulated on cancer cells upon genotoxic stress, indicating a role for DNAM-I in NK cell-mediated anti-tumor immunity *in vivo* [144,204,205]. Of note, the DNAM-I ligands CD112 and CD155 can also interact with the two inhibitory NK receptors CD96 (TACTILE) and TIGIT, which can oppose DNAM-I activation [199,203]. DNAM-I has been further implicated in adhesion and migration of monocytes and shown to interact with the β-2-integrin LFA-1 [195,206].

Tumor necrosis factor receptor superfamily (TNFRSF)

The members of the tumor necrosis factor receptor superfamily (TNFRSF) are widely expressed among lymphoid and non-lymphoid tissues in mice and humans, and are critical for modulating the immune response against pathogens by regulating cell death and survival [207,208]. The TNFR superfamily is subdivided into two groups, the death domain (DD)-containing receptors and TRAF (TNF receptor-associated factor) binding receptors. The DD-containing 'death receptors', such as Fas (CD95), TRAIL-R1, TRAIL-R2, and TNFR1, associate with the adapters Fas-associated DD protein (FADD) and TNFR-associated DD protein (TRADD) leading to caspase activation and apoptosis [207,209,210].

The second subgroup of TRAF binding receptors include OX40 (CD134), 4-1BB (CD137), HVEM, CD27, CD30, CD40, and GITR (CD357). Most receptors of the TNFRS family are not constitutively expressed on NK cells but can be induced upon activation [211–215]. They generally operate as co-stimulatory receptors and their function in T cell activation is well

defined. On T cells, TNFRSF members are upregulated after sequential TCR and CD28 or CD40 stimulation. They signal via recruitment of TRAF adaptor proteins resulting in activation of the NF-κB pathway and enhanced proliferation and cell survival [213,216,217]. Ligands for TNFRSF members (TNFSF) are primarily expressed on APCs but also by non-immune cells as smooth muscle and endothelial cells. Additional immune cells such as NK cells, conventional activated T cells, lymphoid tissue inducer (LTi) and other ILCs can express ligands depending on the stimulatory environment [212,213,217–219]. Thus, under defined conditions, NK cells can express either the receptors and/or the ligands of the TNFRSF, which enables an extensive cross-talk with other immune cells during an ongoing immune response.

4-1BB (CD137) and Ox40 (CD134), the two TNFRSF members examined in this study, are absent on resting NK cells but can be induced upon cytokine activation and are binding to 4-1BBL and Ox40L, respectively [214,216,217,220,221]. A co-stimulatory role has been described for T cells, but their function in NK cells might be ambiguous and depending on the cytokine milieu, the environment and nature of the ligand [214]. Agonistic antibodies for both receptors are currently tested in clinical trials to enhance T cell anti-cancer immunity [222–226].

3.3.3. Inhibitory receptors

The best characterized inhibitory receptors are the MHC-I binding Ly49 receptors in mice and the killer cell immunoglobulin-like (KIR) family in humans (see section 3.4), and the lectin-like heterodimer CD94/NKG2A in both species [110,111,227,228]. The downregulation of HLA-I expression on virally-infected or transformed cells impairs inhibitory signals and therefore confers enhanced NK cell activation (‘missing self’ recognition, see section 3.3) [19,118]. Both families of KIR and CD94/NKG2 have inhibitory as well as activating members. Under steady state conditions, the ligand binding affinity of the inhibitory receptors is higher compared to their activating receptor counterparts, allowing the inhibitory signal to dominate [229–232]. Although they are quite divers in their extracellular domains, all inhibitory NK receptors transmit signals via cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) [83,128,233]. The inhibition is based on the recruitment of phosphatases such as SHIP, SHP-1 and SHP-2, which dephosphorylate and

inactivate the signaling molecules downstream of activating receptors. Thereby ITIM signaling interferes with ITAM signaling and blocks effective NK cell activation [234–238].

CD94/NKG2A

The inhibitory C-type lectin like receptor NKG2A is expressed as a disulfide-linked heterodimer with CD94 on human and mouse NK cells and on subsets of memory $CD8^+$ and γδ-T cells [157,160,239,240]. NKG2A contains two ITIMs and thus can transmit inhibitory signals by recruiting the phosphatases SHP-1 and SHP-2 [241–243]. Like its family member NKG2C, NKG2A/CD94 binds to the non-classical MHC-I ligand HLA-E in humans and to the homologous mouse Qa-1b, albeit with a significantly higher affinity than NKG2C/CD94 [162,163,229,244]. HLA-E and Qa-1b present peptides derived from the leader segments of other MHC class I proteins, directly reflecting the overall MHC-I expression of the cell [239,244–246]. Since the surface expression of HLA-E and Qa-1b is dependent on the availability of classical MHC-I molecules, this provides a mechanism to monitor the total expression of MHC-I on tissues which is often impaired upon viral infection or malignant transformation.

TIGIT

In addition to inhibitory receptors recognizing self-MHC class I, other inhibitory receptors can negatively regulate NK responsiveness such as the T cell immunoreceptor with Ig and ITIM domains (TIGIT). It is a co-inhibitory immunoglobulin superfamily receptor of the CD28 family and was discovered by bioinformatic approaches of different laboratories [200,247–249]. TIGIT is present on effector and memory T cells, regulatory T cells (T_{regs}), follicular helper T cells and NKT cells [200,247,248,250–252]. It is constitutively expressed on human naïve NK cells, but not on mouse NK cells where it is inducible upon activation [249,253]. TIGIT proteins form homodimers on the cell surface and contain an ITIM motif and an additional immunoglobulin tail tyrosine (ITT)-like motif in their cytoplasmic tail [247–249,253]. The importance or redundancy of both motifs for the inhibitory function of TIGIT is not well understood and might differ between species and cell types [249,253–255]. The ITT-like motif recruits SHIP-1 through the cytoplasmic adaptor Grb-2 (growth factor receptor-bound protein 2) leading to dephosphorylation and abrogation of PI3K and MAPK signaling pathways [254,255]. Additionally, the ITT-like motif can impair NF-κB signaling and combined effects result in strong inhibition of NK cell cytotoxicity and cytokine production [254]. TIGIT binds to the nectin family members CD112 (Nectin-2) and CD155

(polio virus receptor, PVR) which are broadly distributed on T cells, APCs and a variety of non-hematopoietic tissues [200–203,247–249]. Their expression is upregulated upon DC maturation and can be induced on cancer cells upon genotoxic stress [203,204,256–258]. TIGIT shares its cellular ligands with another inhibitory receptor, CD96 (TACTILE), and with the co-activating receptor DNAM-I (CD226), but TIGIT was shown to possess a higher affinity and to compete with DNAM-I in a dose dependent manner [203,247–249,253]. The interactions of TIGIT/CD96 and DNAM-I closely resemble the CD28/CTLA-4 pathway, where the co-stimulatory receptor DNAM-I shares ligands with the high-affinity co-inhibitory receptors TIGIT and CD96 [199,247,259]. TIGIT can not only counterbalance DNAM-I–mediated activation of NK cells through competition for its ligand, but also by directly interacting with DNAM-I in cis, thereby disrupting its homodimerization and functionality [249,251,260,261]. In addition, TIGIT can also indirectly suppress the immune response through interaction with CD155-expressing DCs. CD155 itself contains an ITIM motif which induces a transformation to a tolerogenic DC phenotype [248]. In the tumor microenvironment, TIGIT is expressed on murine and human tumor-infiltrating lymphocytes (TILs) which exhibit a dysfunctional phenotype and co-express other inhibitory checkpoint molecules such as PD-1, Tim-3, and Lag-3 [257,260,262]. Although its inhibitory role in anti-tumor functionality of $CD8^+$ T cells and T_{regs} is well defined, the influence of TIGIT on NK cell-mediated tumor control is less clear [258,263,264]. However, the importance of DNAM-I, its competing co-stimulatory receptor, in NK cell-mediated immunity has been demonstrated *in vivo* [199,205,265,266]. Moreover, suppression of NK cell functionality by MDSCs (myeloid-derived suppressor cells) was shown to depend on TIGIT-CD155 interactions, supporting the development of therapeutic strategies targeting TIGIT for cancer immunotherapy [267].

3.3.4. Cytokines and their receptors

Cytokines and chemokines are soluble mediators of the immune system and are involved in the activation, regulation and communication between different cell types of innate and adaptive immunity. The cytokine milieu determines the nature of the NK cell effector function and also modulates the extent of the general immune response. Cytokine signaling is essential for efficient NK cells activation in inflammation but also in their development, differentiation, homeostasis and migration or homing at steady state [268]. Especially pro-inflammatory cytokines such as interleukin-2 (IL-2), IL-15, IL-12, IL-18, IL-21 and type-I

interferons (IFNs) have been demonstrated to play pivotal roles in NK cell biology [109,269] The combination of different cytokines together with cellular ligands can have synergistic effects on NK cell reactivity as e.g. when cytokine concentrations are limited *in vivo* [270,271]. Due to the importance of IL-2, IL-15, IL-12 and IL-18 in this thesis, these cytokines, their receptors and their impact on NK cell function are described in more detail below.

Common gamma-chain cytokines

Cytokines sharing the common cytokine receptor gamma-chain (γc or CD132) are crucial for development and homeostasis of lymphoid cells as γc-deficient mice have defective B, T and NK cell compartments [272–275]. The γc family consists of several members with a similar protein structure and comprises IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. The high affinity heterodimeric receptors are formed by a cytokine specific α-chain and the γc-chain [276,277]. The only exceptions are IL-2 and IL-15 binding to a heterotrimeric receptor complex. The γc is the essential signaling component for these cytokines. It associates with Janus tyrosine-kinase (JAK)-3 which phosphorylates and activates different STAT molecules (signal transducer and activator of transcription) [278]. Phosphorylated STAT dimers translocate to the nucleus where they serve as transcription factors. IL-4 preferentially activates STAT-6, whereas IL-2, IL-7, IL-9 and IL-15 mostly act through STAT-5, and IL-21 signals via STAT-1 and STAT-3 [272,273,279].

The impact of IL-2 and IL-15 on NK cell function and survival is well studied. Both cytokines share the same IL-2/15Rβ (CD122) and the and γc (CD132) and thus have overlapping signaling pathways and functions [56,280,281]. Both cytokines only interact with intermediate affinity to the heterodimeric βγ-receptor complex, requiring nanomolar concentrations. IL-2 and IL-15 have distinct high affinity α-chains, which accounts for the different biological functions of IL-2 and IL-15 [282,283]. The high affinity IL-2Rα chain (CD25) is incorporated into a heterotrimeric IL-2Rαβγ receptor complex, highly increasing the affinity to picomolar concentration of IL-2 [284,285]. CD25 is constitutively expressed on $CD4^+$ T_{regs} and on $CD56^{bright}$ NK cells, which is associated with their high proliferative capacity in response to picomolar IL-2 concentrations [286,287]. CD25 can be induced on NK and T cells upon various stimulations and is therefore often used as activation marker [288–291]. Soluble IL-15 can directly bind with low affinity to the IL-15Rβγ, but high affinity binding requires its trans-presentation via the IL-15Rα. The high affinity IL-15Rα is primarily expressed on

macrophages and dendritic cells and trans-presents membrane-bound IL-15 to the IL-15Rβγ complex on the surface of NK or T cells [292–295].

Signal transduction downstream of the shared IL-2/15Rβγ involves activation of different signaling cascades such as the MAPK/ERK pathway or STAT-3/5 phosphorylation by JAK1/3 [296]. Furthermore, signaling via the PI3K-AKT-mTOR pathway leads to the induction of anti-apoptotic Bcl-2 promoting cell survival [297–300].

Both cytokines enhance NK cell proliferation and effector functions such as cytokine production and cytotoxicity and can improve NK-mediated anti-tumor responses [74,272,283,298,301]. However, *in vivo* administration of IL-2 has been shown to simultaneously expand $CD25^+$ regulatory T cells competing for the availability of IL-2 [302–305]. Although both cytokines enhance NK cell effector functions and proliferation, the different phenotypes of IL-2 or IL-15 knockout mice have suggested unique roles for both cytokines in immune cell homeostasis [56,306–309]. This is also reflected in the different sources of both cytokines as IL-2 is mainly produced by activated T cells, illustrating its importance for crosstalk during an activated immune response [71]. In contrast, IL-15 is provided at steady state by a variety of APCs and non-immune cells [295,310]. IL-15 signaling is indispensable for NK cell development, as mice deficient for STAT-5, IL-15 or IL-15R have impaired NK cell compartments [294,307,311]. IL-15 has been shown to promote NK cell development from hematopoietic precursors *in vivo* and was capable of differentiating $CD34^+$ hematopoietic progenitor cells into NK cells *in vitro* [55,77,268,297,312]. Furthermore, IL-15 is essential for NK cell homeostasis and survival in the periphery [58,275,294].

IL-12 and its receptor

IL-12 is a heterodimeric molecule with two subunits (p35 and p40) and belongs to the monokine family [313–315]. It is produced by activated DCs and phagocytotic cells in response to pathogen encounter [316–319]. The cognate receptor is composed of IL-12Rβ1 and IL-12Rβ2 chains and is primarily expressed on activated NK and T cells as well as on resting NK cells, facilitating rapid immune responses without prior activation [320–324]. Ligand binding and dimerization of the IL-12R results in JAK2 and TYK2 activation and phosphorylation of downstream STAT-3, STAT-4 and STAT-5 [324–326]. IL-12 was shown to be a strong inducer of IFN-γ and TNF-α and to improve NK cell cytotoxicity by upregulating granzyme and

perforin levels [313]. The effects of IL-12 on NK cell mediated IFN-γ production and enhanced cytotoxicity have been mainly attributed to STAT-4 signaling [321,325,327].

IL-12 synergizes with other activating stimuli such as cytokines or activating receptors, resulting in robust NK cell effector responses [328–332]. IL-2 and IL-15 can augment IL-12 signaling by regulation of the IL-12R and STAT-4 [329,332] and combinatorial stimulation of IL-2/IL-15, IL-12 or IL-18 leads to a dose-dependent and synergistic effect on IFN-γ secretion [74,271,288,333,334]. An essential mechanism of the synergy between IL-12 and IL-18 is based on the STAT-4 dependent upregulation of the IL-18 receptors and vice versa [335–338]. In T cells, IL-12 acts synergistically with IL-2 signaling and TCR or CD28 stimulation for induction of IFN-γ production [328,331]. In this way, NK and T cells can tune their responsiveness to the extent of inflammation. Furthermore, IL-12 has been shown to favor a T_H1 response and to be instrumental in resistance against bacterial infections and intracellular parasites [316,317,339–342].

IL-18 and its receptor

IL-18 belongs to the IL-1 family and is synthesized as a biological inactive precursor protein (Pro-IL-18) which has to be processed and activated by caspase-1 or proteinase-3 [343–346]. As a monokine, IL-18 is produced by activated monocytes, macrophages and dendritic cells upon TLR stimulation but also by non-immune cells such as keratinocytes or osteoblastic stromal cells [347–350]. The IL-18R is composed of a ligand binding chain (IL-18Rα) and an accessory signaling protein (IL-18Rβ) [351,352]. The IL-18R complex primarily signals via recruitment of MyD88 (Myeloid differentiation primary response gene 88) and IRAK (interleukin-1 receptor-associated kinase) / TRAF6 (TNF receptor associated factors) leading to NF-κB activation [353]. In addition, induction of STAT-3 and MAPK transduction pathways has been reported in NK cells and neutrophils [354–356]. IL-18Rα is constitutively expressed on resting NK cells, naïve T and mature T_H1 cells as well as on other immune cells [338,357–360]. Free IL-18 can also be bound by the IL-18–binding protein (IL-18BP) in the circulation, preventing its receptor binding and its biological function [361]. IL-18 stimulation alone only induces minor effects due to the low constitutive expression levels of the IL-18R. However, IL-18 can function synergistically with type-I IFNs, IL-12 and IL-15, particularly because IL-12 signaling upregulates the expression of the IL-18R [337,338]. In synergy, IL-18 can stimulate potent proliferation and IFN-γ production in NK cells and upregulation of perforin and FasL dependent cytotoxicity in NK cell and $CD8^+$ T cells [74,270,271,288,333,362–365].

IL-18 has further been implicated in the priming of NK cells during their maturation in the periphery since NK cells deficient for IL-18 have impaired IFN-γ production and cytotoxicity [365,366]. For T cells, IL-18 together with IL-12 plays an important role in T_H1 differentiation, providing another link between innate and adaptive immune responses [343,365,367]. In contrast, IL-18 alone or in combination with IL-2 rather stimulates a T_H2 response [368–370], whereas synergy with IL-23 induces T_H17 differentiation [371].

3.4. The killer immunoglobulin-like receptor system

Activation of NK cells is counterbalanced by inhibitory receptors like the KIR family and the lectin-like NKG2A receptors, both binding to MHC class I molecules expressed on all nucleated cells [110,227]. Therefore, both receptor families largely contribute to NK self-tolerance towards autologous healthy cells preventing uncontrolled activation and immunopathology. Upon perturbation of MHC-I expression during viral infection or malignant transformation, inhibition through these receptors is attenuated, rendering the target cell susceptible to NK cell mediated killing ('missing-self') [19,118]. In the human system, the killer cell immunoglobulin-like receptor (KIR) family comprise the biggest and best studied group of inhibitory receptors and recognizes HLA-A, -B, and -C alleles. KIR receptors were first described and cloned in the 1990s by several groups and were subsequently shown to function as inhibitory receptors for different HLA class I molecules [372–383].

KIRs are not detectable in mice, where the structurally different Ly49 receptors recognize MHC class I and perform analogous functions [384,385]. Ly49 and KIR molecules have evolved independently and KIR genes have diversified rapidly in primates and humans [386,387]. Similar as their polymorphic MHC-I ligands, the human KIR and mouse Ly49 loci are both highly polymorphic in gene content and allelic polymorphism [388,389]. The complexes for KIR/Ly49 and MHC genes are located on different chromosomes and independent segregation results in variable receptor-ligand combinations. Interaction between KIR receptors and HLA-I variants have been shown to influence the potency of an immune response and certain KIR-HLA combinations have been associated with autoimmunity, reproductive complications as well as resistance or susceptibility to viral infections and cancer [390,391].

In addition to NK cells, KIRs are expressed on divers T cell subsets including NKT cells, γδ-T cells and on memory/effector αβ-T cells [392–397]. KIR expression on T cells occurs after TCR rearrangement and is considered to protect from activation-induced cell death as the KIR$^+$ subset increases with age [393,398–400].

3.4.1. Structure and nomenclature of KIR molecules

KIR proteins are type-I transmembrane glycoproteins belonging to the immunoglobulin-like superfamily and are composed of a different number of extracellular Ig-like domains ("D"), indicated in their nomenclature as KIR2D or KIR3D [111,401,402]. They express either a long or a short cytoplasmic tail involved in signal transduction [83,128]. Inhibitory KIRs with a long cytoplasmic tail ("L") contain ITIM motifs (immunoreceptor tyrosine-based inhibitory motifs) thus transferring inhibitory signals [83,128]. Binding of inhibitory KIRs to their cognate ligands results in inhibitory signaling and in suppression of NK effector functions. ITIM signaling involves recruitment of SHP-1/2, leading to dephosphorylation and inhibition of Vav-1 [234–237]. ITIM-mediated inhibition dominates over activating signals by blocking signaling at a proximal step and thus preventing downstream stimulatory signals [83,236]. In contrast, activating KIRs harbor a short cytoplasmic tail ("S") that signals via recruitment of the ITAM-bearing adapter DAP12, leading to activation of NK cells [83,134,135].

The last digit of KIR nomenclature indicates the number of the gene encoding the molecule in the leukocyte receptor complex (LRC). The denotation "P" refers to non-expressed pseudogenes [111,401,402]. Exemplarily, KIR2DL1 encodes an inhibitory receptor containing a long cytoplasmic tail and two Ig domains, whereas KIR3DS1 represents an activating KIR with three Ig domains and a short cytoplasmic tail.

KIR2DL4 represents an interesting exception, harboring a long cytoplasmic tail with only a single ITIM and weak inhibitory potential [403]. In contrast, KIR2DL4 contains a charged arginine residue allowing the receptor to couple with FcεRIγ resulting in high IFN-γ production but low cytotoxicity [404,405]. In addition, KIR2DL4 transcripts are ubiquitously detected in all NK cells but do not always correlate with protein surface expression [406].

3.4.2. Ligand binding specificity of KIR receptors

KIR bind HLA-A, -B and -C alleles and the ligand-receptor interactions for most inhibitory KIRs have been clearly defined (Figure 3.3) [407,408]. Most KIR receptors recognize specific motifs located at the α1 and α2 helices of HLA-I molecules and certain amino acids within the HLA binding groove define KIR binding specificity [228,407,409,410]. KIR2DL1 recognizes HLA-C C2 epitopes defined by expression of lysine at position 80 of the HLA-C heavy chain (HLA-C Lys80) [374,376,411,412]. KIR2DL2 and KIR2DL3 bind to HLA-C C1 epitopes carrying an Asparagine at position 80 (HLA-C Asn80) [376,411,413]. In addition, KR2DL2 can also detect some C2 allotypes [414]. Of note, the interaction of KIR2DL1 with its C2 ligands results in relatively stronger inhibitory signals compared to KIR2DL2/L3 and C1 interactions [414,415].

Some HLA-A and approximately 40% of all HLA-B alleles possess the HLA-Bw4 serological motif (located at amino acids 77-83), which functions as ligand for KIR3DL1 [375,413,416,417]. Recent studies indicate a peptide dependency of peptides loaded on HLA-I on the KIR binding affinity, which is much broader compared to TCR specificity and is rather mediated by motifs than sequences [418,419]. In particular, the strength of the binding of KIR3DL2 to HLA-A3 and A11 allotypes [420,421] and of KIR3DL1 to the Bw4 motif is highly sensitive to the bound peptide [422–424]. Chrystal structures of KIR–HLA-I interactions could define these binding parameters in molecular detail, demonstrating importance of amino acids at position 7 and 8 in the peptide [228,407,409,410]. The strength of inhibitory and activating signals induced upon KIR–HLA-I interaction is dependent on the presented peptide and thus can be manipulated by viral infection or malignant transformation [418,422,425].

The ligands for activating KIRs have been harder to identify, probably due to high peptide selectivity and lower binding affinity as their inhibitory counterparts [418,425]. Exemplarily, KIR2DS1 binds to the same C2 epitope as the structurally similar inhibitory KIR2DL1 receptor, although with reduced affinity and higher peptide selectivity [415,426,427]. Peptide specific binding to HLA-A*11:01 and only weak binding to HLA-C1 was detected for the activating KIR2DS2 receptor in contrast to the structurally related KIR2DL2 molecule [230,428]. Additionally, KIR2DS2 was shown to directly recognize conserved flaviviral peptides in context of HLA-C1 and to bind cancer cell lines in a β2-microglobulin (β2m)-independent manner [429,430]. The most prevalent KIR2DS4 receptor exhibits a unique binding specificity for HLA-A11 and selective HLA-C1 and C2 alleles [431]. Interaction of KIR3DS1 with HLA-Bw4 alleles has been implicated by epidemiological studies to contribute to HIV resistance and slow AIDS progression [432,433]. Recently, KIR3DS1 has been described to additionally recognize HLA-F open conformers (a non-classical HLA-Ib

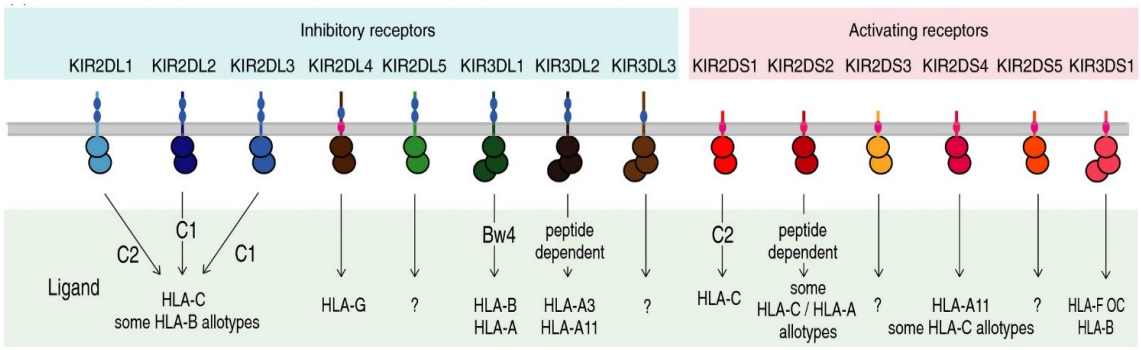


Figure 3.3: HLA class I ligands for activating and inhibitory KIRs. Inhibitory killer immunoglobulin-like receptors (KIRs) harbor a long cytoplasmic tail ('L') with ITIM domains transmitting inhibitory signals. Activating KIRs with short cytoplasmic tails ('S') couple to ITAM containing adapters such as DAP12 for activating signaling. KIRs bind specifically to HLA-A, -B, -C, -F or -G allotypes. The binding motifs for HLA-C are referred to as HLA-C1/C2 and HLA-Bw4 for HLA-A and -B, which comprise specific epitopes characterized by defined amino acid sequences. The interaction between certain KIRs and their ligands are peptide dependent and some ligands still remain elusive. (Adapted from Béziat et al. Immunology 2017) [408]

without association of β2m), which might be expressed on HIV-infected lymphocytes [434,435]. Therefore, activating KIRs might also recognize viral-induced or altered ligands and non-classical HLA-I molecules. Furthermore, the interaction of KIRs to their HLA-I ligands is constantly subjected to viral escape strategies. Up- and downregulation of HLA-I expression could both influence binding to inhibitory and activating KIRs and viral manipulation of selected peptide presentation can further impact KIR binding specificity and affinity [418,422,425,436].

The rather activating KIR2DL4 (see section 3.4.1) binds to HLA-G molecules expressed on trophoblasts mediating tolerance of the fetus and indirectly promoting vascularization of the maternal decidua via IFN-γ production [30,437–439]. Ligands for KIR2DL5, 3DL3, 2DS3 and 2DS5 remain elusive and are currently under intensive investigation.

3.4.3. KIR expression on NK cells

KIRs are encoded within the leukocyte receptor complex (LRC) on chromosome 19q13.4. containing 15 genes and 2 pseudogenes [440,441]. The variable gene content of the LRC locus and allelic nucleotide sequence polymorphism of each KIR gene contribute to the substantial KIR diversity in the human population [388,442–444]. In an individual, KIRs are expressed in a stochastic and variegated manner and therefore each NK cell clone

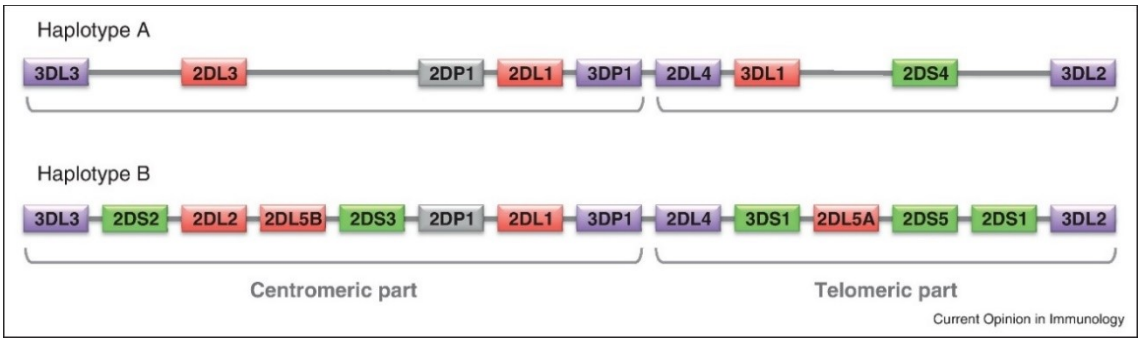


Figure 3.4: Human haplotypes A and B at the KIR locus. Two examples for each haplotype are shown. The gene content of each haplotype can vary giving rise to a high diversity of KIR haplotypes that are either AA, AB or BB. Activating KIR genes are indicated as green and inhibitory KIR genes as red boxes. Conserved genes present in both haplotypes are depicted in purple and pseudogenes in grey. (Thielens et al. Curr Opin Immunol 2012) [693]

expresses a different combination of the encoded KIRs, leading to a diverse KIR receptor repertoire on individual NK cell clones [373,406,442,444,445]. The frequency of an NK cell expressing a certain KIR as well as the levels of its surface expression can vary between individuals depending on e.g. the copy number of the KIR gene ('gene-dose' effect) or the allelic variation at the locus (KIR alleles with high or low protein surface densities) [446–449]. These factors contribute to the broad range of functionally distinct NK populations enabled to detect expression changes of highly polymorphic MHC class I molecules on target cells.

Co-expression of different KIR genes within one NK cell clone is mainly predicted by the product rule, meaning that the probability of co-expression is predicted by the product of the individual expression frequencies of each KIR, which are independent events [442,445,450]. Reports in mice indicate a negative correlation of the expression frequency of MHC-I ligands and their inhibitory receptors Ly49 and CD94/NKG2A, suggesting a role for MHC-I in shaping individual KIR repertoires [451–453]. The interaction of MHC-I with its cognate inhibitory receptor would suppress the co-expression of a second cognate receptor, limiting the number of self-specific inhibitory receptors. Thus, the expression of KIR/Ly49 during development would be dependent on a MHC-I–based selection [454–457]. In humans, however, this influence is still under debate and might be reflected as an imprint from previous infectious challenges [443,445,458,459].

Two KIR haplotypes can be distinguished by the composition of their KIR genes. Group A haplotypes mostly comprise inhibitory KIRs, whereas group B haplotypes harbor additional genes encoding activating KIRs [443,444,460]. Combination of these haplotypes

results in KIR genotypes with ‚AA', ‚AB' or 'BB' content. Studies indicate a correlation between group A haplotypes and improved immunity against pathogens whereas group B haplotypes are associated with productive fitness [388,390,391].

NK cells acquire KIR expression late during maturation and terminal differentiation. KIR expression is weak or absent in $CD56^{bright}NKG2A^{+}CD94^{hi}CD16^{-}$ cells and increases gradually during maturation with highest expression in terminally differentiated $CD56^{dim}NKG2A^{-}CD94^{lo}CD16^{+}$ NK cells [64–66,81].

3.4.4. Regulation of KIR gene expression

KIRs are expressed in a stochastic manner on individual NK cell clones during development, but the molecular mechanism defining which KIR gene is expressed and which is silenced remain poorly defined. Although the regulatory regions of most KIR genes are highly conserved, the individual KIR alleles are regulated independently resulting in variegated and clonally restricted expression patterns [440]. The regulatory regions of KIR2DL4 and KIR3DL3 mark exceptions consistent with their distinct expression characteristics (ubiquitously and non-expressed, respectively) and are driven by a different type of promoter [461,462]. Acquisition of KIR expression during development has been shown to be a complex process involving epigenetic DNA modifications, multiple promoter elements, intergenic fragments, bidirectional transcription and anti-sense RNA [463,464].

Epigenetic mechanisms controlling KIR expression

In hematopoietic stem cells, the KIR locus is epigenetically silenced by DNA methylation and histone condensation accompanied with a closed chromatin state. In order for KIR gene transcription to take place, transcription factors need to gain access to the promoter regions. As hematopoietic progenitor cells commit to the NK cell linage, initiation of KIR gene transcription thus first requires chromatin opening via histone acetylation. In a two-step model, euchromatic histones represent a prerequisite for subsequent DNA demethylation and gene transcription, since active as well as untranscribed KIR genes exhibit a high degree of histone acetylation associated with an open chromatin signature [465,466].

It has been shown by several groups that the clonally restricted expression of highly homologous KIR genes is stably maintained by epigenetic mechanisms [465,467,468] and expression frequency and intensity are passed on during cell division [442,455,458]. *De novo* KIR expression and generation of the clonal KIR repertoire are established by DNA demethylation during development [465,467,468]. The methylation status of CpG island clustered in an upstream region of the KIR transcriptional start site was found to correlate with transcriptional activity of the KIR gene. Highly methylated CpG islands corresponded to non-expressed and silenced KIR genes and vice versa [467]. Chan and colleagues demonstrated that DNA methylation ensured a predominant mono-allelic expression of highly homologous KIR genes indicating an individual control of the regulatory regions [465,468]. Exposure of mature NK cells to 5-aza-2'-deoxycytidine (5Aza-dC, a DNA methyltransferase inhibitor causing demethylation) was shown to induce *de novo* expression of previously silenced KIR genes and conversely, *in vitro* methylation of CpG islands resulted in repression of reporter gene transcription [465,467].

KIR promoter elements

To date, several promoter elements are described that are shared by most KIR genes, such as a distal promoter Pro-D), a bidirectional proximal promoter switch (Pro-S), an intron 2 promoter and an intermediate promoter element (Pro-1) [463,464]. A model illustrates how these different promoters orchestrate KIR expression during development (see Figure 3.5) [464,469].

Anti-sense transcripts originating from an intron 2 promoter are restricted to early progenitor and hematopoietic stem cells but have not been detected in mature NK cells as the intron 2 promoter activity declines during NK cell linage commitment [466,470]. These lncRNAs (long non-coding RNAs) may play a role in establishing epigenetic marks such as DNA methylation or histone modifications.

The distal promoter element (Pro-D) exerts a low transcriptional activity early during NK cell development due to non-methylated transcription factor binding sites. During lineage specific transition from the pro- to the immature NK cell stage, basal Pro-D activity contributes to the 'priming' of KIR gene expression through recruitment of chromatin modifying enzymes that increase DNA accessibility [471,472]. As developing NK cells acquire IL-15 responsiveness via CD122 (IL-15Rβ) expression at the $CD56^{bright}$ stage [28,48], IL-15 drives full transcriptional activity of the KIR distal promoter element [464,472]. Transcripts

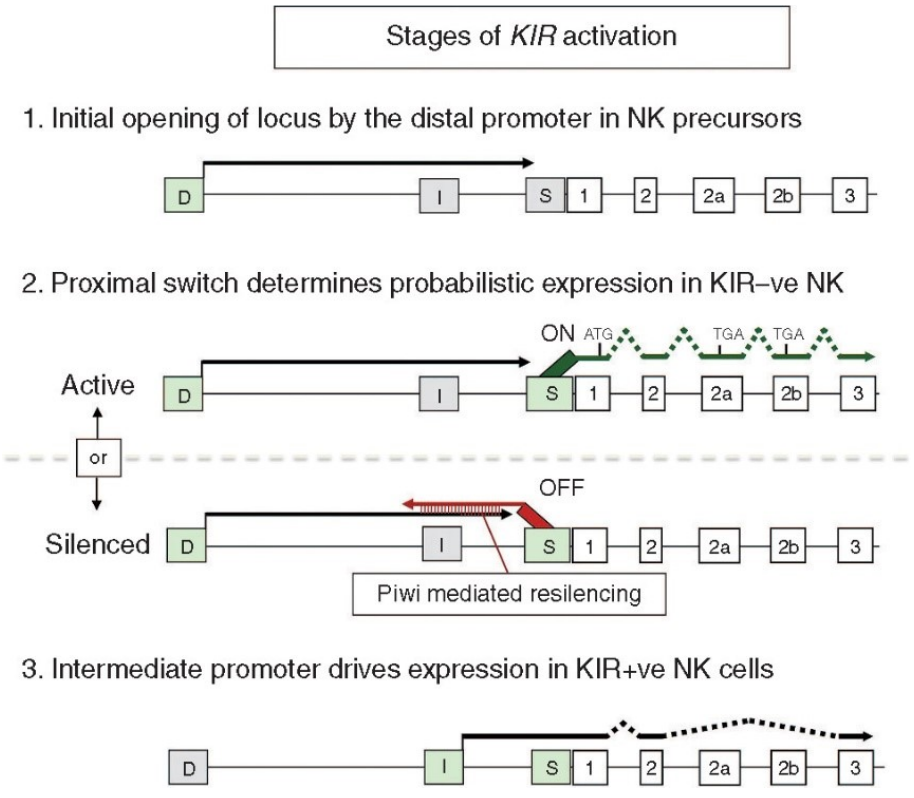


Figure 3.5: Model of successive regulation of KIR acquisition during development by different promoter elements. The scheme depicts the 5′ control region and the first three exons encoding KIR genes with 'ATG' labeling the initiation of protein translation. Promoter elements are indicated by filled rectangles. Three main promoter elements mark the three phases of KIR gene transcription with green rectangles highlighting the promoters that define the proposed phase of expression. Black arrows represent sense transcripts and red arrows show antisense transcription with dsRNA displayed as red box. (Adapted from Anderson, Mol Ther Nucleic Acids 2014) [474].

from the distal promoter span the downstream proximal promoter region, which leads to initial opening of the chromatin structure allowing access of transcription factors that promote demethylation such as Runx/AML [464,473].

The proximal promoter has a probabilistic, bidirectional activity and is active in both the forward and reverse direction and is therefore often referred to as probabilistic promoter switch (Pro-S) [473,474]. Probabilistic transcription from the bidirectional proximal promoter in CD56^{dim} NK cells defines whether a certain KIR gene is expressed and accounts for the stochastic and clonal KIR distribution [449,473]. It harbors a multitude of transcription factor binding sites and their relative affinity of inducing sense vs antisense promoter activity determines the probability of sense vs antisense transcription [449,461,473]. The anti-sense

transcripts spread the upstream distal promoter generating a double-stranded (ds)RNA, which is processed into a 28bp piwiRNA involved in epigenetic re-silencing of the locus [475]. A higher ratio of sense to anti-sense transcripts thus allows a higher chance of stable gene transcription and expression of the particular KIR allele. Polymorphisms in KIR promotor sequences that alter transcription factor binding sites can impact KIR expression and can account for expression differences in different alleles as shown for KIR3DL1 [449,473]. A mouse study investigating the highly homologous Ly49 bidirectional promoter further suggests a role of activating receptor signaling during NK cell development in induction of proximal promoter sense-activity, thus increasing the probability of KIR expression [476].

Once the clonal KIR gene expression is established, the regulation of the surface expression frequency or intensity of an expressed KIR are poorly defined. A recently identified intermediate promoter element (Pro-1) upstream of the proximal promoter has been reported to be involved in regulating the expression of actively transcribed KIR genes in mature $CD56^{dim}KIR^{+}$ NK cells [477,478]. Sense transcription from the proximal promoter was shown to be not sufficient but additional activity of the intermediate promoter was required to drive KIR protein expression in mature NK cells.

Figure 3.5 illustrates a successive model of KIR expression during different developmental stages [474,477]: KIR gene expression is epigenetically silenced in early progenitor cells by DNA methylation and condensed histone signatures but is transformed into euchromatin as NK cells progress during development. Upon transition to the $CD56^{bright}$ stage, transcription from the distal promoter (Pro-D) opens the downstream proximal promoter region (Pro-S). The sense to anti-sense ratio of transcripts from the bidirectional switch (Pro-S) determines the stochastic clonal expression patterns of KIR alleles, while the intermediate promoter (Pro-1) controls expression in mature KIR^{+} NK cells.

3.5. NK cell education

The complexes for KIR/Ly49 and MHC genes are located on different chromosomes and independent segregation results in variable receptor-ligand combinations. KIR expression of NK cells is a stochastic event and genetically unlinked to the HLA haplotype of the individuum, resulting in NK cells expressing multiple or no KIRs on the surface [442,443]. In

line with this, some NK cells lack expression of a receptor for binding of self MHC-I molecules.

Ligand specificity of KIR/Ly49 receptors for self-MHC class I is essential for NK cells in order to recognize and eliminate 'missing-self' target cells with downregulated or altered self–MHC-I expression [19,117–119]. To prevent autoimmunity, self-tolerance of NK cells lacking any inhibitory receptors for self-HLA-I is generated in a process termed 'education' or 'licensing' [479,480]. NK cells are educated by the interaction of self–MHC-I and their cognate inhibitory receptors resulting in acquisition of full responsiveness [481–483]. Thereby, educated NK cells are functional competent but remain tolerant towards self–MHC-I positive cells because they express at least one inhibitory receptor. Conversely, NK cells that fail to bind self–MHC-I are hyporesponsive to ligation of activating receptors and thus preserve tolerance towards self. NK cells developing in the absence of interactions with self-MHC class I molecules, as shown in MHC deficient mice, are hyporesponsive and defective in killing of MHC-I negative target cells [481–485]. In addition to KIRs, also other inhibitory receptors are involved in licensing of NK cells. Among others, these include the CD94/NKG2A heterodimer, which recognizes the non-classical MHC-I molecule HLA-E, and the signaling leukocyte activating molecule (SLAM) family receptors 2B4 and SLAMF6 [486–489]. Furthermore, not only interactions of inhibitory self-receptors with their ligands on adjacent cells (in trans) contribute to NK cell tolerance but several studies have demonstrated a role for cis interactions (on the same cell) in the education process [490–494].

Currently, several models exist that describe the molecular mechanism leading to NK education upon engagement of a self–MHC-I specific receptor with its cognate ligand [480,495–497]. In the arming model, NK cells only acquire functional competence after encountering self–HLA-I and are actively endowed with effector potential [481,495]. The disarming model postulates that initially functional NK cells are disarmed through constant activation-induced anergy to preserve tolerance if the activation is not balanced by inhibitory signals [483,497]. In general, both licensing mechanisms seem to require functional ITIM signaling upon inhibitory receptor engagement, which might act through an additional signaling pathway than dephosphorylation of Vav-1 [235,481]. The rheostat model proposes that NK effector potential is calibrated by the quantitative and qualitative interactions between inhibitory receptor and their ligands [487,498–502]. The density of inhibitory self-receptors correlates with the strength of responsiveness of individual NK cells tuning the magnitude of education to environmental MHC. The different models could be rather

complementary than mutually exclusive and experimental evidence supports the concept that NK cell education could be adaptable to changes in the MHC-I environment of the host [503]. Chronic exposure of mature or developing cells to activating ligands could tolerize NK cells, rendering them hyporesponsive even towards unrelated stimuli. The reversibility of the anergic state indicates an ongoing calibration of NK cell education to maintain tolerance to constantly changing environments [504–507]. Adoptive studies in mice have further demonstrated plasticity in the education status of mature NK cells. Uneducated and hyporesponsive NK cells regain their functional competence when transferred into a MHC-I sufficient host and vice versa [508,509]. However, studies in humanized mice and HSCT in patients have indicated that transferred NK cells remain educated in the different HLA-I environment of the recipient [492].

However, uneducated NK cells are not completely dysfunctional but rather exhibit a higher threshold for excitability by activating receptors and inefficient target cell recognition. They are still capable of robust effector function when stimulated with pro-inflammatory cytokines or with pharmacological stimuli such as PMA/Ionomycin [481,510]. Furthermore, *in vitro* studies using human NK cell and *in vivo* tumor models in mice revealed that anergy of unlicensed NK cells was reversible and responsiveness could be restored in the presence of inflammatory cytokines such as IL-2, IL-12 or IL-18 [510–512]. Marçais et al. recently demonstrated that the level of education and responsiveness correlated with mTOR activity which was restored in hyporesponsive NK cells upon cytokine treatment resulting in re-educated NK cells [513]. Thus, mTOR operates as a molecular rheostat of NK cell responsiveness and education. Unlicensed NK cells may thus be functional during inflammatory conditions, suggesting that the cytokine milieu may overcome strict MHC-dependent licensing requirements which primarily affects contact-dependent NK cell activation. Moreover, it was shown that infection with *Listeria monocytogenes* as well as virus-induced inflammation was capable of breaking self-tolerance in mice [483,514]. Recent studies suggested distinct roles for licensed and unlicensed NK cells in viral infection, anti-tumor immunity and engraftment of allogeneic transplantations [515–517]. Unlicensed cells dominate the protection against MCMV infection and are superior effectors compared to licensed NK cells, probably because they become functional in the pro-inflammatory milieu and are enabled to expand preferentially in absence of restraining inhibitory self-receptors [518]. Furthermore, hyporesponsiveness of uneducated NK cells could be overcome by robust CD16 signaling resulting in a beneficial role of these uneducated NK cells in

mediating ADCC in therapeutic settings of MHC-I–expressing neuroblastoma or lymphoma [519–521]. Taken together, NK cell licensing provides an individual activation threshold that is adjusted to perturbations in the peripheral environment such as transplantation or inflammation with specialized functions of educated NK cells in providing immunity against MHC-deficient tumors and uneducated NK cells protecting against viral infections or MHC-I sufficient tumors.

3.6. Memory in NK cells

Immunological memory is defined as the generation of a faster and more potent recall response upon subsequent challenge with the same antigen as during the initial encounter. The formation of immunological memory was classically attributed to the adaptive immune system. T and B cells encountering their cognate antigen undergo clonal expansion and differentiation into potent effector cells, expanding the pool of cells specific for a particular antigen. After clearance of the infection, cell numbers decrease in the contraction phase, leaving some cells to differentiate into long-lived memory cells that are capable of fast and enhanced effector responses upon re-challenge with the same antigen [522]. Antigen specificity is one of the main characteristics of adaptive immunity and a crucial hallmark of immunological memory.

Since NK cell activation is facilitated by germline encoded receptors without somatic gene re-arrangement, NK cells were considered to lack antigen specificity and immunological memory. However, a growing body of experimental evidence suggests that NK cells as well as other innate immune cells can possess adaptive features such as clonal expansion, long-term persistence and superior recall responses [10,11,24,25].

Several ways have been described that can induce the formation of NK cell memory [523]. Memory-like NK populations with robust recall responses have been reported during viral infection [23,524] and contact hypersensitivity reactions [525], indicating an antigen-specific immunologic memory of NK cells (Figure 3.6). Stimulated NK cells can undergo a robust clonally expansion followed by a contraction phase and creation of a long-lived memory pool persisting for several month. Upon re-challenge cells are capable of a more robust recall response, mediating greater effector functions than conventional NK cells. Besides antigen-specific memory, short pre-activation with the pro-inflammatory cytokines

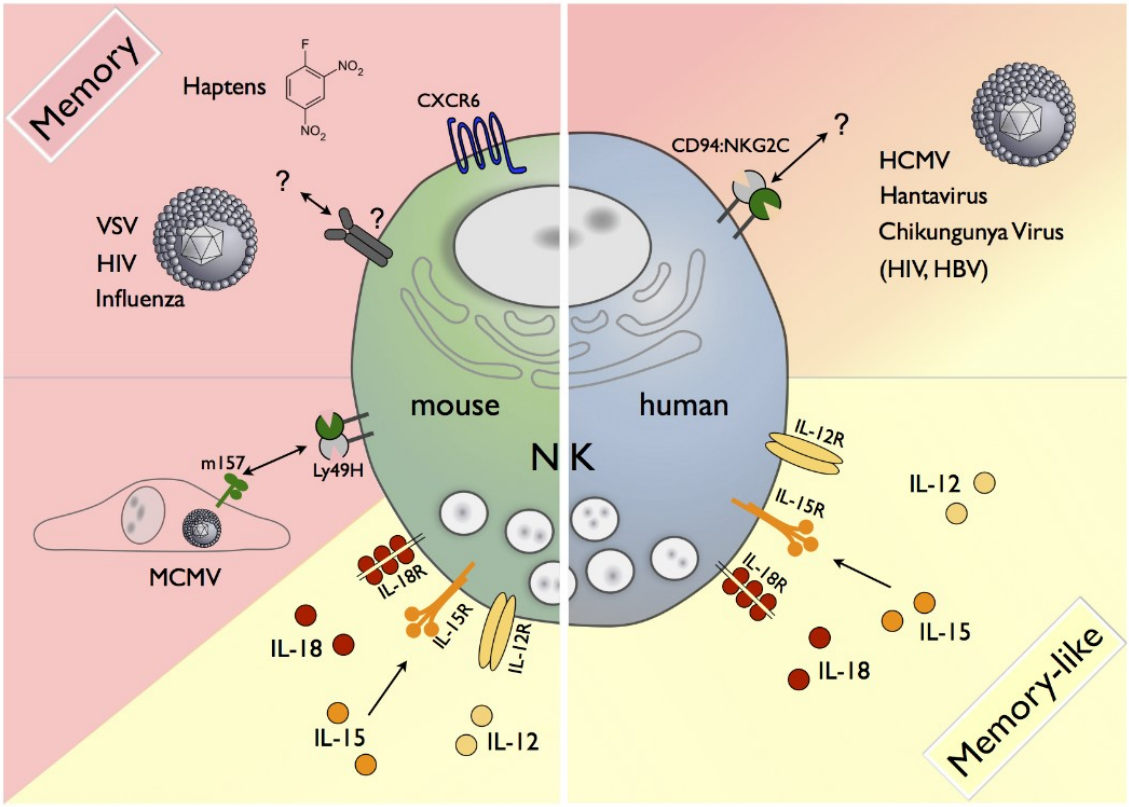


Figure 3.6: Memory and memory like cells in mice and human. A variety of factors contribute to the generation of memory or memory-like cells. Antigen-specific memory is created in mice against haptens and the MCMV m157 protein. The human NK subset expressing the activating CD94/NKG2C receptor expands in HCMV, Hantavirus, Chikungunya Virus, HIV, and HBV infection. Memory-like NK cells in both mice and human can be generated by short pre-activation with IL-12/15/18. (Adapted from Rölle et al. PLOS pathogens 2013) [524].

IL-12/15/18 has been shown to induce an antigen-unspecific memory-like phenotype with sustained effector function persisting for several weeks *in vitro* and even for up to 3 month *in vivo* [288,289,333,334,526].

3.6.1. Liver-restricted memory NK cells

The first observations of NK cells exerting antigen-specific recall responses have been described in a model of hapten-specific contact hypersensitivity (CHS). In 2006 O'Leary et al. reported NK cell dependent specific memory in response to haptens in T and B cell deficient RAG$^{-/-}$ mice [525]. The mice exhibited pronounced recall responses to a secondary challenge with the same hapten, but not to a related molecule with similar chemical

structure. Hapten-specific contact hypersensitivity responses persisted for 4 weeks and could be transferred from hapten-sensitized mice into naïve mice. Antigen-specific responses were shown to be mediated by $CXCR6^{+}CD90^{+}$ liver NK cells and to be induced additionally by various viral components [527].

3.6.2. NK memory in viral infections

MCMV-induced memory

NK cells with adaptive features have also been detected in murine and human cytomegalovirus (MCMV and HCMV) infections [524,528]. MCMV infection in mice were reported to drive the expansion of NK cells expressing the Ly49H receptor recognizing the viral glycoprotein m157. Expansion of $Ly49H^{+}$ NK cells was followed by a contraction phase and generation of a pool of long-lived memory cells [529,530]. Upon re-challenge with MCMV, the persisting $Ly49H^{+}$ NK cells were capable of conferring protection against the infection and exhibit robust recall response such as secondary expansion and superior effector functions [529,530]. In addition to m157 antigen-specificity and Ly49H-DAP10 signaling, signaling via the pro-inflammatory type-I IFNs, IL-18R–myD88, IL-12R and STAT-4 was indispensable for the NK cell anti-viral response [531–533]. Moreover, critical roles for microRNA-155, DNAM-I and the transcription factor Hopx have been reported [534–536]. The contraction phase was shown to be driven by Bim-mediated apoptosis after $Ly49H^{+}$ subset expansion [537] and to involve mitophagy to generate MCMV-specific memory [538]. Moreover, the transcription factors IRF8 and Zbtb32 were demonstrated to be induced upon IL-12 and STAT-4 signaling and to maintain a high proliferative potential of MCMV-specific NK cells in part by antagonizing pro-apoptotic Blimp-1 [539,540].

HCMV-induced memory

In line with observations in the murine CMV infection model, memory-like properties of NK cells were detected in human CMV infections. Increased frequencies of NK cells expressing the activating receptor CD94/NKG2C correlated with a positive serostatus for HCMV of healthy individuals [541]. Preferentially expansion of the $NKG2C^{+}$ NK cell subset was detected during acute HCMV re-infection and in an *in vitro* co-culture system of peripheral blood leukocytes (PBLs) with HCMV-infected fibroblasts [164,542]. A study of HCMV re-activation after hematopoietic stem cell transplantation (HSCT) reported enhanced recall responses

NKG2C+ NK cells in HCMV-seropositive recipients in contrast to HCMV-seronegative patients [543,544]. High numbers of NKG2C+ NK cells were still detectable one year after transplantation, presenting evidence for adaptive features of an antigen-specific NK cell subset resembling the observations in murine CMV infection. Moreover, clinical studies suggest a beneficial role for HCMV re-activation after HSCT in protection against leukemia relapse indicating anti-leukemia properties of adaptive NKG2C+ NK cells [545–548]. Recently, *ex vivo* expansion of adaptive NKG2C+ NK cells using HLA-E–expressing feeder cells has been reported, resulting in effector cells with high anti-tumor potential against ALL (acute lymphoblastic leukemia) blasts [549].

In addition to HCMV infection, expansion of NKG2C+ NK cells has been observed in other viral infections such as Hantavirus and Chikungunya virus infection and also in chronic HIV-1 and HBV infections [550–554]. Of note, NKG2C+ subset expansion has only been detected in HCMV co-infection or in HCMV seropositive patients, supporting the hypothesis that HCMV primes NK cells for memory formation. Interestingly, antigen-specific NK cell memory responses were detected in simian immunodeficiency virus (SIV) infected rhesus macaques, depending onNKG2C and NKG2A and persisting for up to five years [555].

Rölle and colleagues from our laboratory recently demonstrated that HLA-E expression on infected fibroblasts is responsible for driving the expansion of the NKG2C+ subset in HCMV infection [556]. Using an *in vitro* co-culture system of PBMCs and HCMV-infected fibroblasts, our laboratory further reported a crucial role for IL-12 produced by inflammatory monocytes in expansion of NKG2C+ NK cells and for the CD2-CD58 axis in functionality of adaptive NKG2C+ cells [556,557]. Expanded NKG2C+ NK subsets have been described to co-express self-specific KIRs and the maturation marker CD57 [552,558,559]. A recent study indicated a role for activating KIRs, for instance KIR2DS2 and KIR2DS4, in HCMV specific memory formation in the absence of NKG2C expression in NKG2C deficient patients [560,561]. reported HCMV-specific NK cell expansion in the absence of NKG2C expression in patients carrying a homozygous deletion in the NKG2C gene [561]. Importantly, these NK cells were shown to express activating KIRs, including KIR2DS2 and KIR2DS4 involved in activation [560].

The elevated functionality of adapted NKG2C+ NK cells has been attributed to epigenetic imprinting driven by HCMV infection such as high demethylation of the IFN-γ locus [562]. Furthermore, adaptive NKG2C+ NK cells in HCMV-seropositive individuals were found to

be negative for the CD16 adaptor molecule FcεRIγ [563,564]. FcεRIγ-deficient NK cells possessed enhanced antibody-dependent responses compared to conventional NK cells against HCMV-infected antibody-coated target cells [564]. Furthermore, FcεRIγ-deficient NK cells were shown to harbor several deficiencies of proteins classically attributed to innate immunity, including signaling molecules and transcription factors such as SYK or EAT-2, leading to altered cytokine responsiveness [565,566]. FcεRIγ-deficient NK cells in HCMV-seropositive individuals were associated with distinct genome-wide DNA methylation patterns, resembling rather adaptive than innate immune cells [566].

3.6.3. Cytokine-induced memory-like NK cells

The importance of pro-inflammatory cytokines, especially of IL-12, for driving NK cell memory has been demonstrated in viral MCMV and HCMV infection [531,556], suggesting a mechanistic link between pro-inflammatory cytokines and adaptive NK cells. Indeed, short overnight pre-activation of mouse and human NK cells with a combination of the pro-inflammatory cytokines IL-12, IL-15 and IL-18 were demonstrated to generate NK cells with sustained effector functions termed cytokine-induced memory-like NK cells [333,567]. IL-12/15/18–pre-activated NK cells showed high longevity and persistence for up to 3 month after adoptive transfer and were able to reject established tumors in mice [288,333]. Moreover, cytokine-induced memory-like NK cells exhibited high proliferative capacity and pronounced anti-tumor responsiveness after an *in vitro* resting phase in IL-2 or IL-15 as well as after adoptive transfer into tumor-bearing mice [288,289,334,526,568,569]. The strong proliferative capacity of IL-12/15/18–stimulated NK cells has been assigned to enhanced expression the high affinity IL-2R α-chain (CD25), facilitating strong expansion and persistence in response to low-dose IL-2 [288,289]. Furthermore, pre-activation with IL-12/15/18 has been shown to induce epigenetic remodeling of the IFN-γ locus upon adoptive transfer in mice, contributing to their superior IFN-γ production upon re-activation [569].

Recently, the safety and feasibility of adoptive transfer of IL-12/15/18–pre-activated NK cells has recently been reported in a phase-I clinical trial against AML [526]. In this first-in-human trial, the adoptively transferred cytokine–pre-activated NK cells exhibited substantial proliferation and long persistence in the recipient. Importantly, upon *ex vivo* re-stimulation 7 days post-infusion, IL-12/15/18–pre-activated NK cells showed potent anti-

leukemia responses correlating with improved patient survival. Of note, cytokine-induced memory-like NK cells lack antigen specificity and respond to a variety of stimuli. Until now, no specific marker could be identified to discriminate these cells from activated or conventional NK cells.

3.7. Tumor immunology

Cancer is a life-threatening disease with high mortality and growing incidence worldwide. Cancer development is a complex process involving multiple steps of malignant transformation and de-regulation of previously healthy cells. Malignant transformation can be induced through irradiation, chemicals, viruses or chronic inflammation. Through accumulation of genetic mutations, normal body cells lose regulatory circuits controlling proliferation, DNA repair, apoptosis and cellular motility. During cancer progression, uncontrolled proliferation of de-regulated cells results in formation of a solid tumor mass. Dissemination of single cancer cells can lead to metastasis at distant sites in the body, which are often fatal for the cancer patient. Although cancer is a complex, heterogeneous and multi-factorial disease, most cancer types share several common properties that can be summarized as the hallmarks of cancer (Figure 3.7) [570,571].

3.7.1. Hallmarks of cancer

In 2000 Hanahan and Weinberg proposed the six hallmarks of cancer, describing shared capabilities enabling cancer formation, which were extended by 4 additional features in 2011 [570,571].

Sustained proliferative signaling. During cancer progression, tumor cells receive sustained proliferative signaling, enabling uncontrolled proliferation and growth. In healthy tissue, proliferation is carefully controlled through growth-promoting signals and several checkpoints in the cell division cycle. Tumor cells acquire the competence of producing their own growth factors or induce growth-factor production in the tumor microenvironment and the tumor-associated stroma. In addition, mutations in growth factor receptors or downstream signaling pathway components can render tumor cells

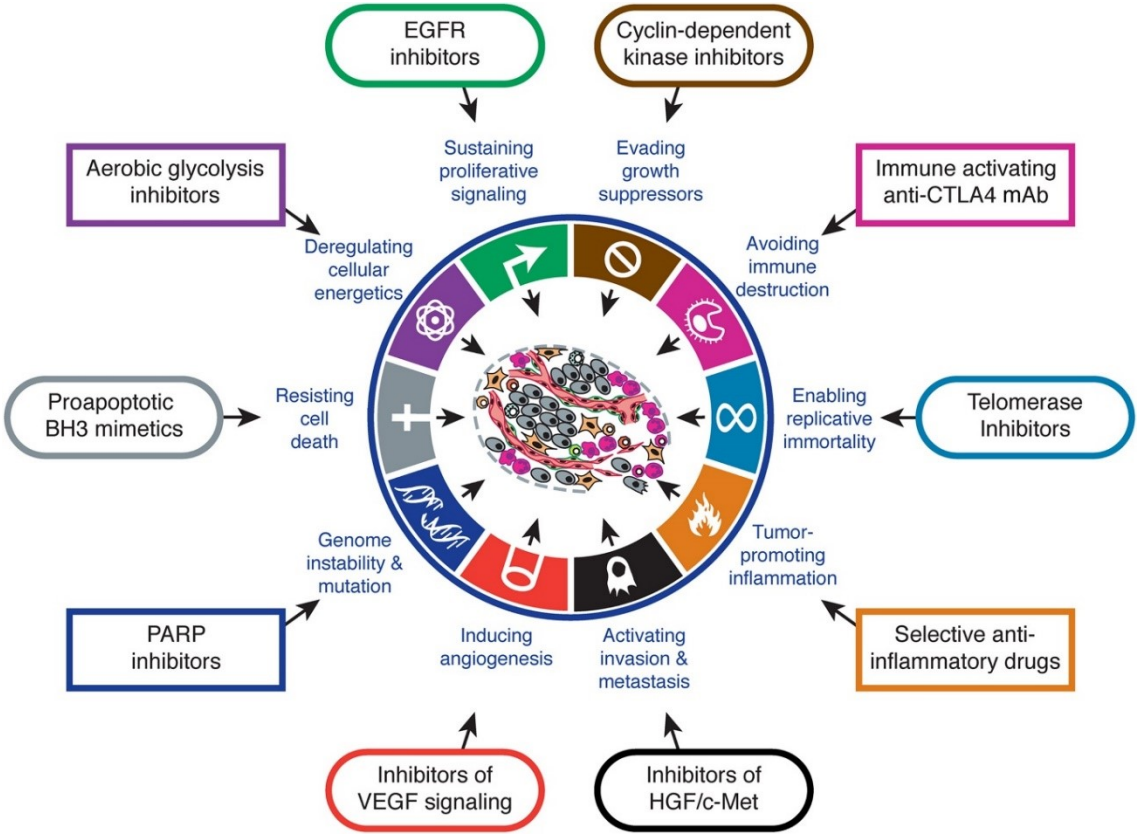


Figure 3.7: The hallmarks of cancer and their therapeutic targeting. The hallmarks of cancer describe ten common characteristics inherent to all tumors. For each hallmark, potential strategies for tumor therapy are indicated. (Hanahan and Weinberg, Cell 2011) [570].

independent of growth signals. Tumor growth promoting oncogenes include for instance *MYC, RAS, BRAF* and components of the MAPK pathway [570,572].

Evading growth suppressors. Tumor cells avoid negative regulatory mechanisms controlling cell division and growth. This often involves mutations in tumor suppressor genes such as retinoblastoma protein (Rb) and tumor protein 53 (p53), which act as central checkpoints of the cell cycle regulating the induction of proliferation, senescence or apoptosis. In addition, the 'contact-inhibition' through neighboring cells, usually controlling aberrant growth in tissues, is de-regulated in tumorigenesis [570].

Resisting cell death. Cancer cells acquire resistance to apoptosis-mediated cell death, which usually controls the removal of malignantly transformed cells and is induced in response to DNA damage or cellular stress. Resistance to apoptosis is often accompanied with loss of function of the DNA damage-sensing protein p53, by downregulation of pro-

apoptotic factors such as Bim or Bax and overexpression of anti-apoptotic proteins such as Bcl2 [570].

Enabling replicative immortality. During tumorigenesis, cancer cells gain an unlimited replication potential through overexpression of telomerase and by preventing the induction of senescence [570].

Induction of angiogenesis. Tumor cells are able to induce an 'angiogenic switch' through the formation of new vessels and a tumor-associated vasculature. Thereby, an increased supply of nutrients and oxygen reaches the neoplasia via the blood stream to cover the increased demand of the constantly growing tumor mass. In this process, cancer cells or cells in the tumor microenvironment induce angiogenesis through production of pro-angiogenic factors like vascular endothelial growth factor-A (VEGF-A) [570].

Invasion and metastasis. Malignant cells can disseminate and invade into distant tissues, forming often fatal metastasis in vital organs. Invasion into tissues involves alternations in matrix proteases and adhesion molecules. Furthermore, cytokines and chemokines, which are expressed by the tumor or the tumor microenvironment, can influence metastatic dissemination, [570,571].

Genome instability and mutation. Additional hallmarks of cancer include characteristics enabling the acquisition of the previous hallmarks. Mutations are considered to be the main driver of tumorigenesis and accumulation of mutations results in destabilization of the gene copy numbers [573,574]. Commonly, inactivation of tumor suppressor genes promotes tumorigenesis, especially of proteins involved in DNA damage recognition and repair [575].

Tumor-promoting inflammation. Tumor-infiltrating immune cells can cause chronic inflammation resulting in the induction of angiogenesis and invasion. In addition, chemicals like reactive oxygen species (ROS) secreted by inflammatory cells can further promote mutagenesis [570,576].

Reprogrammed energy metabolism. Modification and reprogramming of the glucose metabolism from aerobic oxidative phosphorylation towards the faster anaerobic glycolysis, indicated as 'glycolytic switch' and 'aerobic glycolysis', facilitates unrestricted expansion of cancer cells [570]. Due to their uncontrolled growth, tumors often contain hypoxic areas with high levels of the hypoxia-inducible (HIF)-1/2α transcription factors,

which can induce the expression of glucose transporters and glycolytic enzymes and thereby upregulate glycolysis [570,577].

Evasion from immune destruction. The immune system is constantly monitoring tissues, recognizing and killing virus-infected, stressed and transformed cells, a process called tumor immunosurveillance [578]. Therefore, tumor cells evade immune-mediated destruction for instance by reducing the expression of immune recognition structures on their surface and by creating an immunosuppressive tumor microenvironment [570,578].

3.7.2. The immune system and cancer

The immune system has a two-sided role in tumorigenesis and possesses both tumor-suppressive and tumor-promoting functions. In a process called immunosurveillance, the immune system identifies and eliminates malignantly transformed and stressed cells before a tumor mass can establish and become clinically apparent. On the other hand, under certain conditions immune cells can promote tumor progression through chronic inflammation or the creation of an immunosuppressive milieu. Thus, the immune system can shape the tumor during cancer progression by influencing its growth and immunogenicity characterized in three distinct stages of immunoediting: elimination, equilibrium and escape [578–580].

In the elimination phase, immune cells recognize and eliminate emerging tumor cells via the release of perforins/granzymes, type-I IFNs, pro-inflammatory cytokines or damage-associated molecules (DAMPs). Tumor cells express tumor-specific antigens detected by T and NKT cells and stress-induced ligands for activating NK cells receptors such as NKG2D [15,581]. If complete elimination by the immune system fails, the system reaches a balance of tumor cell destruction and tumor growth, the equilibrium phase. Killing of immunogenic tumor cells can facilitate the outgrowth of tumor cell clones with low immunogenicity, defined by low or no expression of tumor-specific antigens, downregulation of MHC-I and of activating NK cell ligands. These clones are hardly detected by the immune system and are thus able to escape the immunosurveillance. In this escape phase, the immune system is no longer capable of controlling the tumor. Moreover, tumor cells that leave their functional state of dormancy become clinically apparent as primary tumor or as metastasis [582].

In addition, the anti-tumor potential of immune cells is hampered by the immunosuppressive microenvironment containing high levels of immunosuppressive mediators, such as TGF-β, IL-10, arginase-1, PGE-2 (prostaglandin-2) or IDO (indolamin-2,3-dioxygenase involved in tryptophan metabolism) [125]. The inhibitory character of the tumor microenvironment is shaped through the recruitment of suppressive immune compartments such as regulatory T cells (T_{regs}), myeloid derived suppressor cells (MDSCs) and tumor-associated M2 macrophages (TAMs). T_{regs} are the main source of the immunosuppressive cytokines TGF-β and IL-10 and additionally express inhibitory molecules such as CTLA-4 or PD-1 [583,584]. Additionally, they compete with T and NK cells for the availability of IL-2 due to their constant expression of the high affinity IL-2Rα-chain (CD25) [302,303]. Their accumulation in the tumor site is often correlated with poor prognosis in the clinics [585]. MDSCs are a heterogeneous population of immature myeloid cells with immunosuppressive activity via the depletion of arginine and the secretion of IL-10, TGF-β and ROS [586,587]. Moreover, suppression of NK cell functionality by MDSCs has been shown to depend on TIGIT-CD155 interactions, supporting the development of strategies that target TIGIT for cancer immunotherapy [267]. Furthermore, they have been shown to directly support tumor growth and to be associated with resistance to immunotherapy [588–590]. To boost anti-tumor immunity in cancer patients, many of these escape strategies are targeted in tumor immunotherapy [578,580,590].

3.8. NK cell-based immunotherapy

Multiple components of the immune system have been implicated to play important roles in cancer immunosurveillance and immunotherapy of cancer has been recently honored as the breakthrough of the year [591]. Based on the 'missing-self' hypothesis of target recognition, NK cells are especially promising options for therapy of HLA-I negative tumors [592]. Partial or complete loss of MHC-I expression, to avoid T cell–mediated recognition, occurs in a wide spectrum of tumor types. In these clinical settings, T cell therapy is compromised through lack of recognition, but NK cells are potently activated.

NK cells deficiencies and defective functionality have been associated with higher incidences of cancer and recurring viral infections in patients and in mouse models [1,593–597]. Conversely, high NK cell infiltration into tumor tissue has been correlated with better

disease outcome in different solid tumor entities [598–601]. *In vivo*, NK cells have been shown to control tumor growth and metastasis of many transplanted, induced or spontaneous tumors in various mouse models [122,123,578,602–605]. Evidence for NK cell cancer immunosurveillance in patients has been derived from several clinical studies supporting their therapeutic potential in the clinic to participate in tumor eradication [34,100,606–608].

Their capacity to distinguish healthy cells from stressed, altered and malignant cells (missing self/induced self, see section 3.3) can be exploited as a promising tool in cancer therapy [592,609,610]. Furthermore, cytokines and chemokines secreted by NK cells were shown to contribute to the anti-tumor immune response of tumor infiltrating T cells and myeloid cells [5,6,104,611,612].

NK cells are particularly effective against metastasis, minimal residual disease and hematological cancers such as acute myeloid leukemia (AML) or multiple myeloma (MM) [605,613–615]. However, the effectiveness of NK cell therapies depends on the type of malignancy as solid tumors (e.g. colorectal cancer or breast cancer) are poorly infiltrated by NK cells [598,599,616]. Several strategies have been designed for the therapeutic use of NK cells in the clinics [100,609,617].

3.8.1. Adoptive transfer of NK cells

Clinical data from adoptive transfer of NK cells and from hematopoietic stem cell transfer (HSCT) have indicated safety and clinical effectiveness of NK cell-based immunotherapy [618,619]. Various approaches can be applied for adaptive cellular transfer of NK cells, differing in the source, degree of maturity, number and activation status of the transferred NK cells [620]. Cells can be obtained from a healthy donor (allogeneic) or directly from the patient (autologous). NK cells for adoptive transfer can be derived from different sources such as peripheral blood (PB), umbilical cord blood (UCB), bone marrow (BM), human embryonic stem cells (hESC) or induced pluripotent stem cells (iPSC) [585,621–623]. Other sources include NK cell lines (e.g. NK-92), which exhibit high cellular cytotoxicity, lack inhibitory KIRs and can be easily genetically modified under GMP conditions [624–627].

NK cells can be infused as part of HSCT, transferred alone as purified and expanded mature NK cells or as additional treatment shortly after HSCT. For a successful therapeutic approach, high numbers of transferred NK cells but also high purity and functionality are

required. Standard GMP protocols include enrichment of NK ells and $CD3^+$ T cell depletion, which is important to prevent the induction of GvHD [628,629]. Numerous methods have been developed for long-term *ex vivo* expansion of NK cells using different kinds of cytokines such as IL-15 or IL-21 or feeder cells expressing membrane bound cytokines and co-stimulatory molecules (e.g. 4-1BB) [630–633]. Automated long-term expansion protocols can generate high numbers of clinical grade NK cells under standardized conditions [634–636]. Major approaches for adoptive NK cell therapy include infusion of autologous or allogeneic NK cells or as part of HSCT treatment.

Adoptive transfer of autologous NK cells

The first clinical application of NK cells involved infusion of autologous PBMCs that were *ex vivo* activated with IL-2 (lymphokine activated killer cells, LAK cells) and mainly contained NK cells and $CD8^+$ T cells [637–639]. Although the IL-2 stimulated cells were highly activated and functional at the time of transfer, only limited clinical efficacy of LAK cells was observed. Transferred cells persisted in the recipient, but anti-tumor activity could only observed upon re-stimulation with IL-2 [640]. To sustain expansion and activity of the transferred cells, daily administration of IL-2 was applied but clinical efficacy was limited by the toxicity of high dose IL-2 [641]. In addition, administration of low-dose IL-2 has been shown to induce expansion of IL-2Rα–expressing (CD25) T_{reg} cells that hamper NK cell activity [642–646].

To maintain NK cell expansion in vivo and to prevent T_{reg} cell induction, application of IL-15 is currently explored in clinical trials, which preferentially stimulates NK cells and $CD8^+$ T cells in the absence of T_{reg} activation [647–649]. For high biological activity, IL-15 has to be trans-presented by IL-15Rα in the body [292]. Therefore, superagonists combining IL-15 and IL-15Rα in heterodimeric fusion proteins such as hetIL-15 (IL-15/sIL-15Rα) or the IL-15–IL-15Rα–Sushi-Fc fusion complex (ALT-803) hold promise as potent activator of NK cell proliferation *in vivo* [650–652].

However, the functionality of transferred NK cells is limited in autologous settings by the inhibitory signals transmitted through KIR receptors interacting with self–HLA-I on cancer cells. Hence, novel approaches are needed to preserve NK cells activation and to improve their anti-cancer efficacy.

Adoptive transfer of allogeneic NK cells

A well-established example of allogenic NK cell transfer is hematopoietic stem cell transplantation (HSCT) in patients with hematological malignancies such as leukemia, resulting in disease free survival and reduced relapse rates [607,653]. In allogeneic settings, hematopoietic stem cells or bone marrow grafts can be obtained from unrelated (mismatched) or related, haploidentical (partially matched) donors. T cells should be carefully removed to prevent graft-vs-host disease (GvHD), because donor-derived T cells can recognize the healthy tissue of the recipient as foreign cells and induce tissue damage of life threatening extent [654]. To avoid rejection and to allow engraftment of the transplanted donor cells, pre-conditioning of the recipient's immune system is required by preparative regiments such as total body irradiation or chemotherapy [607]. Blood NK cells have been shown to recover early after transplantation and to exert a strong graft-vs-leukemia (GvL) effect without induction of GvHD [655–657]. A seminal study by Velardi and colleagues provided evidence for the importance of haploidentical donor-derived NK cells in successful HSCT, observing significant lower relapse rates of AML patients that received allogeneic bone marrow transplants [606]. A prerequisite for the successful treatment was the KIR/ligand mismatch of donor and host cells. In these settings, the recipient's tumor cells should lack one or more cognate HLA-I ligands for donor inhibitory KIRs. Tus, the alloreactivity of donor NK cells is based on KIR/ligand-mismatched graft NK cells not inhibited by host HLA-I, resulting in high cytotoxicity against leukemic blasts in spite of HLA-I expression [658,659]. Genetic studies further showed that the presence of KIR B haplotypes in HSCT grafts, possessing high gene content of activating KIRs, correlated with lower relapse rates and improved survival [660–663].

Moreover, purified and expanded allogeneic NK cells with high GvL potential can be adoptively transferred in addition to HSCT or as individual therapy [664–666]. This approach, also called donor lymphocyte infusion (DLI), represents an effective treatment option upon relapse after HSCT or in combination for high-risk leukemias [667,668]. In several studies, infusion of expanded and IL-2–activated NK cells resulted in favorable responses in patients with various hematological malignancies [646,669–671]. It is based on the infusion of T cell depleted and highly purified haploidentical NK cells, which can be pre-activated *ex vivo* with IL-2 and are potent effector cells.

To potentiate their anti-tumor activity, NK cells can be stimulated with various cytokines *ex vivo* before adoptive transfer [288,511,526,669]. *Ex vivo* manipulations presents the possibility to use cytokines or drugs that might be otherwise harmful for *in vivo* application [109,672]. The

combination of IL-12, IL-15 and IL-18 for *ex vivo* activation is of particular interest as it is involved in the generation of long-lived memory-like NK cells with high proliferative capacity and sustained effector functions (see section 3.6.3) [673]. Short pre-activation of NK cells with IL-12/15/18 has been shown to induce expression of the high affinity IL-2R α-chain (CD25) [288,289], facilitating strong expansion and persistence in response to low-dose IL-2 after adoptive transfer in mice [288]. Furthermore, cytokine-induced memory-like NK cells exhibit pronounced anti-cancer responsiveness *in vivo* and *in vitro* [288,334,526,568]. Safety and feasibility of adoptive transfer of cytokine-induced memory-like NK cells has recently been demonstrated in a clinical trial with AML patients [526].

However, the adoptive transfer of allogenic NK cells alone or as HSCT requires the availability of a matching donor and might be hampered by short persistence of the transferred NK cells in the patient [645]. Moreover, it might bear the risk of promoting GvHD, as recent studies reported severe GvHD upon adoptive allogeneic NK cell transfer in combination with HSCT, which was probably exerted by contaminating alloreactive T cells [666,674–678].

3.8.2. Strategies to improve NK cell anti-tumor potential

NK cell anti-tumor efficacy in clinical trials has been limited by various mechanisms such as inhibitory KIRs, low persistence of transferred cells, weak tumor infiltration and attenuation of their activation status through the immunosuppressive tumor microenvironment [34,582,679]. Current approaches aim to improve effectiveness of NK cell-based therapies through increasing NK cell numbers by novel expansion protocols, improving their activation state and trafficking to the tumor site and by transfer of selected subpopulations with desired functional capacities [680–682]

The application of therapeutic antibodies or antibody-related constructs to bolster anti-tumor efficacy of NK cells experiences currently intensive investigations in cancer immunotherapy [101]. Therapeutic antibodies can specifically redirect immune cells to tumor-restricted antigens expressed on cancer cells and have been shown to greatly improve the outcome of cancer patients [100,101]. Antibodies with different specificities for various cancer entities are widely used in the clinics targeting for instance CD19, CD20 (rituximab), Her2/neu (trastuzumab), GD2 or EGFR (cetuximab). Their clinical efficacy has

been mainly contributed to the exertion of ADCC mediated via engagement of CD16 (FcγRIIIA) through the Fc part of the antibody. CD16 triggering can elicit strong activation of NK cells without the need of additional co-activation, resulting in potent effector cells [12,98].

Bispecific antibodies and bispecific killer engagers (BiKEs) are bivalent molecules containing two antigen binding specificities, designed to bind a tumor-specific antigen with one arm while engaging an activating NK receptor (e.g. CD16) with its second specificity [683]. Simultaneous binding of both antigens cross-links NK cells with the tumor target cell and can elicit potent NK cell activation and effector function. TriKEs (trispecific killer engagers) can simultaneously bind either two antigens, two receptors or can incorporate e.g. IL-15 to additionally stimulate NK cells [684]. The anti-tumor efficacy of therapeutic antibodies or bispecific constructs can be enhanced by combination with cytokines that stimulate and expand NK cells in vivo [109,673,684].

Activating chimeric antigen receptors (CARs) contain an antibody-related receptor for a tumor-specific antigen on the surface, coupled to potent intracellular signaling modules such as CD3ζ, CD28 or 4-1BB. Antigen-specific binding thus results in strong activation of the effector cell [685]. CAR-expressing T cells yielded promising results in the clinics but also raised severe safety issues [686]. The generation of CAR-expressing lymphocytes has been previously focused on T cells, but also protocols for genetically engineering NK cell lines and primary NK cells are currently exploited [687–690].

Similar to checkpoint blockade of exhausted T cells, blocking antibodies against inhibitory checkpoint molecules are currently explored for NK cell immunotherapy [691,692]. The anti-tumor effector function of autologous NK cells is substantially diminished by inhibitory signals delivered through the interaction of inhibitory KIR molecules with cognate HLA-I ligands on tumor cells. Checkpoint blockade of inhibitory receptors such as KIR or NKG2A therefore represents a promising strategy to reduce the threshold for NK cell activation and to increase their responsiveness towards self–HLA-I positive tumors [693,694]. Blocking of pan-KIR2D receptors with the blocking anti-KIR2D antibody Lirilumab (1-7F9/IPH2101/IPH2102) has been shown to enhance NK cell activity *in vitro* and in transgenic mouse models against HLA-I–expressing tumor cells [695–698]. In addition, an anti-KIR3DL2 blocking antibody is currently under clinical development [699]. The safety and efficacy of KIR blockade has been reported for the treatment of patients with AML or

multiple myeloma [700–705]. Recently, clinical investigations of KIR checkpoint blockade have been additionally extended for various other hematological malignancies and for solid tumors [706]. However, a recent study indicated contraction and functional detuning of KIR2D positive NK cells upon single therapy with Lirilumab in smoldering multiple myeloma patients [707]. Therefore, combination of anti-KIR therapy with other approaches are currently considered, for instance together with anti–CTLA-4 or anti–PD-1 blockade [682,708] or with ADCC inducing therapeutic antibodies [697,698,709].

4. AIM OF THE STUDY

Cancer immunotherapy based on NK cells is a promising field in the treatment of hematological malignancies. However, one major obstacle represents the attenuated activation of NK cells in the patient by the interaction of inhibitory killer immunoglobulin-like receptors (KIRs) with self–HLA-I on autologous tumor cells. Several approaches are currently applied for circumventing KIR-mediated inhibition to fully unleash NK cell anti-tumor efficacy in immunotherapy. For instance, adoptive transfer of haploidentical KIR/ligand-mismatched NK cells has been shown to greatly improve the clinical outcome of leukemia patients compared to matched NK cells that are inhibited by KIR/self–HLA-I interaction. Moreover, Lirilumab, a therapeutic antibody blocking inhibitory KIR2D molecules on NK cells, is currently tested in clinical trials. However, in contrast to autologous infusions, adoptive transfer of allogeneic cells is limited by the selection of a matching donor, low persistence of transferred NK cells in the adoptive host and the risk of graft-vs-host disease (GvHD). Therefore, it is of great importance in the field to develop new strategies to further improve the anti-cancer potency of NK cells against HLA-I–expressing tumors in autologous transfer.

Short pre-activation of NK cells with the pro-inflammatory cytokines IL-12/15/18 has been implicated in the generation of NK cells with sustained effector functions, high longevity and memory-like features. Recently, a clinical trial in AML patients employed adoptive transfer of IL-12/15/18 pre-activated allogeneic NK cells, which showed superior memory-like anti-tumor activity one week after transfer and mediated remission in a subset of patients. However, no characterization of the cytokine-stimulated NK cells was performed at the time of infusion into the patient, evaluating their immediate anti-cancer potential.

In the present study, we wanted to uncover the mechanisms underlying the potent effector functions of IL-12/15/18–activated NK cells to further improve treatment options for autologous cell transfer. We therefore performed a comprehensive phenotypical and functional analysis of NK cells at different time points after IL-12/15/18 stimulation, focusing on the balance of activating and inhibitory NK cell receptors. We aimed to gain mechanistic insights into the superior competence of IL-12/15/18–activated NK cells against transformed cells that express self–HLA-I. To study the implication of the

modulated KIR/HLA-I axis on NK cell functionality, we performed redirected lysis assays with specific antibody-mediated stimulation of single activating and inhibitory receptors. Furthermore, we assessed the impact of IL-12/15/18 stimulation on NK cell cytotoxicity against tumor targets bearing cognate HLA-I molecules. Finally, we employed an *in vitro* HCMV infection model to evaluate our phenotypical observations in a physiological setting of pro-inflammatory cytokines.

5. MATERIALS AND METHODS

5.1. Materials

5.1.1. Cells and cell lines

Table 5.1: Cells and cell lines

Cells or cell line	Cell type	Medium
Primary PBMCs	Human peripheral blood mononuclear cells	RPMI, 10% FCS, 1% P/S
Primary NK cells	Human natural killer cells	SCGM, 10-20% human serum, 1% P/S
K562	Human myelogenous leukemia cell line	RPMI, 10% FCS, 1% P/S
P815	Mouse mastocytoma cell line	RPMI, 10% FCS, 1% P/S
MRC-5	Human fetal lung fibroblasts	DMEM, 10% FCS, 1% P/S
721.221	Human EBV transfected B lymphoblastoid cell line	RPMI, 10% FCS, 1% P/S, Gln 2mM
721.221_HLA-Cw03	Human EBV transfected B lymphoblastoid cell line	RPMI, 10% FCS, 1% P/S, Gln 2mM

5.1.2. Cell culture products

Table 5.2: Cell culture products

Product	Company
Biocoll separating solution	Biochrom
Dimethylsulfoxide (DMSO) Hybri-Max	Sigma Aldrich
Dulbecco's modified Eagle's Medium (DMEM)	Sigma-Aldrich
Dulbecco's phosphate buffered saline (PBS)	Sigma-Aldrich
Fetal Calf Serum	Gibco
Geneticin (G418)	Gibco
GolgiPlug	BD Biosciences
GolgiSTOP	BD Biosciences
Human serum, type AB, converted	PAA
Ionomycin	Sigma Aldrich

Product	Company
L-Glutamin 200mM (100x)	Gibco
Penicillin/Streptomycin	Sigma-Aldrich
Phorbol-12-myristate-13-acetate	Sigma Aldrich
RPMI 1640	Sigma Aldrich
Stem cell growth medium (SCGM)	CellGenix
Trypan Blue	Sigma Aldrich
Trypsin-EDTA	Sigma-Aldrich

5.1.3. Chemicals

Table 5.3: Chemicals

Chemicals	Company
^{51}Chromium	Perkin Elmer
Batimastat	Merck Millipore
Cytofix™ Fixation Buffer	BD Biosciences
EDTA	Ambion
Ethanol	Sigma-Aldrich
Nuclease-free water	Ambion
Phosflow™ Perm Buffer III	BD Biosciences
Triton X-100	Sigma-Aldrich
β-mercaptoethanol	Sigma-Aldrich

5.1.4. Solutions

Table 5.4: Solutions

Solution	Ingredients
FACS buffer	PBS, 3% FCS, 0.05% (v/v) NaN_3
Freezing medium	FCS, 10% DMSO
MACS buffer	PBS, 3% FCS, 0.5 mM EDTA

5.1.5. Cytokines

Table 5.5: Cytokines

Cytokine	Company
IL-12	Peprotech
IL-15	Peprotech

Cytokine	Company
IL-18	MBL
IL-2	NIH

5.1.6. Kits

Table 5.6: Kits

Product	Company
EasySep™ Human NK cell isolation kit	Stemcell Technologies
Foxp3 / Transcription Factor Staining Buffer Set	eBioscience / Invitrogen
Human IFN-γ ELISA	BioLegend
Human IL-18 ELISA	R&D
Human NK cell isolation kit	Miltenyi Biotech
LightCycler 480 SYBR Green I Master	Roche
LS Columns	Miltenyi Biotech
MACSplex human Cytokine 12	Miltenyi Biotech
MojoSort™ Human NK cell isolation kit	BioLegend
ProtoScript M-MuLV First Strand Synthesis Kit	NEB
QIAshredder	Qiagen
RNeasy Mini Kit	Qiagen
TURBO DNA-free Kit	Invitrogen

5.1.7. Antibodies

Table 5.7: Primary antibodies for flow cytometry

Antigen	Clone	Company	Dilution
CD107a	H4A3	Biolegend	1:100
CD134 (OX40)	Ber-ACT35 (ACT35)	Biolegend	1:40
CD137 (4-1BB)	4B4-1	Biolegend	1:40
CD14	HCD14	Biolegend	1:80
CD16	3G8	Biolegend	1:50
CD19	HIB19	Biolegend	1:80
CD25	BC96	Biolegend	1:50
CD3	HIT3a	Biolegend	1:50
CD56	HCD56	Biolegend	1:50
CD57	HCD57	Biolegend	1:60
CD69	FN50	Biolegend	1:50

Antigen	Clone	Company	Dilution
DNAM-I	DX11	Biolegend	1:40
HLA-ABC	W6/32	Biolegend	1:100
IFN-γ	4S.B3	Biolegend	1:40 - 1:50
KIR2DL1	143211	R&D Systems	1:10
KIR2DL1/S1/S3/S5	HP-MA4	Biolegend	1:40 - 1:50
KIR2DL2/L3/S2	DX27	Biolegend	1:40 - 1:50
KIR2DL2/L3/S2	GL183	Beckman Coulter	1:25
KIR2DL3	180701	R&D Systems	1:10
KIR3DL1	DX9	Biolegend	1:40
NKG2A	REA110	Miltenyi Biotec	1:20
NKG2C	134591	R&D Systems	1:10
NKG2D	1D11	Biolegend	1:40
NKp30	P30-15	Biolegend	1:15
NKp44	P44-8	Biolegend	1:20
NKp46	9E2/NKp46	Biolegend	1:40
NKp80	5D12	Biolegend	1:10
phospho-STAT-5	47/Stat5(pY694)	BD Biosciences	1:5
TIGIT	MBSA43	eBioscience	1:20

Table 5.8: Isotype controls

Antigen	Clone	Company
mIgG1	MOPC-21	Biolegend
mIgG2a	MOPC-173	Biolegend
mIgG2b	MPC11	Biolegend

Table 5.9: Assay antibodies

Antigen	Clone	Company	Concentration
CD16	3G8	Biolegend	1 µg/ml
IFN-α/β-R	MMHAR-2	PBL Interferon Source	5 µg/ml
IL-12	Polyclonal Goat IgG	R&D Systems	5 µg/ml
KIR2DL2/L3/S2	GL183	Beckman Coulter	25 µg/mL

5.1.8. Cell dyes

Table 5.10: Cell dyes for flow cytometry

Dye	Company	Dilution
Fixable Viability Dye Zombie Aqua	Biolegend	1:200
7AAD	Biolegend	1:50
Annexin V	Biolegend	1:50
Carboxyfluorescein succinimidyl ester (CFSE)	Sigma-Aldrich	1 µM

5.1.9. Oligonucleotide qRT-PCR Primers

Table 5.11: RT-qPCR Primers

Primer	Sequence 5' - 3'
KIR2DL1 *fwd*	TTGGGACCTCAGTGGTCATC
KIR2DL1 *rev*	GGTCCATTACCGCAGCATT
KIR2DL3 *fwd*	CTTCGGCTCTTTCCGTGACT
KIR2DL3 *rev*	AGAACATGCAGGTGTCTGGG
β-2microglobulin *fwd*	TCAGATCTGTCCTTCAGCAA
β-2microglobulin *rev*	CATGTCTCGGTCCCAGGTGA
β-actin *fwd*	AAACTGGAACGGTGAAGGTG
β-actin *rev*	AGAGAAGTGGGGTGGCTTTT

5.2. Methods

Part of this section has been published in Ewen et al. EJI 2018 [710].

5.2.1. Cell culture methods

The 721.221 cell line and its transfectants were kindly provided by C. Watzl (Technical University Dortmund, Germany) and were cultured in RPMI medium supplemented with 10 % FCS and 2 mM Glutamin. The K562 and P815 cell lines were cultured in RPMI medium supplemented with 10 % FCS. MRC-5 fibroblasts were cultured in DMEM medium supplemented with 10 % FCS. All media contained 100 U/mL penicillin and 100 mg/mL streptomycin and cell lines were routinely tested for mycoplasma infection by RT-PCR. All sera were heat-inactivated at 56°C for 30 minutes prior to usage. The transfected

721.221_HLA-Cw03 cell line was selected by adding in 0.5 mg/ml G418 (Geneticin) to the cell culture medium.

5.2.2. Human NK cell isolation and culture

PBMC isolation

Buffy coats were provided by DRK-Blutspendedienst Mannheim, collected according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all human subjects prior to blood donation and ethical approval 87/04 was granted by the Ethik Kommission II of the Medical Faculty Mannheim, Germany. Isolation of peripheral blood mononuclear cells (PBMCs) was performed by Biocoll density-gradient centrifugation. Peripheral blood was diluted 1:4 with pre-warmed PBS and 35 ml of diluted blood was carefully layered on top of 15 ml Biocoll separation solution. The tubes were centrifuged without brake for 30 min at 1800 rpm. The lymphocyte ring was collected, and cells were washed 3 times with warm PBS. PBMCs were cultured as $2x10^6$/ml in RPMI supplemented with 10% FCS.

Magnetic-activated cell sorting (MACS)

NK cell isolation from PBMCs was performed by negative selection (human NK cell isolation kits) according to the manufacturer's protocol, although only 75 % of the recommended reagents were used. Purity of isolated CD3- CD56+ NK cells was > 90 % as determined by flow cytometry. Freshly isolated NK cells are indicated as naïve throughout the manuscript and analyzed immediately. NK cells were cultured in SCGM medium containing 10 % human serum (heat-inactivated) or 20 % human serum for stimulation. NK cells were activated with a combination of IL-12 (10 ng/mL), IL-15 (20 ng/mL) and IL-18 (100 ng/mL) or IL-15 alone (20 ng/mL) for 48 h or as indicated. After stimulation, NK cells were washed and re-cultured in the presence of 100 U/mL recombinant IL-2 and medium supplemented with IL-2 was exchanged every two days.

CFSE labeling

Freshly isolated NK cells or PBMCs were washed with PBS and adjusted to $1x10^7$ cells/ml. 3.3 µl/ml of 300 µM CFSE solution was added while shaking the cells to achieve a final CFSE concentration of 1 µM. After 10 min incubation at 37°C in the incubator, the reaction was

stopped by adding 5 ml of pure FCS and 5 ml of medium. Cells were washed twice with medium containing 10 % FCS and used in further experiments.

HCMV Co-cultures

One day before starting the co-cultures, $1x10^5$/well MRC-5 fibroblasts were seeded in a 24-well plate. The next day, fibroblasts were infected for 8 h with human cytomegalovirus (HCMV, subtype AD169, kindly provided by Anne Halenius, University of Freiburg) at a multiplicity of infection (MOI) of 10 or were left uninfected. Virus was washed away and fibroblasts were co-cultured together with $2x10^6$ PMBCs per well in complete DMEM supplemented with 20 IU/ml IL-2. Fresh medium containing IL-2 and if applicable blocking antibodies was added after 5 days. After 3, 5 and 7 days, PBMCs were harvested and analyzed for their receptor expression by flow cytometry. For neutralization of IL-12 and blocking of type-I IFN receptor, PBMCs were resuspended in the appropriate amount of antibody (both 5 µg/ml) or isotype control and pre-incubated for 30 min. Cells were filled up with complete DMEM to reach the final cell and antibody concentration per well. Neutralizing agents were added again at day 5.

5.2.3. Flow cytometry methods (FACS)

Extracellular FACS staining

For flow cytometry staining, cells were harvested and washed with PBS. Up to $1x10^6$ cells were stained in a 96-well plate with 15 µl FACS buffer containing appropriate dilutions of all primary antibodies. After 30 min incubation on ice in the dark, cells were washed twice with FACS buffer, resuspended in FACS buffer and analyzed immediately. For co-staining of KIR2DL2/L3 and KIR2DL3 or KIR2DL1/S1/S3/S5 and KIR2DL1, respectively, cells were first incubated for 20 min with the single KIR antibody and the multiple KIR antibody was directly added afterwards for another 20 min. For discrimination of dead cells, 7-AAD was added 5 min prior to analysis. Staining of apoptotic cells was conducted by adding Annexin V diluted in Annexin V-binding buffer 15 min at RT before analysis according to the manufacturer's protocol.

Intracellular FACS staining

If cells were fixed for intracellular staining, the fixable viability dye ZombieAqua was used as dead cell marker instead of 7-AAD. The ZombiAqua dye was diluted 1:200 in PBS and 25 µl were added to the washed cells prior to antibody staining. After 10 min on RT in the dark, 25 µl of 2x concentrated antibody mastermix was directly added and incubated for 30 min on ice in the dark. For intracellular staining, cells were fixed and permeabilized after extracellular staining using the Foxp3/Transcription Factor Staining Buffer kit from eBioscience according to the manufacturer's protocol. 100 µl of fixation/permeabilization concentrate (diluted 1:4) was added to the stained and washed cells and incubated for 30 min on ice. Cells were washed twice with diluted permeabilization buffer and primary antibodies ware added in titrated concentration in 25 µl permeabilization buffer for 30 min on ice. Cells were washed once with diluted permeabilization buffer and once with FACS buffer before analysis.

Phospho-STAT-5 staining

NK cells were either left untreated or stimulated with IL-15 or IL-12/15/18 or for 15 - 60 min as indicated. Cells were washed once with FACS buffer, fixed with BD Cytofix Fixation Buffer for 15 min at RT in the dark and washed. After permeabilization for 30 min on ice with BD Phosflow™ Perm Buffer III, cells were stained for phosphorylated STAT-5 with an anti-Stat-5 (pY694) antibody for 30 min on ice. Cells were washed once with FACS buffer and analyzed immediately by flow cytometry.

FACS sorting

To keep the sorted cells in culture for several days, cells were prepared and sorted under sterile conditions. Freshly isolated NK cells were washed with sterile PBS and stained for appropriate surface markers for 30 min on ice in the dark. Cells were washed twice with sterile PBS, resuspended as $5x10^7$/ml in sterile PBS supplemented with 0.1 % FCS and filtered through a 40 µm cell strainer. NK cells were sorted under sterile conditions through a 100 µm nozzle into complete medium. The 15 ml collection tubes were coated with FCS prior to usage and filled with 1 ml complete medium. NK cells were sorted with a FACSAria Fusion cell sorter (BD Bioscience) and purity of all sorted subsets was >98%. Sorted NK cells were analyzed immediately (naïve) or stimulated with IL-15 or IL-12/15/18 as described in 5.2.2.

FACS analysis

Flow cytometry analyses were performed with a FACSCanto II or FACSCalibur (BD Bioscience) and data was analyzed using FlowJo software (Tree Star). Laser settings and compensation were adjusted using appropriate single stainings of each fluorophore. For staining of surface molecules, cell doublets and dead cells (7-AAD positive) were excluded and NK cells were gated on CD3–CD56+ cells. To assess fluorescence intensity staining of a phenotypic marker expressed on a subpopulation, the median fluorescence intensity (MFI) was determined only for that subpopulation. If the marker was expressed on the entire population, the Δ MFI was calculated by subtracting the MFI of the isotype staining from the MFI of the population.

5.2.4. Functional *in vitro* assays

CD107a degranulation assay

Pre-activated NK cells and 721.221 target cells were harvested, washed and adjusted to $1x10^6$/ml in RPMI supplemented with 10 % FCS and 1 % P/S. Effector NK cells were co-cultured with indicated target cells at an effector:target ratio of 1:1 in a 96-well plate. As a negative control of spontaneous degranulation, effector cells were cultured alone. PMA (50 ng/ml) and Ionomycin (750 ng/ml) were added to the effector cells in one condition as positive control. Cells were co-cultured in the presence of anti-CD107a antibody or respective isotype control and after 30 min 0.5 µl/well GolgiStop (monensin) was added. After 4 h cells were harvested and stained for CD3, CD56, KIR2DL2/L3 and 7-AAD as described in section 5.2.3. Degranulation was assessed by flow cytometry analysis of CD107a co-gated on KIR2DL2/L3 positive or negative NK cells.

^{51}Chromium release assay

Cytotoxic activity of NK cells against 721.221 target cells was measured in a standard 4 h ^{51}Cr release assay. NK effector cells were sorted for KIR2DL2/L3 expression and activated with IL-15 or IL-12/15/18 for 48h. When indicated, interaction of 221_Cw03 and KIR2DL2/L3 was blocked by pre-incubation of NK cells with 25 µg/mL anti-KIR2DL2/L3 mAb (GL183) or respective mIgG1 isotype control. Target cells were resuspended in 500 µl complete RPMI and labeled for 1 h with 100 µCi ^{51}Cr. Target cells were washed three times

and 3×10^3 target cells were added to effector cells ± antibody at the indicated effector / target (E:T) ratios in triplicate in a 96-well plate. Target cells were cultured alone (minimum or spontaneous release) or with 10 % Triton X-100 (maximum). After 4 h incubation at 37°C, supernatants were collected and the release of ^{51}Cr was measured. Specific lysis was calculated as [mean cpm - mean cpm (min)] / [mean cpm (max) - mean cpm (min)].

Redirected lysis assay

Cytotoxic activity of NK cells against antibody coated P815 target cells was measured in a standard 4 h ^{51}Cr release assay as described above. NK cells were sorted according to their KIR2DL2/L3 expression and activated for 48 h with IL-15 or IL-12/15/18 in the presence of 10 µM of the matrix-metalloproteinase inhibitor Batimastat or equivalent amounts of dimethylsulfoxide (DMSO) solvent control. For redirected lysis, FcR-bearing P815 target cells were pre-incubated for 30 min with 1 µg/mL anti-CD16 antibody (clone 3G8) in combination with either 1 µg/mL anti-KIR2DL2/L3 antibody (clone GL183) or respective mIgG1 isotype control.

5.2.5. Cytokine measurement

Re-stimulation

NK cells were pre-activated with IL-15 or IL-12/15/18 for 16 h or 48 h, washed and subsequently re-cultured in IL-2 (100 IU/ml) or IL-15 (1 ng/ml) for additional 5 days. Cells were harvested, washed intensely and setup for re-stimulation in a 96-well plate in 200 µl fresh, cytokine-free medium. NK cells were either re-stimulated with IL-12 (10 ng/ml) and IL-15 (50 ng/ml) or with K562 target cells at an E:T ratio of 1:1. As positive control,

PMA (50 ng/ml) and Ionomycin (750 ng/ml) were added in one condition. NK cells were cultured alone in medium as negative control. For IFN-γ detection via ELISA, supernatants were harvested after 24 h and the negative 'medium' control was subtracted from the raw data. For intracellular staining of IFN-γ, cells were re-stimulated for 6 h in the presence of 1 µl/well Brefeldin A (GolgiPlug) and intracellular IFN-γ was detected by flow cytometry as described in 5.2.3.

Enzyme-linked immunosorbent assay (ELISA)

NK cells were re-stimulated as described above. Cell-free supernatants were harvested after 24 h and either frozen at -20°C ore processed directly. IFN-γ and IL-18 were measured by ELISA according to the manufacturer's instructions.

MACSplex

Cell-free supernatants of HCMV co-cultures were harvested 3 days p.i. and analyzed for secreted cytokines using the MACSplex Cytokine 12 Kit. The protocol was performed by Markus Granzin (Cerwenka lab and Miltenyi Biotech) according to the manufacturer's instructions.

5.2.6. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

RNA isolation & cDNA synthesis

RNA was isolated from NK cells using the RNeasy Mini Kit and QIAshredder with minor changes in the manufacturer's recommended protocol. For cell lysis, min $2x10^5$ NK cells were washed with PBS, lysed in RLT buffer containing 0.1 % (v/v) β-mercaptoethanol and stored at -80°C until further usage. Thawed cell lysates were homogenized using QIAshredder and RNA isolation was continued with the RNeasy Mini Kit according to the manufacturer's recommendations. RNA was eluted in 30 µl nuclease-free water and was treated with DNase using TURBO DNA-free kit to remove possible genomic DNA contamination. RNA concentration (A260) and purity (A260/A280 ratio) were determined with a NanoPhotometer and stored at -80°C. Isolated RNA was reverse transcribed into cDNA using the ProtoScript First Strand cDNA Synthesis Kit and the provided random primer mix according to the manufacturer's instructions. The same amount of RNA was employed to ensure the same cDNA concentration within all samples used for later comparison. Samples without reverse transcriptase (-RT control) were included to exclude possible genomic DNA contamination. Synthesized cDNA was stored at -20°C.

SYBR Green qRT-PCR

Due to low cell numbers and RNA/cDNA concentrations, cDNA was only diluted 1:2 or 1:3 with nuclease-free water. 6 µl of diluted cDNA was added per well to LightCycler480

Mulitwell plates and mixed with 3 µl LightCycler 480 SYBR Green I Master Mix and 0.5 µl of forward and reverse primer, respectively, in a final concentration of 0.5 µM. Each reaction was performed in technical duplicates or triplicates. Nuclease-free water and -RT samples were included as negative controls. The plate was sealed with a foil, shortly centrifuged and the qRT-PCR reaction was performed on a Roche LightCycler 480 instrument with the following program:

Preincubation:		95°C	5 min
Amplification:	Denaturation	95°C	10 s
	Annealing	60°C	15 s
	Extension	72°C	20 s
	45 cycles		
Melting curves:		95°C	5 s
		65°C	60 s
		97°C	-
Cooling:		40°C	10 s

The relative expression of the gene of interest compared to a housekeeping gene (β2m or β-actin) was calculated as $2^{-\Delta Cp}$ with ΔCp = Cp(target gene) - Cp(housekeeping gene). The efficiency of each primer pair was determined in advance using a standard curve of serial diluted cDNA.

5.2.7. Statistical analysis

Statistical significance was calculated by paired two-tailed student's t-test or one- or two-way ANOVA followed by Bonferroni's post test using the GraphPad Prism 6 software: ns, not significant; *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$, if not assigned differently.

6. RESULTS

The results reported in this study have been previously published in their original or modified form in Ewen et al. 2018 Eur. J. Immunol. [710] and in Rölle, Pollmann et al. 2014 J. Clin. Invest. [556]. Text and Figures have been originally designed and written by myself.

6.1. Downregulation of KIR expression on NK cells upon IL-12/15/18 stimulation

6.1.1. Phenotype of IL-12/15/18–stimulated NK cells

To dissect the improved functionality of IL-12/15/18–activated NK cells, we performed an in-depth phenotypical analysis of human cytokine–stimulated NK cells. Therefore, mature NK cells were isolated from PBMCs of healthy donors, stimulated for 60 h with IL-12/15/18 or with IL-15 alone and analyzed for their surface receptor repertoire by flow cytometry. Cytokine stimulation induced changes in the expression of most NK receptors (Figure 6.1), either reflected by the percentage of receptor expressing cells or by receptor surface expression density per cell as indicated by the median fluorescence intensity (MFI). Expression densities of most activating NK receptors were enhanced upon cytokine stimulation (Figure 6.1 A), whereas expression of NKp46 was only marginally changed (Figure 6.1 A). NKp30, NKp44 and NKG2D expression was more potently induced by IL-15, whereas the intensity of DNAM-I expression was increased by IL-12/15/18 alone (Figure 6.1 A). Expression of NKp80 and CD16 on NK cells was substantially reduced upon IL-12/15/18 stimulation, which is in line with previous publications [156,711]. CD25, the IL-2 high affinity receptor α-chain, was highly upregulated on IL-12/15/18 stimulated cells (Figure 6.1 B) as shown previously by our group and others [271,288,289]. Expression of CD69, which is considered to be an activation marker on NK cells, was highly induced by both IL-15 and IL-12/15/18, while NKG2A expression is slightly increased by both cytokine stimulations (Figure 6.1 B). Furthermore, the co-stimulatory TNFRSF members Ox40 and 4-1BB were highly upregulated upon cytokine stimulation with regards to the percentage of positive cells as well as the expression density levels (Figure 6.1 C). Expression densities

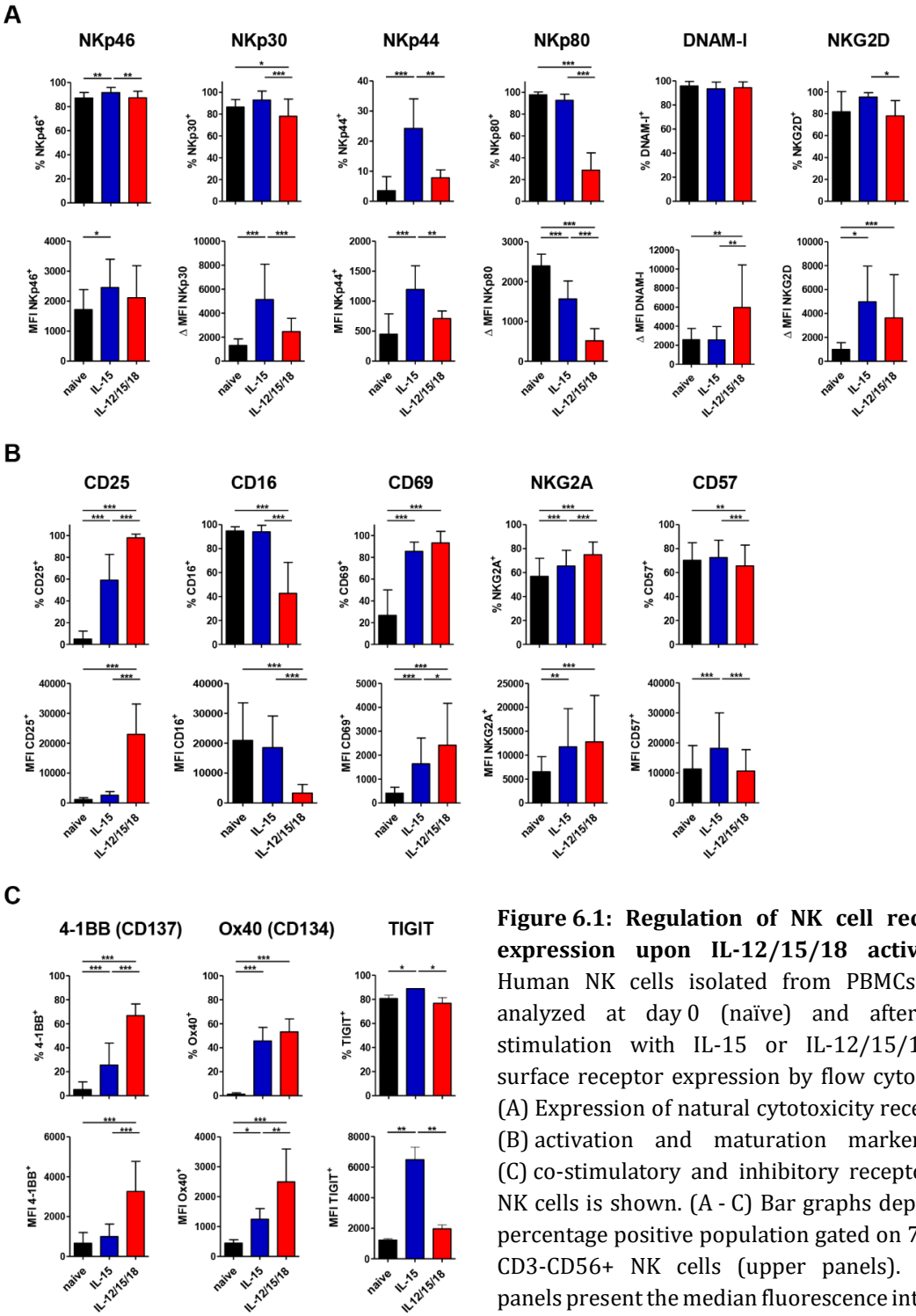


Figure 6.1: Regulation of NK cell receptor expression upon IL-12/15/18 activation. Human NK cells isolated from PBMCs were analyzed at day 0 (naïve) and after 60 h stimulation with IL-15 or IL-12/15/18 for surface receptor expression by flow cytometry. (A) Expression of natural cytotoxicity receptors, (B) activation and maturation marker and (C) co-stimulatory and inhibitory receptors on NK cells is shown. (A - C) Bar graphs depict the percentage positive population gated on 7-AAD-CD3-CD56+ NK cells (upper panels). Lower panels present the median fluorescence intensity (MFI) of the positive subpopulation or the isotype-corrected median fluorescence intensity (Δ MFI = MFI - MFI(isotype)) of the indicated receptors (n = 3 - 27, mean + SD). Statistical analysis was performed with one-way ANOVA followed by Bonferroni's post test.

of TIGIT (Figure 6.1 C) and of the maturation marker CD57 (Figure 6.1 B) were enhanced by IL-15, but not by IL-12/15/18.

6.1.2. Reduced expression of KIR2DL2/L3, KIR2DL1 and KIR3DL1 on IL-12/15/18–stimulated NK cells

Most strikingly, activation of NK cells for 60 h with IL-12/15/18, but not with IL-15 alone, led to a significantly decreased surface expression of the self-HLA–binding killer cell immunoglobin-like receptors (KIRs) (Figure 6.2 A). The percentage of KIR positive NK cells (Figure 6.2 B) as well as the density of surface expression per cell (MFI; Figure 6.2 C) was significantly decreased upon IL-12/15/18 activation compared to IL-15 treated controls. Due to the high similarity in protein structure of KIR molecules, most anti-KIR antibodies cannot discriminate between KIRs with similar extracellular domains. Many antibodies hence cross-react with KIRs sharing the same extracellular Ig-like domain (2D or 3D), which differ only in their intracellular part. The monoclonal antibody clones DX27 and GL183 for example recognize the structurally similar KIR2DL2, KIR2DL3 and KIR2DS2 molecules. In contrast, antibodies specific for either long or short cytoplasmic tails (L & S forms) exist only for few KIR family members, which could facilitate discrimination between activating (S) and inhibitory (L) receptors by flow cytometry. However, similar results were obtained with two different monoclonal antibody clones cross-reacting to KIR2DL2/L3/S2 (clones DX27, GL183) and with an antibody specific for inhibitory KIR2DL3 (clone 180701), indicating regulation of inhibitory KIRs with a long cytoplasmic tail (Figure 6.2 A - C). The antibody clone HP-MA4 cross-reacts with KIR2DL1 and the structurally similar activating family members 2DS1, 2DS3 and 2DS5. Reduced expression of KIR2DL1 was not only observed using the antibody clone HP-MA4 recognizing KIR2DL1/S1/S3/S5 but also by staining with an antibody specific for inhibitory KIR2DL1 (clone 143211), implicating downregulation of inhibitory KIR receptors (Figure 6.2 A - C). Downregulation of KIR3DL1 could be detected with an antibody (clone DX9) specific for the inhibitory molecule with a long cytoplasmic tail (Figure 6.2 A - C). Expression of KIR2DL4, KIR2DL5 and KIR3DL2 was not detected within several healthy donors (data not shown) and was therefore not further investigated.

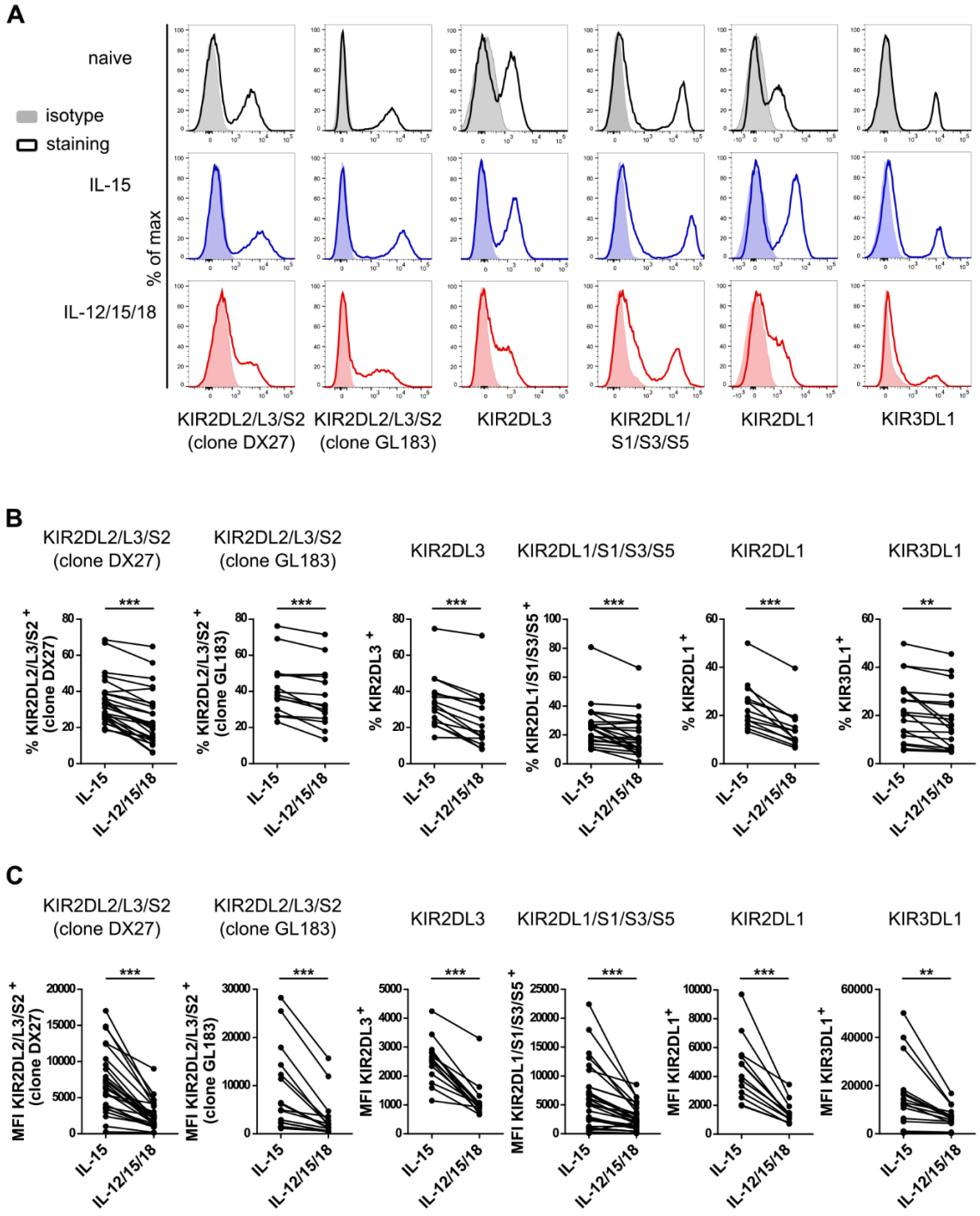


Figure 6.2: IL-12/15/18 stimulated NK cells display reduced levels of KIR2DL2/L3, KIR2DL1 and KIR3DL1 expression. NK cells isolated from PBMCs were analyzed at day 0 (naïve) and after 60 h of stimulation with IL-15 or IL-12/15/18 for the expression of killer cell immunoglobulin-like receptors (KIRs) by flow cytometry. (A) Histograms gated on single 7-AAD-CD3-CD56+ NK cells of one representative donor. (B) Summary of NK receptor expression and (C) median fluorescence intensity (MFI) of the KIR positive subpopulation are depicted. Results from individual donors are connected with a line (n = 13 - 26). Statistical analysis was performed with a paired two-tailed student's t-test.

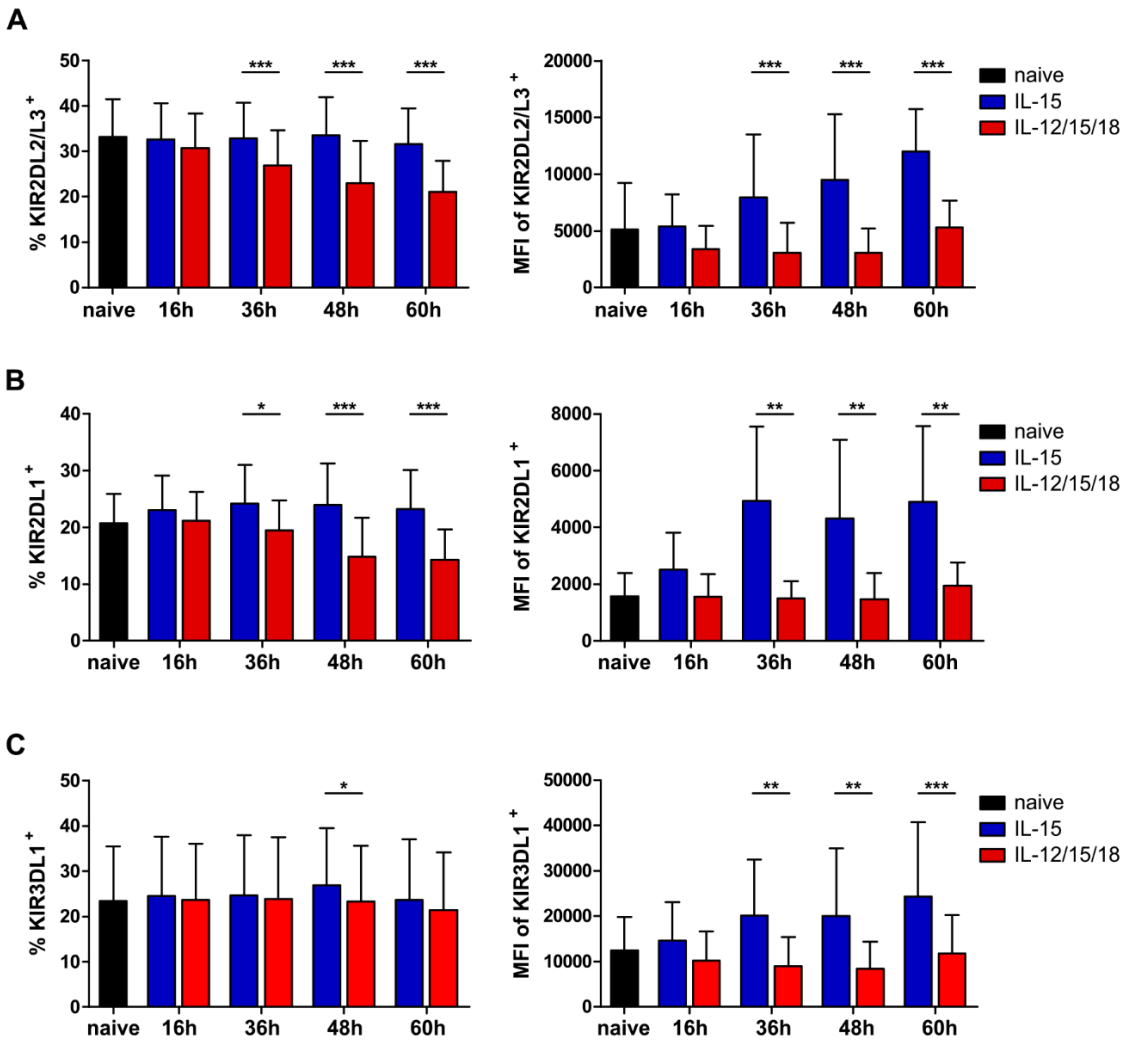


Figure 6.3: KIR downregulation starts after 36 h and is most pronounced after 48 - 60 h of IL-12/15/18 expression. (A) KIR2DL2/L3, (B) KIR2DL1 and (C) KIR3DL1 expression levels on NK cells were determined by flow cytometry at the indicated time points. Bar graphs depict the percentage positive population (left panel) and median fluorescence intensity (MFI) (right panel) of KIR expressing CD3- CD56+ NK cells (n = 6 - 8, mean + SD). Statistical analysis was performed with a two-way ANOVA followed by Bonferroni's multiple comparison test.

Kinetic studies revealed that significant changes in KIR2DL2/L3, KIR2DL1 and KIR3DL1 expression were first detectable after 36 h of IL-12/15/18 stimulation and were most pronounced after 48 h and 60 h (Figure 6.3 A & B). This pattern was reflected in the percentage of KIR positive cells (left panel) as well as in surface expression density levels (MFI, right panel). Downregulation of KIR3DL1 was most evident at surface expression levels (Figure 6.3 C). KIR surface expression was not further decreased upon prolonged stimulation with IL-12/15/18 for up to 6 days (Figure 6.4 A). In contrast, differences

between IL-15 and IL-12/15/18 stimulation were most distinct after 48 h to 60 h and KIR expression levels converged again at later time points. Of note, prolonged stimulation resulted in enhanced apoptosis and reduced cell recovery (Figure 6.4 C & D).

6.1.3. KIR downregulation by IL-12/15/18 is transient and NK cell population intrinsic

Peripheral blood NK cells acquire KIR expression during maturation and differentiation from $CD56^{bright}$ $KIR^{-/dim}$ to $CD56^{dim}$ KIR^{+} subsets. Reduced KIR expression after IL-12/15/18 stimulation could be attributed to regulation of KIR expression on $CD56^{dim}$ KIR^{+} cells or mediated by outgrowth of the $CD56^{bright}$ $KIR^{-/dim}$ NK subset within the whole NK cell population analyzed. Freshly isolated NK cells were FACS-sorted into $CD56^{bright}$ and

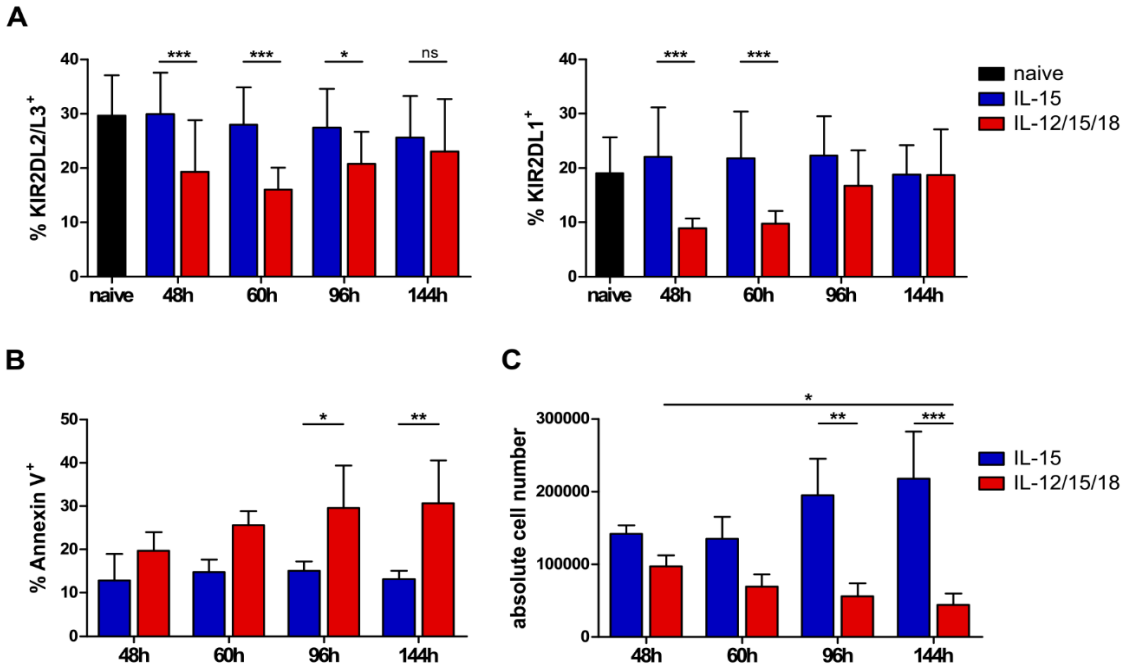


Figure 6.4: Prolonged IL-12/15/18 stimulation results in higher apoptosis and lower cell numbers of NK cells. Human NK cells isolated from PBMCs were stimulated with IL-15 or IL-12/15/18. (A) KIR expression levels were determined by flow cytometry at the indicated time points after stimulation. (B) Annexin V staining for apoptotic cells and (C) absolute cell numbers at the indicated time points after cytokine exposure. $2x10^5$ NK cells were seeded and absolute cell numbers were determined per well. Fresh medium was added to the cultures after 84 h. Statistical analysis was performed with a two-way ANOVA followed by Bonferroni's multiple comparison test (n = 4, mean + SD).

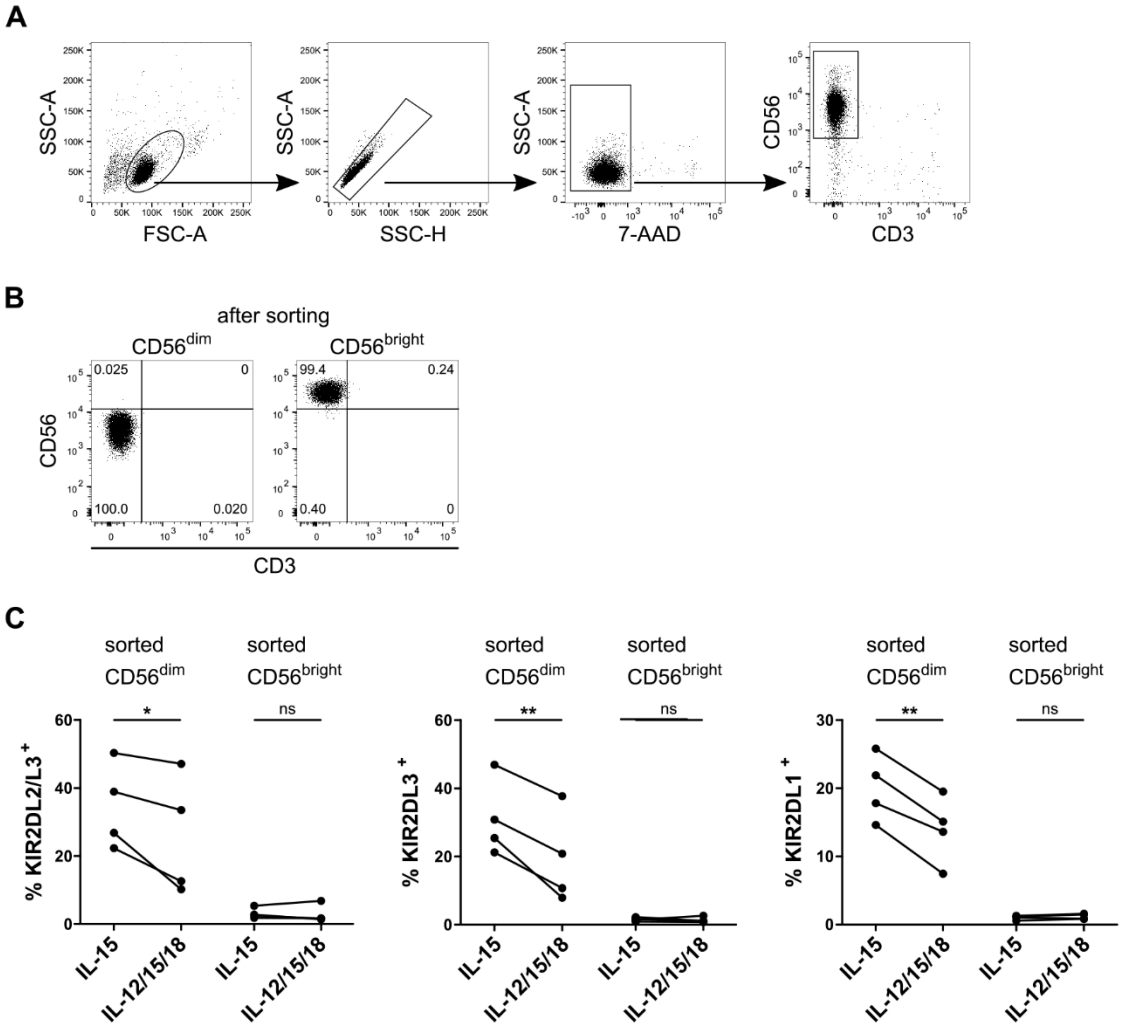


Figure 6.5: KIRs are downregulated on the CD56^{dim} NK cell subset. (A) Gating strategy of NK cells for FACS-sorting and for subsequent flow cytometry analyses. NK cells were distinguished by gating on single, living (7-AAD-), CD3- CD56+ lymphocytes. (B) NK cells isolated from PBMCs were further sorted into CD3- CD56^{dim} and CD56^{bright} subsets. Representative flow cytometry dot plots of naïve NK cells after sorting showing the purity of the sorted populations. Plots were gated on 7-AAD- lymphocytes. (C) Sorted CD56^{dim} and CD56^{bright} subsets were stimulated with IL-15 or IL-12/15/18 for 48 h and analyzed by flow cytometry. Results from individual donors are connected with a line (n = 4 donors). Statistical analysis was performed with a paired two-tailed student's t-test.

CD56^{dim} subsets (Figure 6.5 A & B) and stimulated with IL-15 or IL-12/15/18. Indeed, expression of inhibitory KIR2DL2/L3, KIR2DL3 and KIR2DL1 was downregulated on the sorted CD56^{dim} subset after 48 h activation with IL-12/15/18, whereas naïve CD56^{bright} NK cells exhibited only very low KIR expression that remained unaltered during stimulation (Figure 6.5 C). To determine whether KIR regulation was induced on a distinct NK cell subpopulation or whether it was a result of increased proliferation of the KIR negative

subset, freshly isolated NK cells were sorted according to their KIR expression. Since KIR2DL2/L3 is most frequently expressed in individuals and exhibited pronounced downregulation (Figure 6.5), NK cells were sorted into KIR2DL2/L3 positive and negative subsets and stimulated with IL-15 or IL-12/15/18. Figure 6.5 A and Figure 6.6 A depict the gating strategy for sorting of $CD3^{-}$ $CD56^{+}$ $KIR2DL2/L3^{+/-}$ subsets ensuring high purity of sorted KIR2DL2/L3 positive and negative populations (Figure 6.6 B). KIR2DL2/L3 positive cells could not be isolated by negative depletion but needed to be FACS-sorted after anti-KIR2DL2/L3 antibody staining. Due to the stochastic expression of KIR molecules on NK cells, the KIR2DL2/L3 positive as well as the negative subsets co-expressed other inhibitory self–HLA-I receptors such as NKG2A or various other inhibitory KIRs such as KIR2DL1 (Figure 6.6 C).

Sorted KIR2DL2/L3 positive and negative NK cells were stimulated with IL-15 or IL-12/15/18 for 48 h and analyzed for their KIR expression by flow cytometry. On the

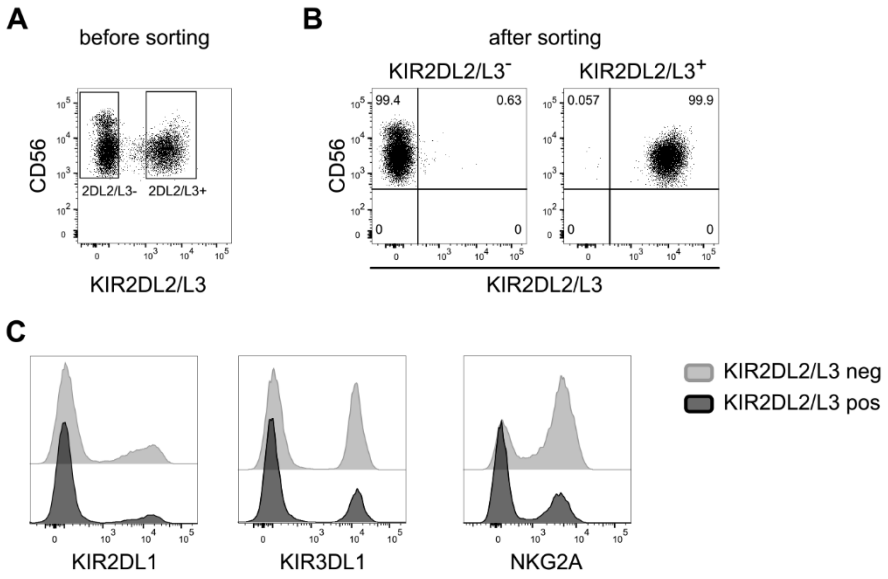


Figure 6.6: FACS-sorting of KIR2DL2/L3 positive & KIR2DL2/L3 negative NK cells. NK cells isolated from PBMCs were further sorted according to their KIR2DL2/L3 expression into KIR2DL2/L3 positive or negative populations using the anti-KIR2DL2/L3 antibody clone DX27. (A - B) Representative flow cytometry dot plots of naïve NK cells (A) before and (B) after sorting showing the gating strategy of KIR2DL2/L3-sorting and the purity of the sorted populations as detected with a different anti-KIR2DL2/L3 antibody clone GL183. Dot plots were gated on 7-AAD– CD3– CD56+ NK cells. (C) Expression of other KIRs and NKG2A on KIR2DL2/L3 positive and negative sorted populations shown as representative histogram overlays.

sorted KIR2DL2/L3 positive subset, KIR2DL2/L3 expression was significantly decreased after IL-12/15/18 activation compared to naïve cells and IL-15–treated controls (Figure 6.7 A & B). Of note, IL-15 stimulation even increased expression density of

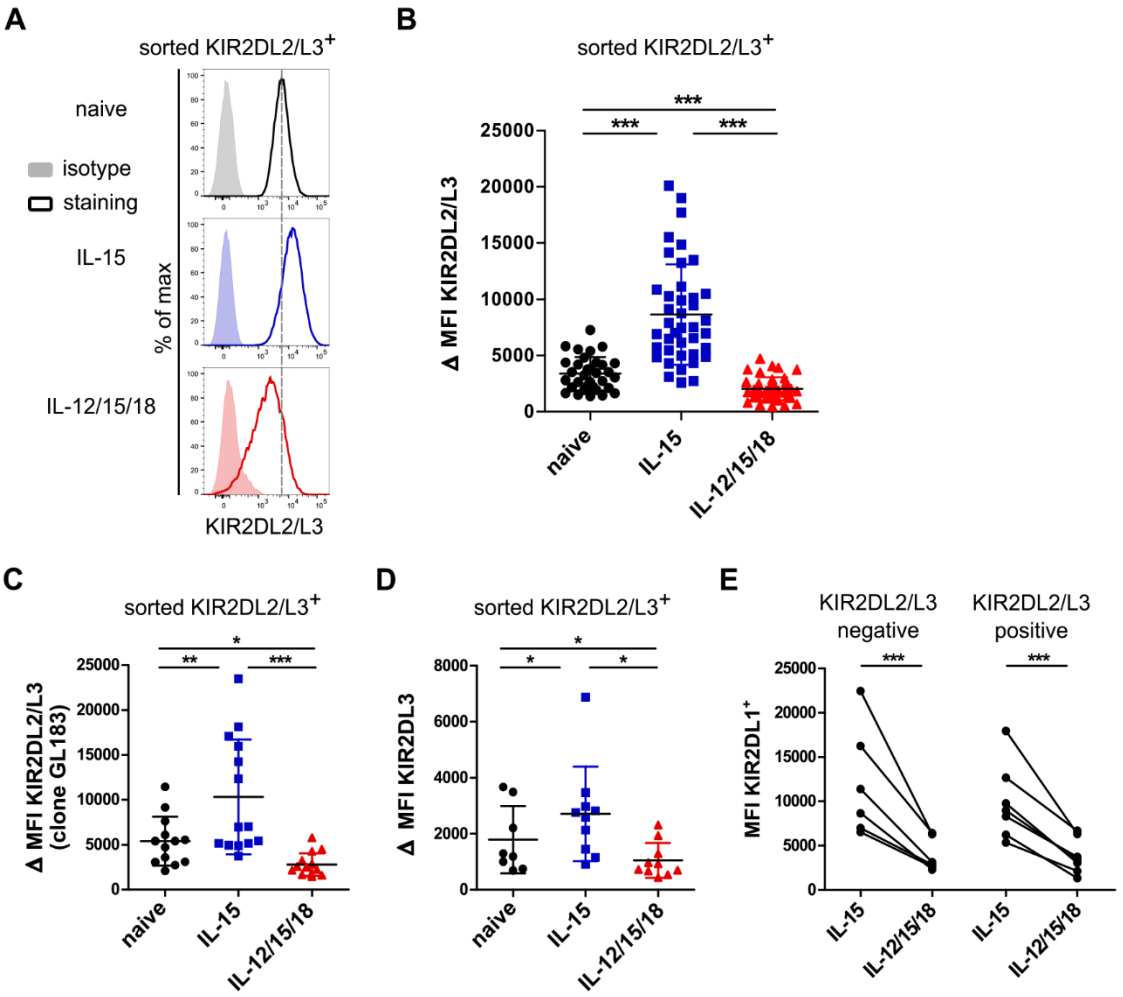


Figure 6.7: IL-12/15/18 drives KIR2DL2/L3 downregulation on sorted KIR2DL2/L3 positive NK cells. NK cells isolated from PBMCs were FACS-sorted into KIR2DL2/L3 positive and negative populations. Cells were analyzed before (day 0, naïve) and after 48 h of stimulation with IL-15 or IL-12/15/18 by flow cytometry. (A) Representative histograms showing KIR2DL2/L3 expression on the KIR2DL2/L3 positive sorted population. (B - D) Summary showing isotype-corrected median fluorescence intensity (Δ MFI) of KIR expression on the KIR2DL2/L3 positive sorted population (mean ± SD). KIR2DL2/L3 expression was assessed by flow cytometry using anti-KIR2DL2/L3 mAb clones (B) DX27 or (C) GL183 and (D) KIR2DL3 expression was detected by anti-KIR2DL3 mAb clone 180701 (n = 37 (B); n = 14 (C) and n = 10 (D)). Statistical analysis was determined by one-way ANOVA followed by Bonferroni's post test. (E) Co-expression and regulation of KIR2DL1 on KIR2DL2/L3 positive and negative sorted NK cells as determined by flow cytometry. The graph depicts the median fluorescence intensity (MFI) of the positive subpopulation. Results from individual donors are connected with a line (n = 7). Statistical analysis was performed with a paired two-tailed student's t-test.

KIR2DL2/L3 compared to expression levels on naïve NK cells. Downregulation of KIR2DL2/L3 was observed with different antibody clones (Figure 6.7 B - D) detecting KIR2DL2/L3 (DX27 & GL183) or KIR2DL3 (clone 180701), indicating regulation of inhibitory receptors with a long cytoplasmic tail. In addition, other inhibitory KIR molecules such as KIR2DL1 were downregulated on both KIR2DL2/L3 positive and negative subsets upon IL-12/15/18 stimulation compared to IL-15 treated controls (Figure 6.7 E). Of note, the sorted KIR2DL2/L3 negative population remained negative (data not shown).

To address the question, whether reduced KIR expression was the result of an outgrowth of the KIR negative subset, proliferation of NK cells was assessed via the fluorescent cell division tracker dye CFSE (carboxyfluorescein succinimidyl ester). The amount of CFSE in the labeled cells is diluted evenly in every cell division, allowing us to track the number of cell divisions of each cell. After 60 h of cytokine stimulation no CFSE dilution and thus no proliferation of KIR2DL2/L3 positive or negative NK cells was detectable (Figure 6.8 A). Moreover, no significant changes in absolute cell numbers or Annexin V positive apoptotic cells were detectable between KIR2DL/L3 positive or negative sorted populations

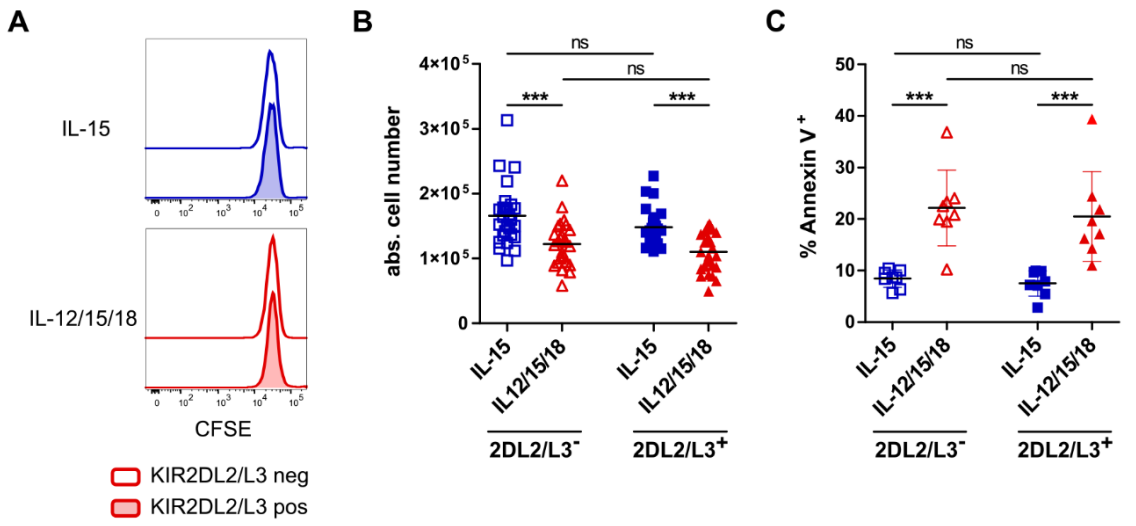


Figure 6.8: Regulation of KIR2DL2/L3 does not involve selective proliferation or apoptosis. (A) Freshly isolated NK cells were stained with the fluorescent cell division tracker dye CFSE and activated for 60 h with IL-15 or IL-12/15/18. Flow cytometry histogram overlays depict one representative donor out of 6 experiments gated on KIR2DL2/L3 positive or negative subsets. (B) Absolute cell numbers determined per well (mean ± SD; n = 26) and (C) Annexin V staining for apoptotic cells (mean ± SD; n = 8) of KIR2DL2/L3 positive and negative sorted subsets stimulated with IL-15 or IL-12/15/18 for 48 h. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post test.

(Figure 6.8 B & C), demonstrating that decreased KIR expression did not originate from preferential cell death of the KIR2DL2/L3 positive population. In general, stimulation of NK cells with IL-12/15/18 led to elevated apoptosis levels and lower cell numbers compared to IL-15 activation, but both subsets were similarly affected and no differences between the KIR2DL2/L3 positive and negative subpopulation were observed.

To evaluate whether KIR regulation was persistent or transient, KIR2DL2/L3-sorted NK cells were pre-activated for 48 h with IL-12/15/18 or IL-15 and subsequently washed and re-cultured in IL-2 for additional 3 to 5 days (Figure 6.9 A). As observed before, stimulation

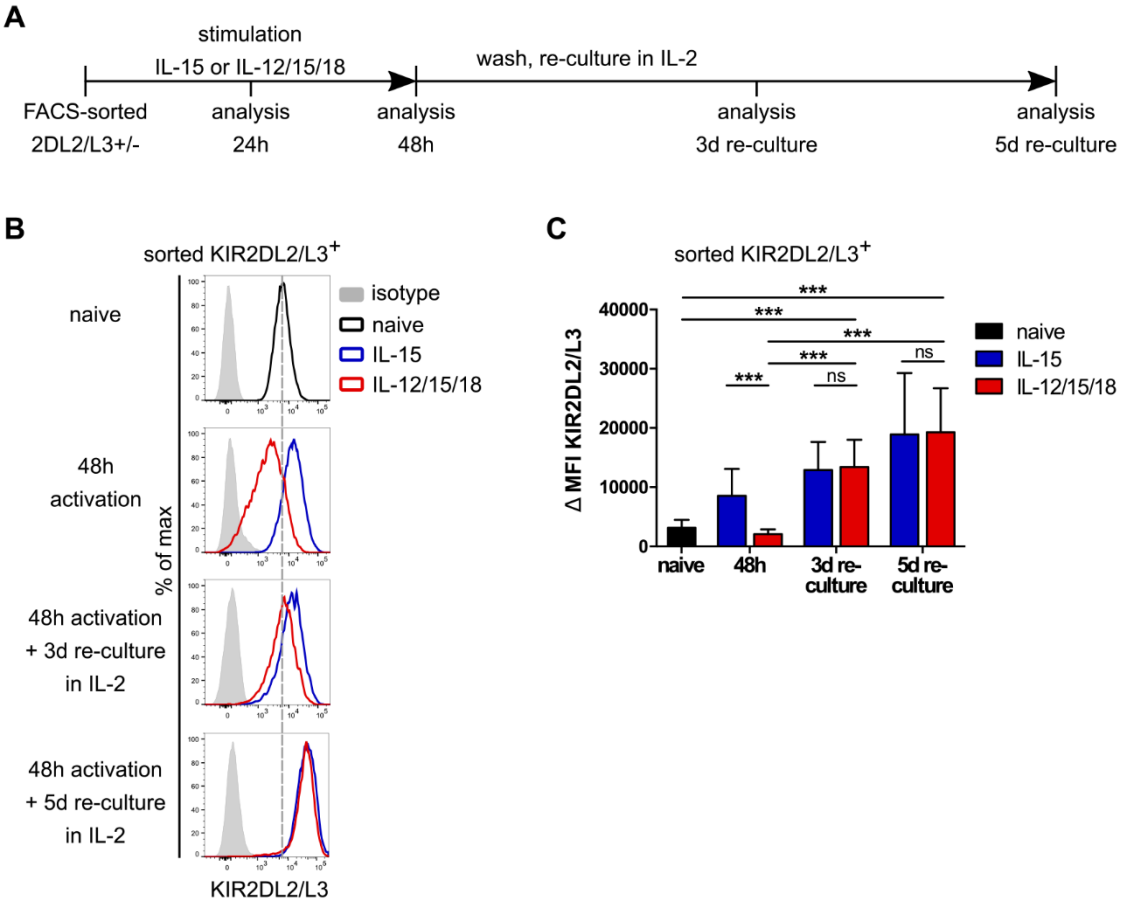


Figure 6.9: KIR downregulation by IL-12/15/18 on KIR2DL2/L3 sorted NK cells is transient. Sorted KIR2DL2/L3 positive and negative NK cells were stimulated with IL-15 or IL-12/15/18 for 48 h, washed and re-cultured in 100 IU/ml IL-2 for additional 3 or 5 days. (A) Schematic overview of the experimental procedure. (B) Representative histograms of KIR2DL2/L3 positive sorted NK cells after 48 h stimulation and after re-culture in IL-2 for 3 or 5 days. (C) Summary of the isotype-corrected median fluorescence intensity (Δ MFI) of KIR2DL2/L3 positive sorted NK cells. Data is shown as mean + SD of 9 - 14 donors. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post test. Part of this figure is a reprint from Ewen et al. 2018 Eur. J. Immunol. [710].

with IL-12/15/18 decreased KIR2DL2/L3 levels after 48 h (Figure 6.9 B & C). However, upon re-culture in IL-2, KIR expression was restored, increasing already after 3 days on sorted KIR2DL2/3 positive cells, indicating that the KIR downregulation was transient (Figure 6.9 B & C). Moreover, expression levels of KIR2DL2/L3 as well as KIR2DL1 were restored when the entire NK cell culture was pre-activated with IL-12/15/18 and re-cultured in IL-2 (Figure 6.10 A & B). Additionally, re-culture of NK cells in IL-15 (Figure 6.10 A & C) was capable of re-inducing KIR expression.

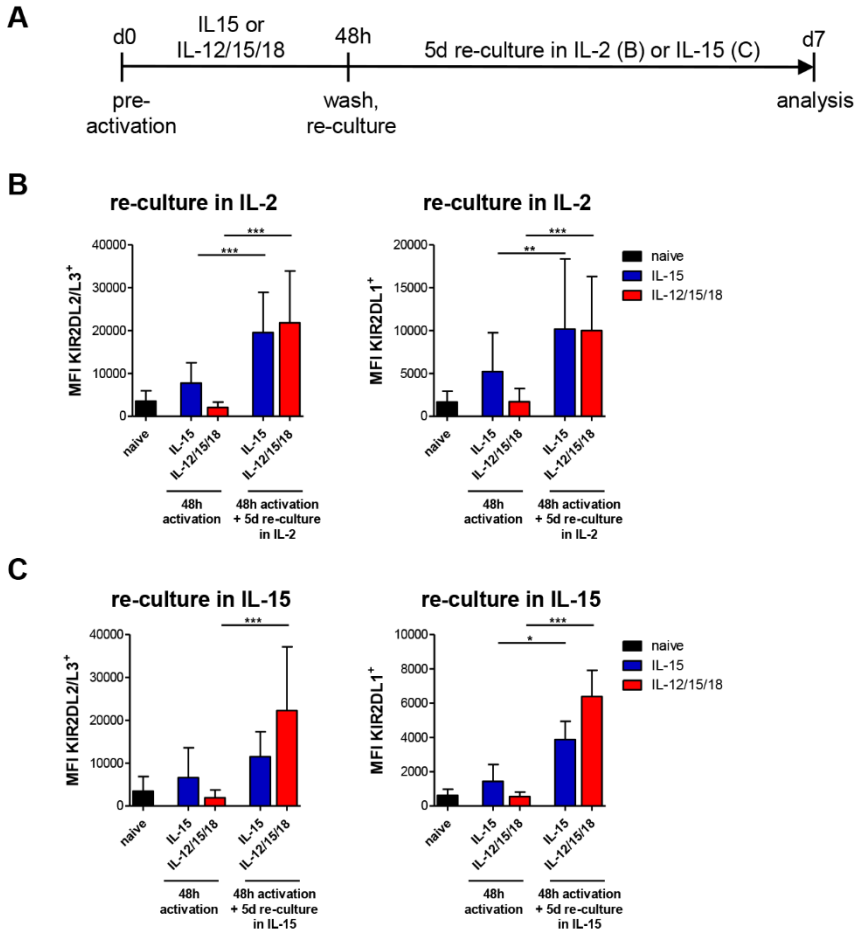


Figure 6.10: IL-12/15/18–induced downregulation of KIR2DL2/L3 and KIR2DL1 is reversible by re-culture in IL-2 or IL-15. Freshly isolated NK cells were stimulated with IL-15 or IL-12/15/18 for 48 h, washed and re-cultured in 100 IU/ml IL-2 or in 1 ng/ml IL-15 for additional 5 days. (A) Schematic overview of the experimental procedure. (B) Summary of all NK cells re-cultured in 100 IU/ml IL-2 for 5 days. Bar graphs depict the median fluorescence intensity (MFI) of KIR2DL2/L3 (left) and KIR2DL1 (right) expressing NK cells (n = 11; mean + SD). (C) Summary of all NK cells after 5 day re-culture in 1 ng/ml IL-15. Bar graphs depict the median fluorescence intensity (MFI) of KIR2DL2/L3 (left) and KIR2DL1 (right) expressing NK cells (n = 4; mean + SD). Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post test.

6.2. Mechanism of IL-12/15/18–induced KIR downregulation

To investigate whether the reduction of KIR protein expression was regulated at the transcriptional level, we analyzed *KIR* mRNA levels by qRT-PCR. Indeed, IL-12/15/18 stimulation of sorted KIR2DL2/L3 positive NK cells resulted in significantly reduced *KIR2DL3* mRNA levels after 24 h and 48 h of cytokine exposure (Figure 6.11 B). IL-15–activated NK cells showed enhanced *KIR2DL3* mRNA levels compared to naïve cells, resembling the pattern of protein surface expression. After cytokine pre-activation, sorted KIR2DL2/L3 positive NK cells were washed and re-cultured in for 3 days in IL-2 (Figure 6.11 A). Upon re-culture in IL-2, *KIR2DL3* mRNA levels were restored after 3 days, demonstrating transient transcriptional KIR regulation by IL-12/15/18, mimicking our protein data (Figure 6.9). qRT-PCR analysis of the whole NK cell culture showed similar tendencies of reduced *KIR2DL1* mRNA levels of IL-12/15/18–stimulated NK cells compared to IL-15–treated controls (Figure 6.11 C). In accordance with our results, we observed decreased mRNA levels of multiple inhibitory KIRs such as *KIR2DL1, KIR2DL2, KIR2DL3, KIR3DL1, KIR3DL3, KIR2DL4 and KIR2DL5A* (Figure 6.11 D) upon analysis of publicly available microarray data [712] comparing IL-2/12/18–stimulated with naïve NK cells. Taken together, we could show that IL-12/15/18–stimulation induced transient downregulation of *KIR* mRNA levels in peripheral blood NK cells.

To dissect the essential cytokines involved in KIR regulation, we stimulated KIR2DL2/L3-sorted NK cells with single or dual cytokine combinations of IL-12, IL-15 and IL-18 (Figure 6.12). KIR2DL2/L3 expression levels were significantly downregulated by IL-12 alone or in combination with IL-18 (Figure 6.12). But the combination of all three cytokines induced the most profound KIR regulation while ensuring survival of the cells (data not shown). Since we observed upregulation of KIR expression by IL-15 in contrast to IL-12/15/18–induced downregulation (Figure 6.7 and Figure 6.11), we tested whether the combination of IL-12/15/18 would diminish IL-15–mediated signaling. Therefore, we assessed differences in phosphorylation of the signaling adapter STAT-5, which gets

activated upon IL-15 signal transduction [296]. KIR2DL2/L3-sorted NK cells were stimulated

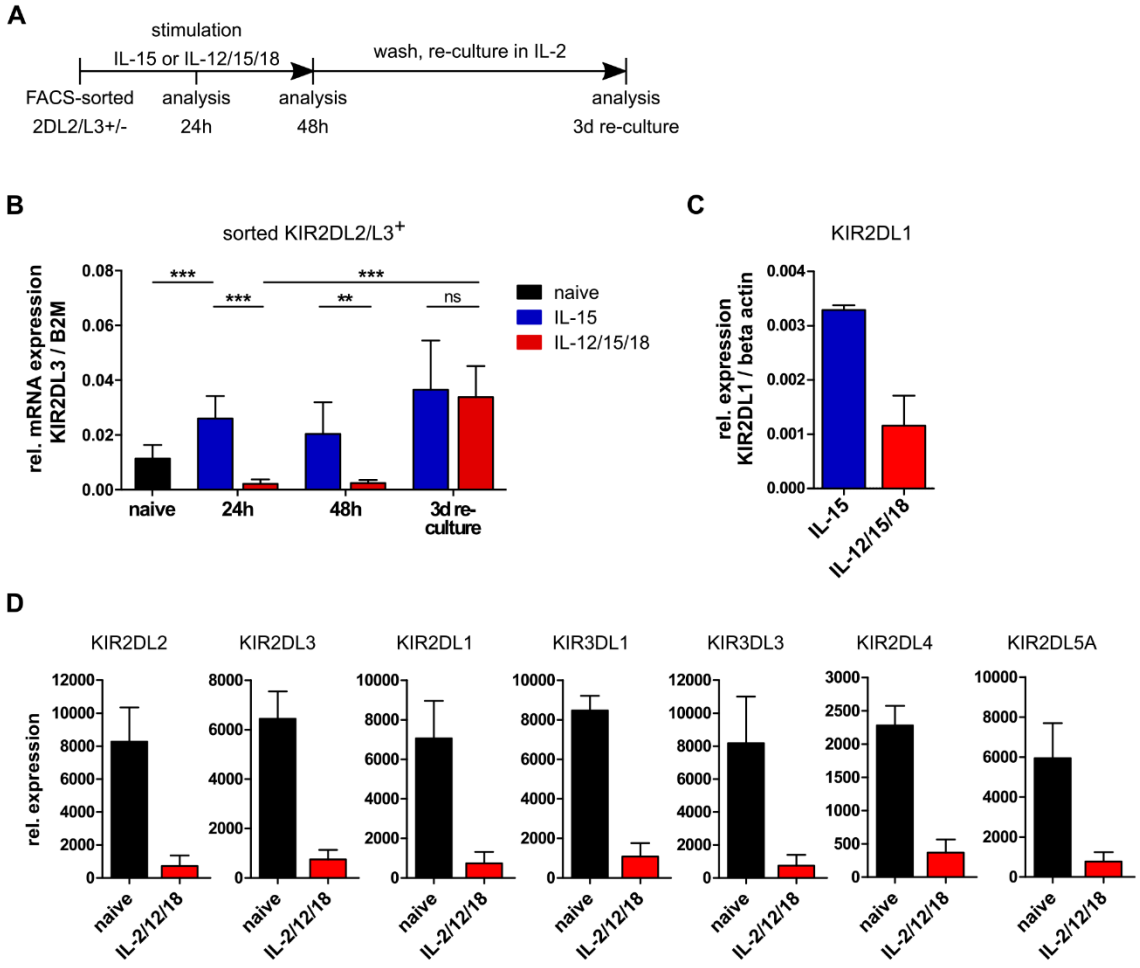


Figure 6.11: *KIR* mRNA levels are transiently downregulated on NK cells upon IL-12/15/18 stimulation. Sorted KIR2DL2/L3 positive and negative NK cells were stimulated with IL-15 or IL-12/15/18 for 48 h, washed and re-cultured in 100 IU/ml IL-2 for additional 3 days. (A) Schematic overview of the experimental procedure. (B) Relative *KIR2DL3* mRNA expression of KIR2DL2/L3 positive sorted NK cells before (naïve), after 24 h and 48 h of cytokine exposure and after 3 days re-culture in IL-2 as described in (A). KIR2DL3 surface expression on NK cells was confirmed by flow cytometry using the anti-KIR2DL3 antibody clone 180701. *KIR2DL3* expression was determined by qRT-PCR and calculated relative to *beta-2-microglobulin (B2M)* as $2^{-\Delta Cp}$ with $\Delta Cp = Cp(KIR2DL3) - Cp(B2M)$. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post test (n = 3 - 9, mean + SD). (C) NK cells isolated from PBMCs were treated as entire NK culture for 24 h with IL-15 or IL-12/15/18 and analyzed for *KIR2DL1* mRNA by qRT-PCR (n = 2; mean + SD). *KIR2DL1* expression relative to *beta-actin* was calculated as $2^{-\Delta Cp}$ with $\Delta Cp = Cp(KIR2DL1) - Cp(beta\ actin)$. KIR2DL1 surface expression on NK cells was confirmed by flow cytometry using the anti-KIR2DL1 antibody clone 143211. (D) Relative *KIR* mRNA expression levels of human NK cells in naïve state or treated for 24 h with IL-2/12/18 (n = 2; mean + SD). Data was extracted from the gene-array data set GEO GSE22919 [712].

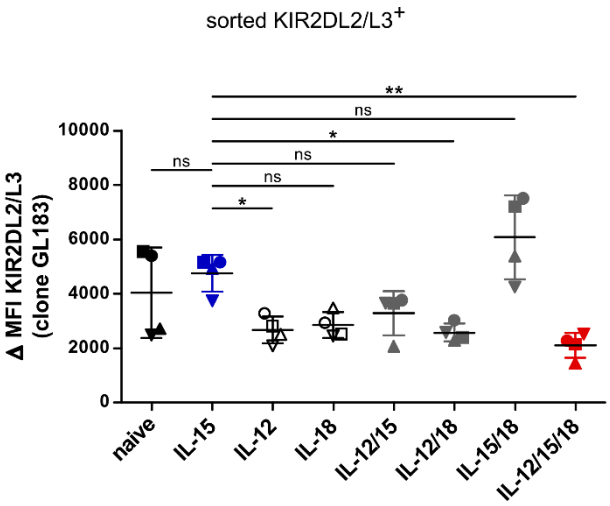


Figure 6.12: KIR expression on NK cells is regulated by different cytokine combinations. NK cells were sorted according to their KIR2DL2/L3 expression and stimulated for 48 h with the indicated cytokine combinations. KIR2DL2/L3 expression on KIR2DL2/L3 positive sorted NK cells was assessed by flow cytometry using the antibody clone GL183. The graph presents the isotype-corrected median fluorescence intensity (Δ MFI) on the KIR2DL2/L3 positive sorted population (n = 4, mean ± SD). Each donor is represented by a distinct symbol. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post test.

for 15 to 60 min with IL-15 or IL-12/15/18 and phosphorylation of intracellular STAT-5 was detected by flow cytometry. Cytokine stimulation was sufficient to induce phosphorylation of STAT-5 after 15 min (Figure 6.13 A) on both KIR2DL2/L3 positive and negative subsets. However, no differential phosphorylation was observed between IL-15 and IL-12/15/18 activated NK cells (Figure 6.13 B). Thus, stimulation with IL-12/15/18 did not mediate KIR downregulation by influencing IL-15–mediated STAT-5 signaling.

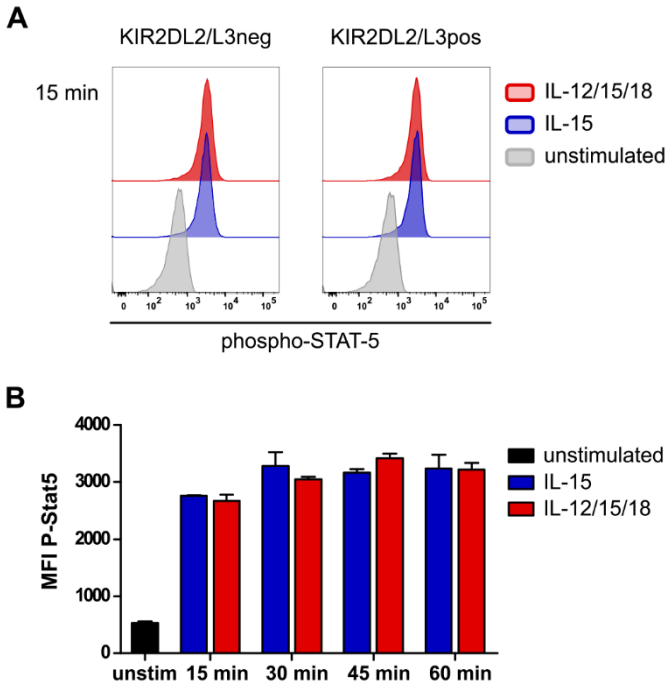


Figure 6.13: No differential STAT-5 phosphorylation is detected after IL-15 or IL-12/15/18 stimulation. KIR2DL2/L3-sorted NK cells were stimulated for the indicated time points with IL-15 or IL-12/15/18 and phosphorylation of intracellular STAT-5 was detected by flow cytometry. (A) Histogram overlays of KIR2DL2/L3 positive and negative sorted NK cells after 15 min of cytokine exposure. (B) Median fluorescence intensity (MFI) of intracellular phospho–STAT-5 after indicated time points of cytokine stimulation (n = 2; mean + SD).

6.3. Improved functionality of IL-12/15/18–activated NK cells

6.3.1. IL-12/15/18–activated NK cells are less sensitive to KIR2DL2/L3-mediated inhibition

Down-regulation of KIR surface expression suggested that NK cells might be less sensitive to KIR2DL2/L3-based inhibitory signals. Hence, we assessed NK functionality in a redirected lysis assay with murine FcR^+ P815 cells, employing NK cell activation via CD16 crosslinking as a defined activating signal. As depicted in the scheme in Figure 6.15 A (left), CD16 was engaged by an anti-CD16 antibody that was cross-linked by the Fc receptor on P815 cells. Antibody-mediated cross-linking of activating receptors such as CD16 is capable of inducing activating signals within the NK cell [713–715]. NK cells were sorted into KIR2DL2/L3 positive and negative subsets, stimulated for 48 h with IL-15 or IL-12/15/18 and used in the redirected lysis assay. Both, IL-15 and IL-12/15/18–activated NK cells were capable of CD16-mediated redirected lysis (Figure 6.14 A, middle), although the latter at a lower extent. This correlated with lower CD16 expression on IL-12/15/18–stimulated cells (Figure 6.1 and Figure 6.14 B, left), which was previously shown to involve matrix-metalloproteinase (MMP) mediated shedding [711,716]. Treatment of both NK cell cultures with the MMP inhibitor (MMPi) Batimastat partially restored CD16 cell surface expression (Figure 6.14 B, right), which resulted in comparable levels of CD16-dependent killing between IL-12/15/18 and IL-15–cultured NK cells (Figure 6.14 A, right panel). Importantly, KIR2DL2/L3 expression was not influenced by the MMPi treatment (Figure 6.14 C). Of note, KIR2DL2/L3 positive and negative NK cells exhibited comparable killing capabilities upon CD16 triggering (Figure 6.14 A). Since KIR2DL2/L3 positive and negative sorted subsets co-expressed other inhibitory self–HLA-I receptors such as NKG2A, KIR2DL1 or KIR3DL1 (Figure 6.6 C), these cells were most likely educated and fully functional.

To investigate whether IL-12/15/18 activation alters KIR-mediated inhibition, KIR2DL2/L3 was engaged with a monoclonal antibody in combination with an anti-CD16 antibody in a redirected lysis assay (Figure 6.15 A, right). Cross-linking of inhibitory KIR molecules by antibody-coated P815 cells has been shown to induce inhibitory signals within NK cells mimicking receptor-ligand interactions [713–715]. as observed before, engagement of CD16 alone induced comparable levels of redirected lysis by IL-15 and IL-12/15/18–activated NK cells (Figure 6.15 B, left). Upon inhibitory KIR co-triggering,

cytotoxicity of KIR2DL2/L3 positive NK cells stimulated with IL-15 was greatly reduced (Figure 6.15 B & C, right), indicating KIR dependent inhibition. Remarkably, the CD16-mediated cytotoxicity of IL-12/15/18–activated NK cells was not affected by KIR2DL2/L3 co-engagement (Figure 6.15 B & C, right). IL-12/15/18–activated NK cells possessed high cytotoxic potential even after co-engagement of inhibitory KIR, which was similar to lysis observed upon CD16 engagement by itself. Redirected lysis by KIR2DL2/L3 negative NK cells was not influenced by KIR cross-linking and comparable between IL-15 and

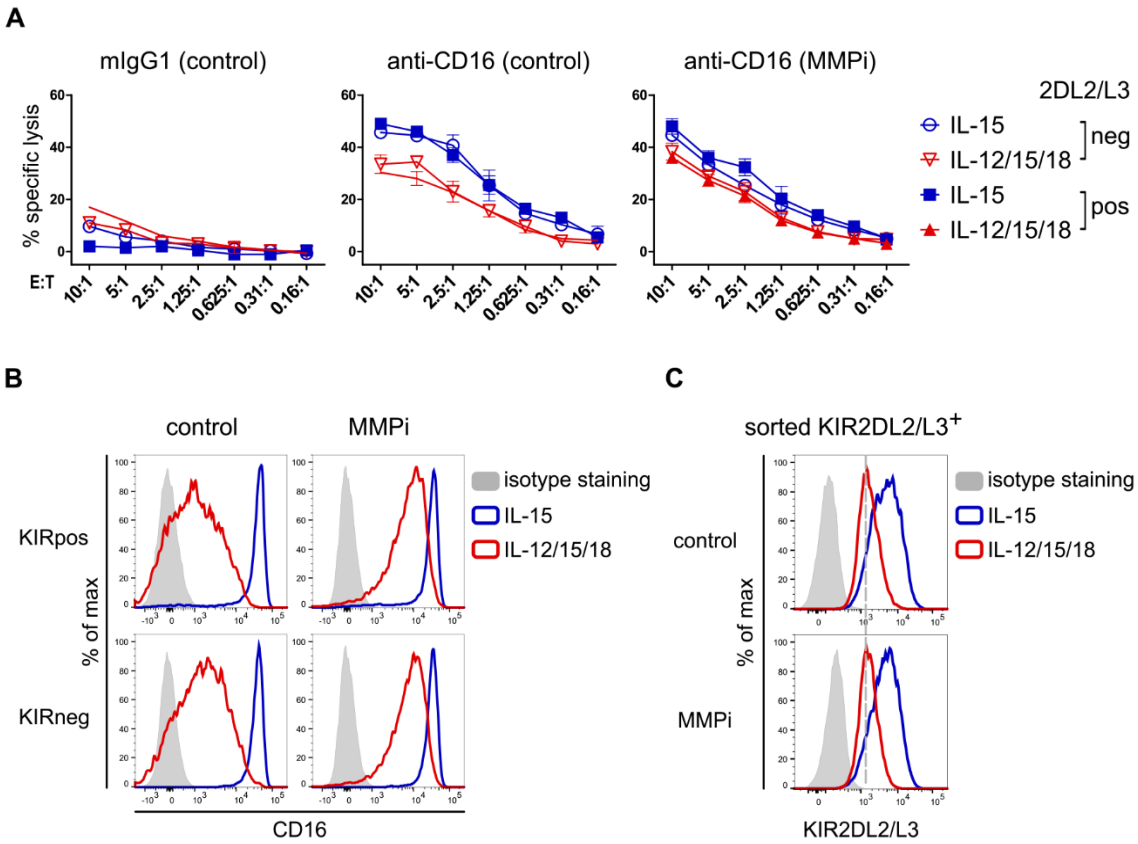


Figure 6.14: NK cell activation with IL-12/15/18 induces MMP-mediated shedding of CD16. NK cells were sorted according to their KIR2DL2/L3 expression and activated for 48 h with IL-15 or IL-12/15/18 in the presence of solvent control (ctrl; DMSO) or treated with the matrix-metalloproteinase inhibitor (MMPi) Batimastat. The CD16-dependent cytotoxicity of NK effector cells was assessed in a redirected lysis assay against antibody-coated P815 target cells, which were pre-incubated with mIgG1 or anti(α)-CD16 antibody (clone 3G8). (A) Representative donor showing different E:T ratios of CD16-mediated redirected lysis of NK cells after control (ctrl; DMSO) or Batimastat (MMPi) treatment. Data is presented as mean ± SD of technical replicates of one representative donor. (B - C) Histogram overlays depict (B) CD16 and (C) KIR2DL2/L3 surface expression of control (ctrl) or MMPi-treated NK cells gated on 7-AAD– CD3– CD56+ NK cells.

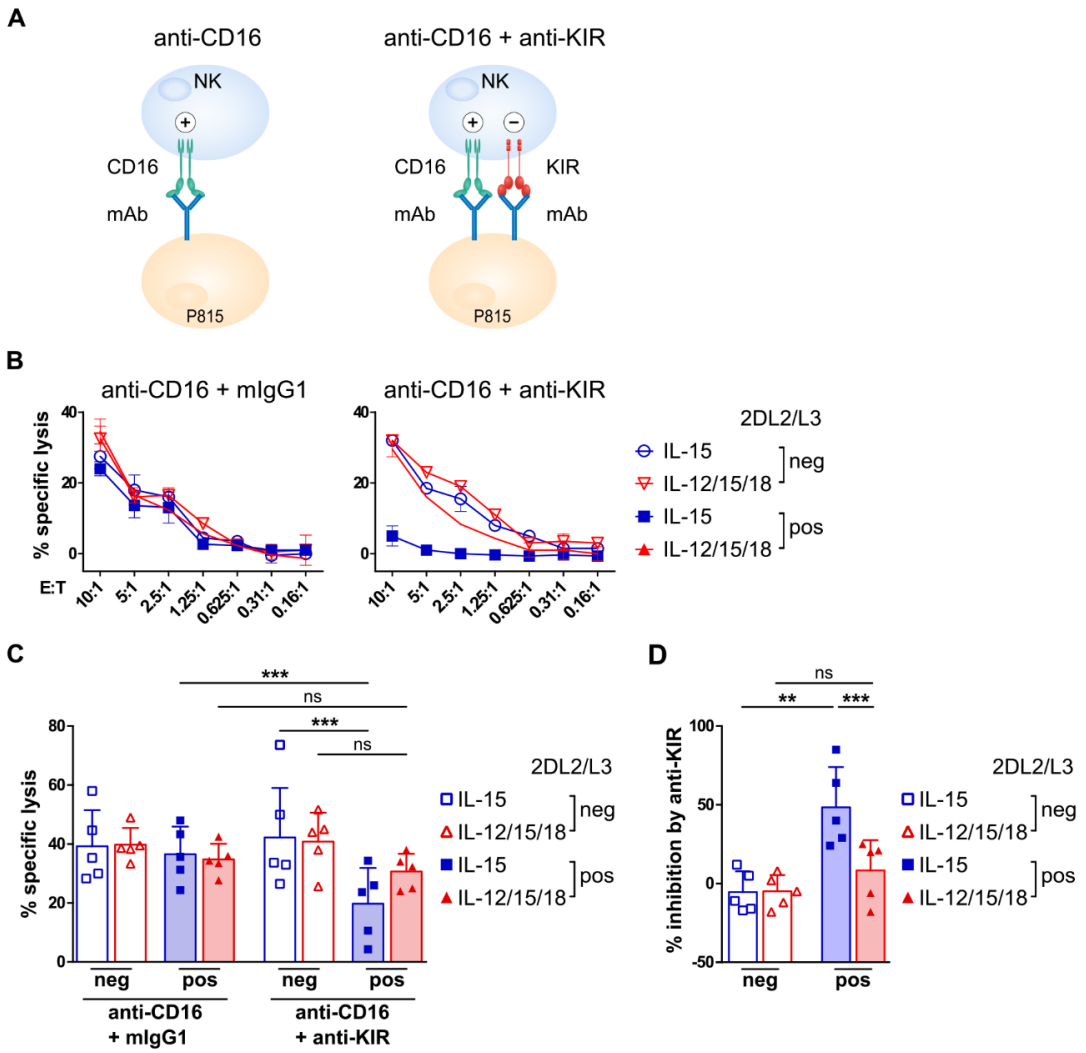


Figure 6.15: IL-12/15/18-induced KIR downregulation leads to reduced inhibition of CD16-mediated cytotoxicity. NK cells were sorted according to their KIR2DL2/L3 expression and activated for 48 h with IL-15 or IL-12/15/18 in the presence of the matrix-metalloproteinase inhibitor (MMPi) Batimastat. Redirected lysis assays were performed against antibody-coated P815 target cells, pre-incubated with anti(α)-CD16 mAb (clone 3G8) together with either anti-KIR2DL2/L3 mAb (clone GL183) or with the respective mIgG1 isotype control. (A) Schematic overview illustrating the redirected lysis assay of CD16 triggering alone (left) and of parallel engagement of CD16 and KIR2DL2/L3 (right). (B) Representative data of redirected lysis against P815 cells pre-incubated with anti(α)-CD16 mAb together with either mIgG1 (left) or anti-KIR2DL2/L3 mAb (right panel). Data is shown at different E:T ratios as mean ± SD of technical replicates. (C) Statistical summary of redirected lysis shown in (B) at E:T ratio 10:1 for 5 donors. Bars are drawn to mean + SD and each symbol represents one donor. (D) Percent inhibition induced by anti-CD16 and anti-KIR2DL2/L3 co-triggering of redirected lysis shown in (C) compared to anti-CD16 alone. Percent inhibition by anti-KIR was calculated as [1 - [specific lysis (anti-CD16 + anti-KIR2DL2/L3) / specific lysis (anti-CD16 + mIgG1)] x 100]. Bars are drawn to mean + SD and each symbol represents one donor (n = 5). Statistical analysis was determined by two-way ANOVA / Bonferroni's multiple comparison test. Part of this figure is a reprint from Ewen et al. 2018 Eur. J. Immunol. [710].

IL-12/15/18 activation (Figure 6.15 B & C), indicating that in absence of KIR engagement both cytokine stimulations elicited the same degree of CD16-dependent cytotoxicity. Lysis of IL-15–treated KIR2DL2/L3 positive NK cells was reduced by approximately 50% if inhibitory KIR2DL2/L3 was engaged, implicating approximately 50% of inhibition by anti-KIR. To further visualize KIR-mediated inhibition, the relative percentage of inhibition induced by KIR2DL2/L3 triggering was calculated for CD16-dependent lysis (Figure 6.15 D), demonstrating that IL-12/15/18–activated NK cells were less sensitive to inhibitory signals compared to IL-15 stimulated cells. Thus, our results indicate that reduced surface expression of KIR2DL2/L3 on IL-12/15/18–stimulated NK cells translated into reduced inhibition upon KIR co-engagement.

6.3.2. IL-12/15/18–activated NK cells show high cytotoxicity against HLA-I–expressing targets

Next, we wanted to investigate whether the downregulation of KIR2DL2/L3 has an influence on the functionality of NK cells during their encounter of targets that express HLA-I. To decipher the effect of the cognate HLA-I ligand, we employed the 721.221 lymphoma cell line, which expresses low levels of HLA-ABC as demonstrated by flow cytometry analysis (Figure 6.16 A). Although they showed weak but positive staining with a pan–HLA-ABC antibody, the 721.221 cells are described to be negative for HLA-C [717], bearing no ligand for KIR2D receptors. In contrast, the daughter cell line 221_Cw03 is transduced with the HLA-Cw03 allele, harboring the C1 epitope specifically recognized by KIR2DL2/L3 (Figure 6.16 A). The interaction of cytokine-stimulated NK cells with HLA_Cw03 positive and negative 221 cells is depicted schematically in Figure 6.16 B. The 221 cell line is negative for cognate ligands for KIR2D receptors and thus NK activation by this cell line should be independent of the KIR/HLA axis. In contrast, the HLA-Cw03 cell line expresses the cognate ligand for KIR2DL2/L3 on NK cells, which inhibits NK activation. Since no ligands for other KIRs are present on both cell lines, this system allows to specifically study the interaction between a certain KIR and its ligand.

Using this system, we first analyzed whether low levels of inhibitory KIR2DL2/L3 on IL-12/15/18–activated cells would affect NK cell degranulation against targets carrying cognate HLA-I molecules. Cytokine-stimulated NK cells were co-cultured for 4 h with HLA_Cw03 positive or negative 221 cell lines and degranulation of NK cells was assessed

in a CD107a mobilization assay. Co-staining of KIR2DL2/L3 and CD107a allowed to determine specific degranulation of KIR2DL2/L3 positive and negative subsets by flow cytometry (Figure 6.17 A). No differences in degranulation of KIR2DL2/L3 positive and negative populations were detectable against 221 target cells in absence of the cognate ligand (Figure 6.17 B, left). Gating on KIR2DL2/L3 positive NK cells stimulated with IL-15 revealed low levels of degranulation against 221_Cw03 targets (Figure 6.17 B, right), implicating inhibition of KIR2DL2/L3 positive NK cells by HLA-Cw03. In contrast, degranulation of IL-12/15/18–activated NK cells against 221_Cw03 cells was improved compared to their IL-15–treated counterparts. In parallel, both KIR2DL2/L3 negative NK populations exhibited comparable degranulation against 221_Cw03 cells, showing NK functionality independent of the KIR/HLA-I axis. Calculation of the percentage of inhibition

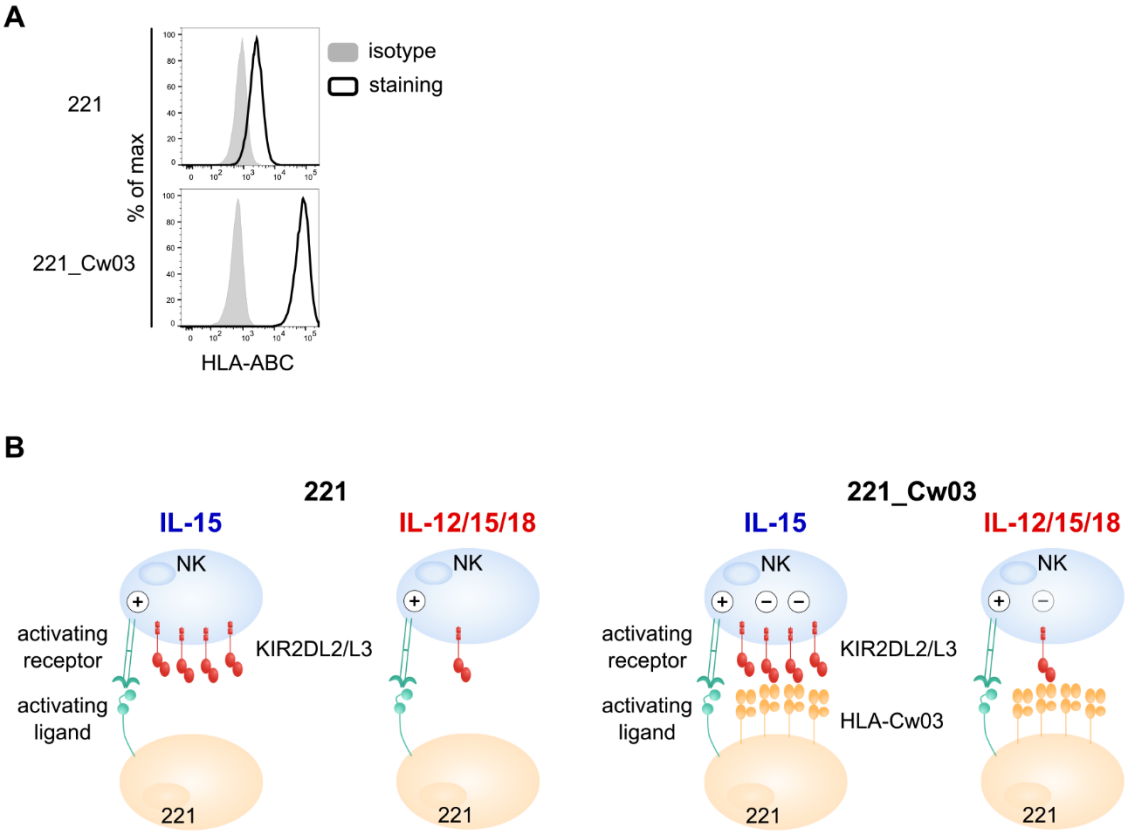


Figure 6.16: HLA-I expression on 221 and 221_Cw03 lymphoma cells. (A) Parental 721.221 lymphoma cells (221) or 721.221 cells transduced with HLA-Cw03, carrying the cognate C1 epitope for KIR2DL2/L3 (221_Cw03), were stained with anti-HLA-ABC mAb (clone W6/32) and analyzed by flow cytometry. Dead cells were excluded by gating on 7-AAD- cells. (B) Schematic overview of the 721.221 cell lines as target cells with matched HLA-I expression. The 221 cell line is negative for cognate ligands for KIR2DL2/L3, which is regulated on IL-15 and IL-12/15/18–stimulated NK cells. The HLA-Cw03–transduced 221_Cw03 cell line expresses the cognate ligand for KIR2DL2/L3 on NK cells. No other ligands for other KIRs are present on both cell lines.

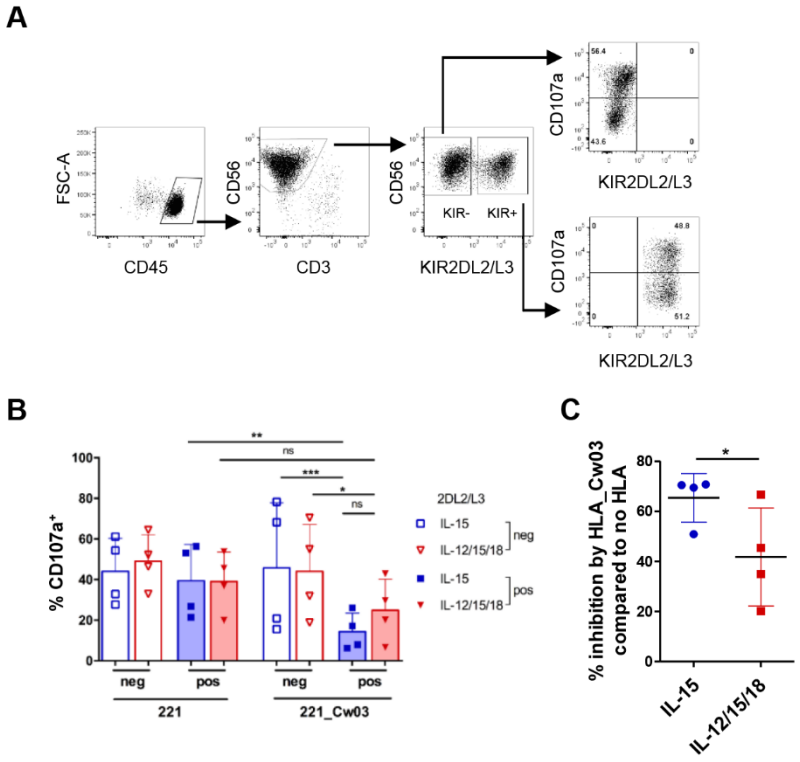


Figure 6.17: IL-12/15/18–activated NK cells exhibit enhanced degranulation towards cognate HLA-I positive target cells. Freshly isolated NK cells were activated for 48 h with IL-15 or IL-12/15/18 and co-cultured for 4 h at E:T ratio 1:1 with 221 or 221_Cw03 target cells, expressing the cognate HLA-I ligand for KIR2DL2/L3. Degranulation was assessed by flow cytometry analysis of CD107a co-gated on KIR2DL2/L3 positive or negative NK cells. (A) Gating strategy for detecting CD107a on KIR2DL2/L3 positive and negative NK subsets. (B) Statistical summary showing the percentage of CD107a positive NK cells. Bars are drawn to mean + SD and each symbol represents one donor (n = 4). Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons test. (C) Percent inhibition of degranulation of KIR2DL2/L3 positive NK cells by the presence of a cognate ligand on target cells (221_Cw03) compared to no HLA (221 cells). Percent inhibition by HLA-Cw03 was calculated as [1 - [%CD107$^+$ (221_Cw03) / %CD107$^+$ (221)] x 100]. Statistical analysis was performed with a paired two-tailed student's t-test (n = 4; mean ± SD).

by HLA-Cw03 compared to targets expressing no HLA-C demonstrated significant less inhibition of IL-12/15/18–stimulated NK cells by HLA-Cw03 compared to IL-15–activated controls (Figure 6.17 C). Thus, our results indicate that NK cells with lower KIR expression levels displayed enhanced degranulation against cognate HLA-I positive targets.

Next, we investigated how IL-12/15/18–mediated KIR downregulation affected NK cell cytotoxicity towards 221 cells carrying cognate HLA-I molecules. The cytotoxicity of KIR2DL2/L3-sorted NK cells was determined in a standard 4 h chromium release assay against HLA-I low (221) and HLA-Cw03–transduced 721.221 cells (221_Cw03), bearing high levels of the HLA-C1 epitope specific for KIR2DL2/L3 (see Figure 6.16). No differences in cytotoxicity of KIR2DL2/L3 positive or negative NK cells was observed towards HLA-I low 221 cells regardless of IL-15 or IL-12/15/18 pre-activation (Figure 6.18 A & B, left panels). In absence of cognate HLA-I on target cells, NK cells stimulated with IL-15 or

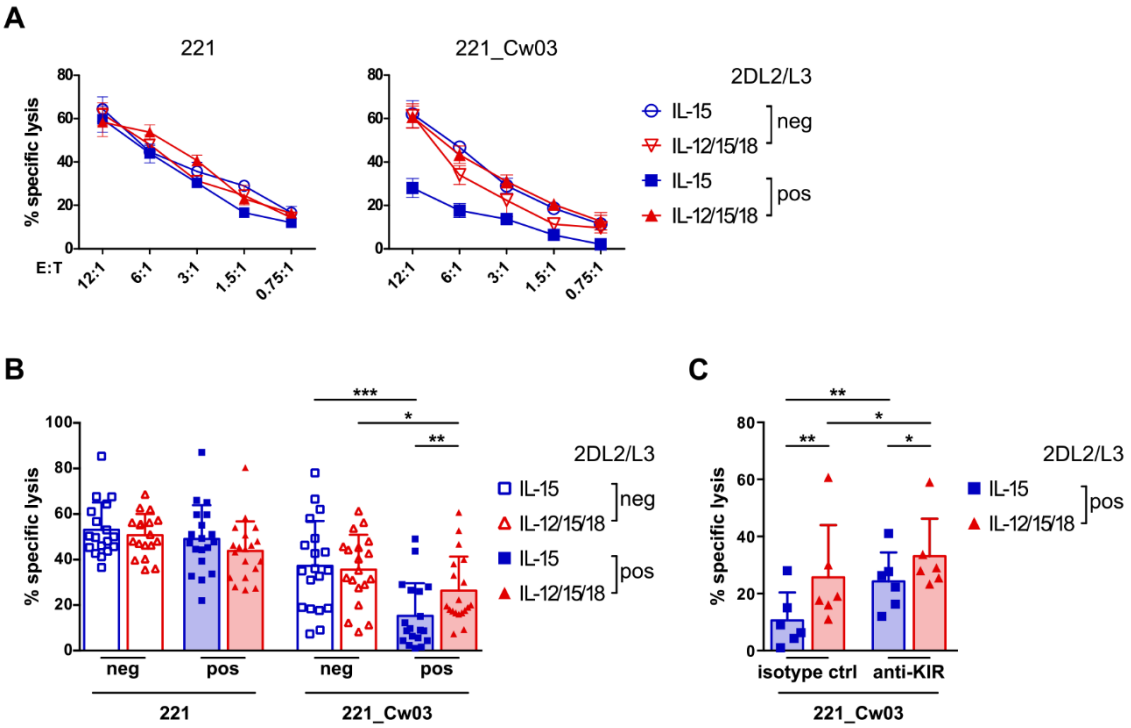


Figure 6.18: IL-12/15/18 stimulation increases NK cell cytotoxicity against cognate HLA-Cw03–expressing lymphoma cells. Cytotoxicity assay using KIR2DL2/L3 positive and negative sorted NK cells activated with IL-15 or IL-12/15/18 for 48 h is shown. Sorted NK cells were incubated with parental 721.221 target cells (221) or with 721.221 cells transduced with HLA-Cw03 (221_Cw3). When indicated, interaction of HLA-Cw03 and KIR2DL2/L3 was blocked by pre-incubation of NK cells with anti-KIR2DL2/L3 mAb (GL183) or respective mouse IgG1 isotype control. (A) Cytotoxicity assay of one representative donor showing specific lysis of 221 or 221_Cw03 cells at several E:T ratios (mean ± SD of technical replicates). (B) Summary of cytotoxicity against 221 and 221_Cw03 cells at E:T ratio 12:1. Bars are drawn to mean + SD and each symbol represents one donor (n = 19). (C) KIR2DL2/L3 positive NK cells were pre-incubated with anti-KIR2DL2/L3 mAb (GL183) or respective mIgG1 isotype control. Cumulative data of specific lysis of 221_Cw03 cells including KIR2DL2/L3 blockade at E:T ratio 12:1. Bars are drawn to mean + SD and each symbol represents one donor (n = 6). Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons test. Part of this figure is a reprint from Ewen et al. 2018 Eur. J. Immunol. [710].

IL-12/15/18 exerted equally potent cytotoxicity at different E:T ratios. Lysis of 221_Cw03 target cells by IL-15–stimulated KIR2DL2/L3 positive NK cells was significantly inhibited by the interaction of KIR2DL2/L3 with its cognate ligand (Figure 6.18 A & B, right panels). Importantly, KIR2DL2/L3 positive NK cells activated with IL-12/15/18 showed superior cytotoxicity towards cognate HLA-I–expressing 221_Cw03 targets compared to IL-15–treated controls (Figure 6.18 A & B, right panels). In parallel, KIR2DL2/L3 negative NK cells exhibited comparable killing capabilities independent of KIR/HLA-I interactions. To study the contribution of the KIR/HLA-I axis, interaction of KIR2DL2/L3 and its ligand HLA-Cw03 was blocked by an anti-KIR antibody (Figure 6.18 C). Indeed, KIR2DL2/L3 blockade increased cytotoxicity of IL-15–treated KIR2DL2/L3 positive NK cells towards 221_Cw03 targets (Figure 6.18 C), demonstrating that cytotoxicity of IL-15–activated NK cells was inhibited by the interaction of KIR2DL2/L3 with its cognate ligand. The high cytotoxicity of IL-12/15/18–stimulated KIR2DL2/3 positive NK cells was preserved in the presence of the blocking anti-KIR antibody. Together, these observations indicate that IL-12/15/18 activation led to downregulation of inhibitory KIR2DL2/L3 receptors, rendering NK cells proficient in killing cognate HLA-I–expressing tumor cells.

6.3.3. IL-12/15/18–pre-activated cells exhibit memory-like functionality

Previous studies reported that human and mouse NK cells can acquire potent memory-like functionality after 16 h pre-activation with IL-12/15/18 [288,289,333,334,526]. Upon re-stimulation, these cells maintained the capacity of high anti-tumor activity and IFN-γ production, both after adoptive transfer *in vivo* and after an *in vitro* resting phase in the presence of IL-2 or IL-15, indicative of memory-like functionality. To address whether prolonged pre-activation for 48 h, as used in this thesis, instead of 16 h would influence the memory-like properties of cytokine-stimulated NK cells, we pre-activated NK cells for 48 h with IL-15 or IL-12/15/18 and compared them to 16 h pre-activation. Directly after cytokine exposure, pre-activated NK cells were re-cultured in IL-2 or IL-15 for 5 days (Figure 6.19 A). To assess NK functionality, cells were re-stimulated with K562 and IFN-γ levels were determined in the supernatant by ELISA. Of note, KIR2DL2/L3 expression was re-induced by IL-2 and IL-15 re-culture as observed before (Figure 6.10) and KIR expression levels were comparable between IL-15 and IL-12/15/18–pre-activated NK cells at day 5. In accordance with previous studies [288,289,334], NK cells pre-activated with

IL-12/15/18 produced more IFN-γ compared to IL-15–treated counterparts (Figure 6.19 B). IL-12/15/18 pre-activated NK cells exhibited improved IFN-γ secretion both after re-culture in IL-2 (Figure 6.19 B, left) as well as after re-culture in IL-15 (Figure 6.19 B, right). Importantly, as compared to 16 h stimulation of NK cells, pre-activation for 48 h resulted in similarly enhanced IFN-γ production of IL-12/15/18–stimulated NK cells upon re-stimulation with K562. Thus, also longer IL-12/15/18 stimulation could prime for generation of NK cells with memory-like properties.

Additionally, we determined the percentage of IFN-γ producing cells upon 6 h re-stimulation with K562 via intracellular flow cytometry staining (Figure 6.20 A). Flow

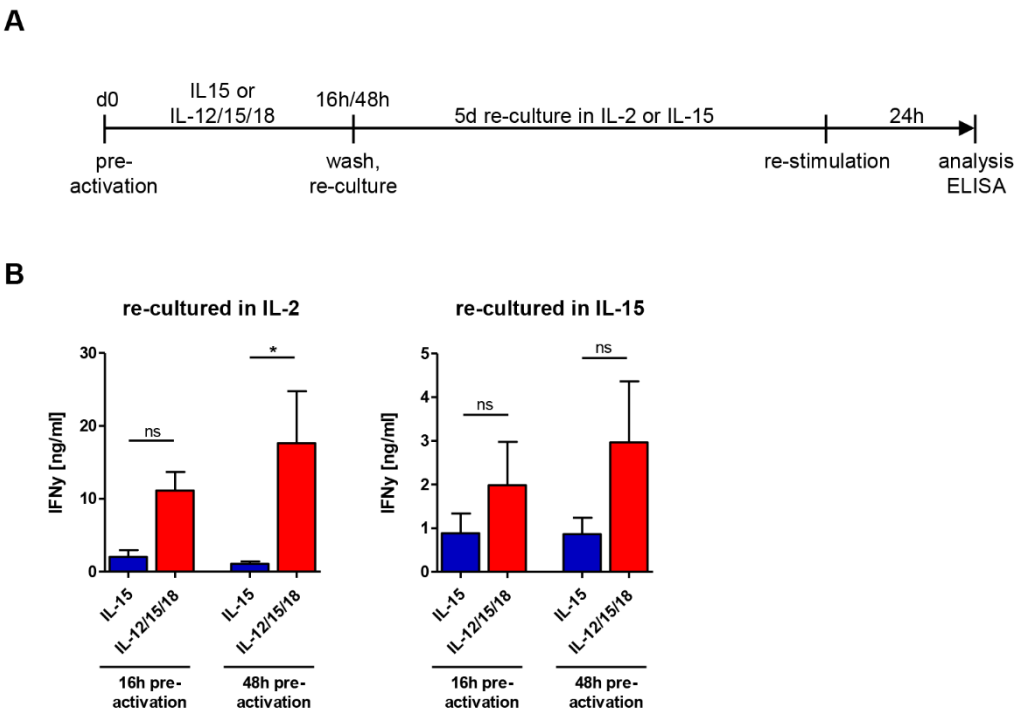


Figure 6.19: Enhanced IFN-γ secretion by IL-12/15/18–pre-activated NK cells upon re-stimulation with K562. Freshly isolated bulk NK cells were stimulated with IL-15 or IL-12/15/18 for either 16 h or for 48 h, washed and re-cultured in 100 IU/ml IL-2 or in 1 ng/ml IL-15 for additional 5 days. For re-stimulation, NK cells were co-cultured at E:T ratio 1:1 with K562 for 24 h, supernatants were harvested and analyzed by ELISA. (A) Schematic overview of the experimental procedure. (B) IFN-γ concentration of IL-2 (left) and IL-15 (right) re-cultured NK cells upon re-stimulation with K562. Both 16 h and 48 h pre-activated NK cells are shown (n = 4; mean + SD). Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons test.

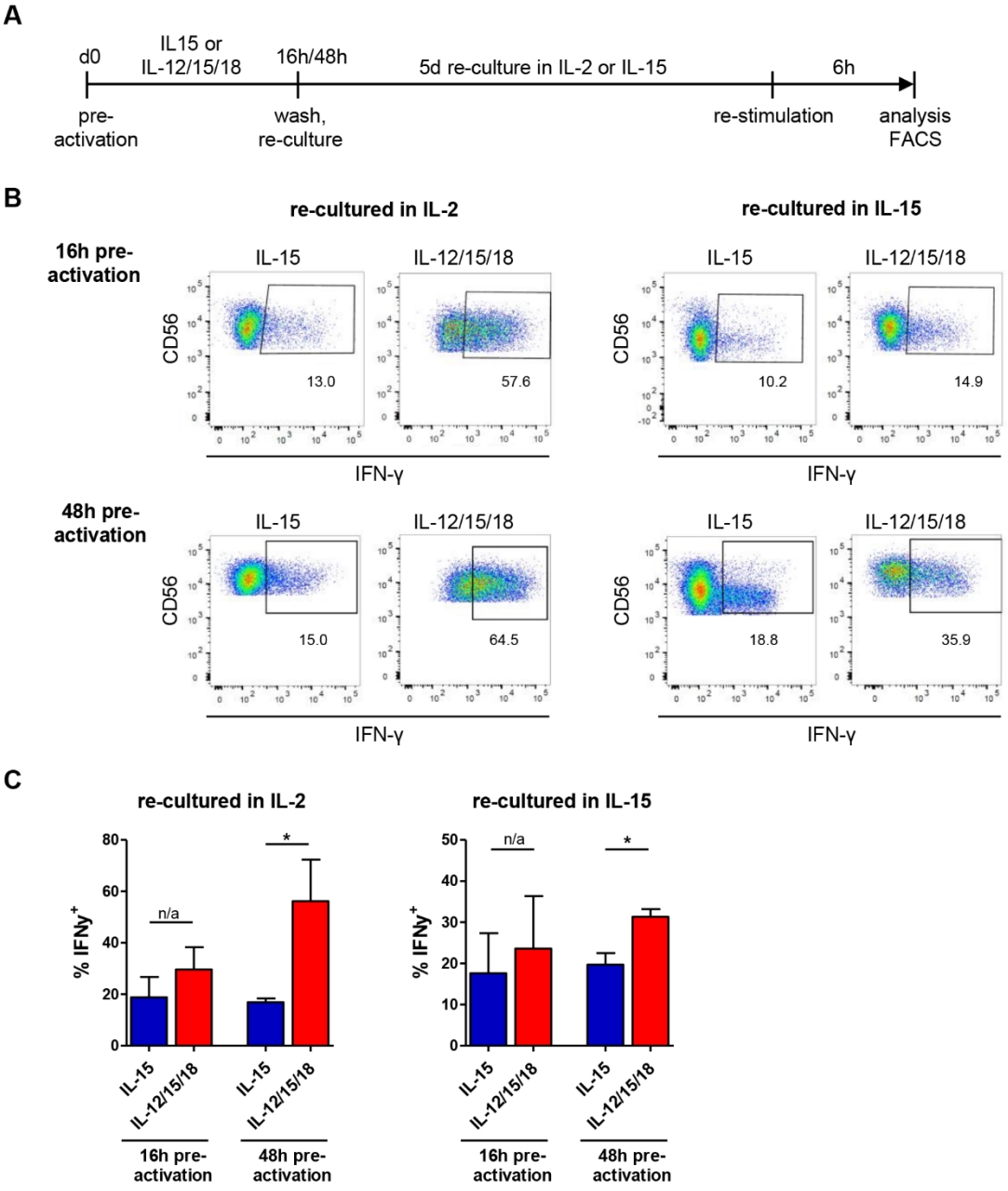


Figure 6.20: IL-12/15/18–preactivated cells exhibit improved IFN-γ expression upon re-stimulation with K562. Freshly isolated bulk NK cells were stimulated with IL-15 or IL-12/15/18 for either 16 h or for 48 h, washed and re-cultured in 100 IU/ml IL-2 or in 1 ng/ml IL-15 for additional 5 days. For re-stimulation, NK cells were co-cultured at E:T ratio 1:1 with K562 for 6 h in the presence of Brefeldin A and intracellular IFN-γ was detected by flow cytometry. (A) Schematic overview of the experimental procedure. (B) Representative dot plots of intracellular IFN-γ staining detected by flow cytometry. NK cells pre-activated for 16 h (upper panel) and 48 h (lower panel) are shown. (C) Percentage of IFN-γ producing NK cells after re-culture in IL-2 (left) or IL-15 (right) NK cells. (n = 2-3; mean + SD). Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons test.

cytometry-based quantification showed a clear tendency of enhanced IFN-γ expression

after IL-12/15/18 pre-activation (Figure 6.20 B & C). IFN-γ production of IL-12/15/18–stimulated NK cells was similarly enhanced both after 16 h and 48 h pre-activation. Of note, since IL-12/15/18 pre-activated NK cells produced more IFN-γ at steady state without re-stimulation, all values were corrected to baseline production. In general, re-culture of pre-activated NK cells in IL-2 resulted in increased IFN-γ secretion upon re-culture (Figure 6.19 B) and in higher percentages of IFN-γ expressing cells (Figure 6.20 B & C) compared to NK cells re-cultured in IL-15, but also in increased background IFN-γ production without re-stimulation. Thus, pre-activation for 48 h with IL-12/15/18 was equally capable of generating memory-like NK cells with high capacity of sustained IFN-γ production.

Taken together, we could show that stimulation of NK cells with IL-12/15/18 induced downregulation of KIR surface expression and mRNA levels, which was reversible by re-culture in IL-2 or IL-15. Reduced expression of inhibitory KIR receptors translated into reduced inhibition and improved NK functionality against cognate HLA-I–expressing tumor cells. Furthermore, IL-12/15/18 pre-activated NK cells exhibited sustained IFN-γ production compared to IL-15–treated cells upon re-stimulation with K562, indicative of memory-like functionality.

6.4. Downregulation of KIR expression on NK cells in an HCMV infection model

6.4.1. KIR2DL2/L3, KIR2DL1 and KIR3DL1 are downregulated upon HCMV infection

Mouse and human cytomegalovirus (MCMV and HCMV) infection have been implicated in the formation of memory-like NK cells with expansion of specific NK subsets [24,718]. A strong pro-inflammatory cytokine milieu is created during these viral infections and in particular IL-12 has been shown to be involved in expansion of the NKG2C$^+$ NK subset [556]. Therefore, we wanted to investigate the impact of HCMV infection and of the cytokines produced during infection on KIR expression. To model HCMV infection, we employed a co-culture system of PBMCs together with infected fibroblasts, which was established in our laboratory [556]. As schematically illustrated in Figure 6.21 A, MRC-5 fibroblasts were left

uninfected or were infected with the HCMV strain AD169 for 8 h. Virus was washed away and fibroblasts were cultured together with freshly isolated PMBCs for 7 days. Co-culture of PBMCs with HCMV-infected fibroblasts resulted in significant downregulation of KIR2DL2/L3, KIR2DL1 and KIR3DL1 molecules on NK cells (Figure 6.21 B) compared to uninfected cultures at day 3 p.i. (post infection). The percentage of NK cells expressing a certain KIR (Figure 6.21 B, upper panel) as well as the surface density of KIR expression (lower panel) was significantly decreased in HCMV-infected co-cultures, resembling the

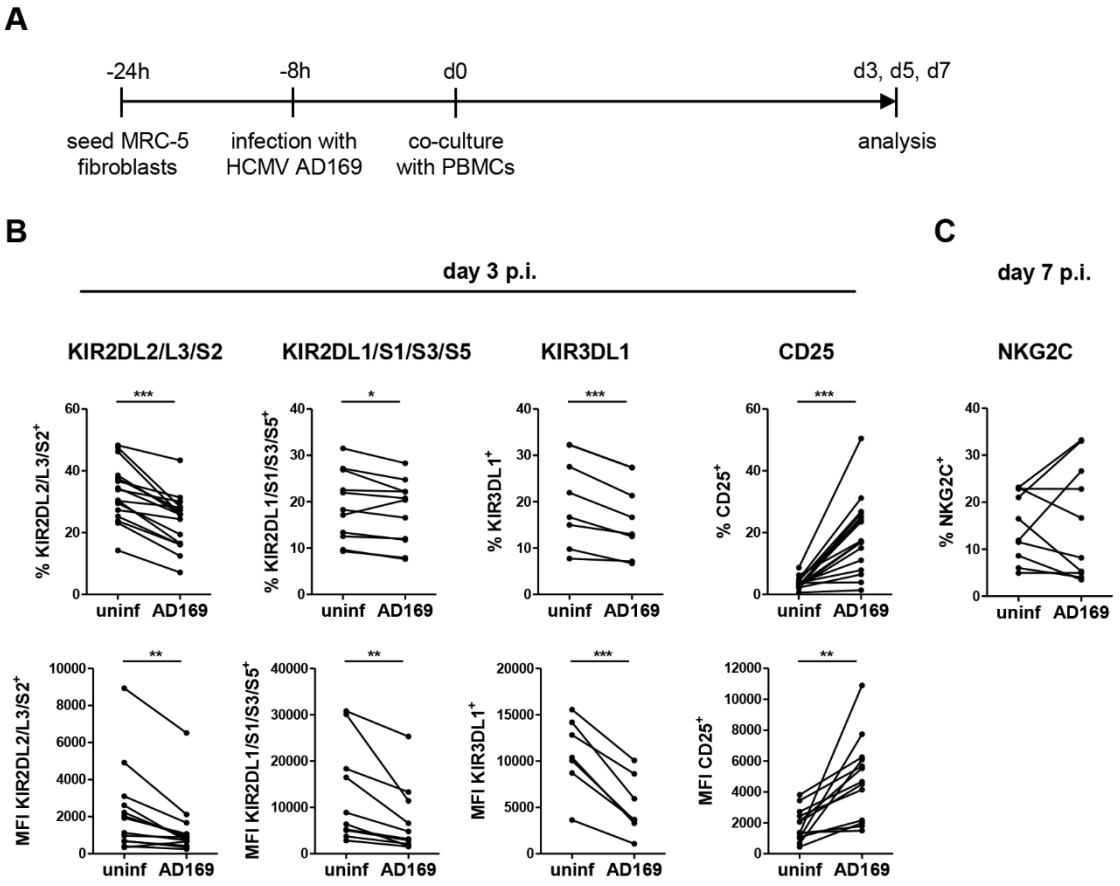


Figure 6.21: Downregulation of KIRs on NK cells in an HCMV infection model system. NK cells were analyzed in a co-culture system of PBMCs together with infected fibroblasts modelling an HCMV infection. (A) Schematic description of experimental procedure of HCMV co-culture system. MRC-5 fibroblasts were infected with human cytomegalovirus (HCMV, subtype AD169) at MOI of 10 for 8 h or were left uninfected. Virus was washed away and fibroblasts were cultured together with PMBCs for 7 days. PBMCs were analyzed for their receptor expression after 3, 5 and 7 days by flow cytometry. NK cells were defined by gating on 7-AAD– CD3– CD19– CD14– CD56+ lymphocytes. (B) Expression of indicated receptors (upper panel) and median fluorescence intensity (MFI, lower panel) on CD56+CD3– NK cells in the HCMV co-culture system at day 3p.i. (post infection). (C) NKG2C expressing NK cells are shown after 7 days p.i. (B – C) Results from individual donors are connected with a line (n = 10 - 16). Statistical analysis was performed with a paired two-tailed student's t-test.

IL-12/15/18–induced KIR downregulation (Figure 6.2). Of note, KIR expression was detected using antibody clones cross-reacting with long and short intracellular domains (e.g. anti-KIR2DL1/S1/S3/S5 clone HP-MA4). Thus, no clear conclusion about regulation of inhibitory or activating KIRs could be drawn. In addition, CD25 was highly upregulated in HCMV-infected co-cultures (Figure 6.21 B), similar to our observations upon IL-12/15/18 stimulation of NK cells (Figure 6.1 B). HCMV infection was described to drive expansion of a NKG2C$^+$ NK subset compared to uninfected co-cultures [556,719]. However, our individual co-cultures displayed a remarkable donor-to-donor variability after 7 days p.i. and resulted in heterogenous expansion or even in reduction of the NKG2C$^+$ subsets (Figure 6.21 C). Kinetic studies revealed, that KIR2DL2/L3 expression is reduced 3 days after HCMV infection compared to uninfected cultures (Figure 6.22). However, KIR expression was restored and even further increased in both uninfected and infected co-cultures after 7 days. In contrast, CD25 expression was constantly increasing upon HCMV infection and highly upregulated 7 days p.i. (Figure 6.22).

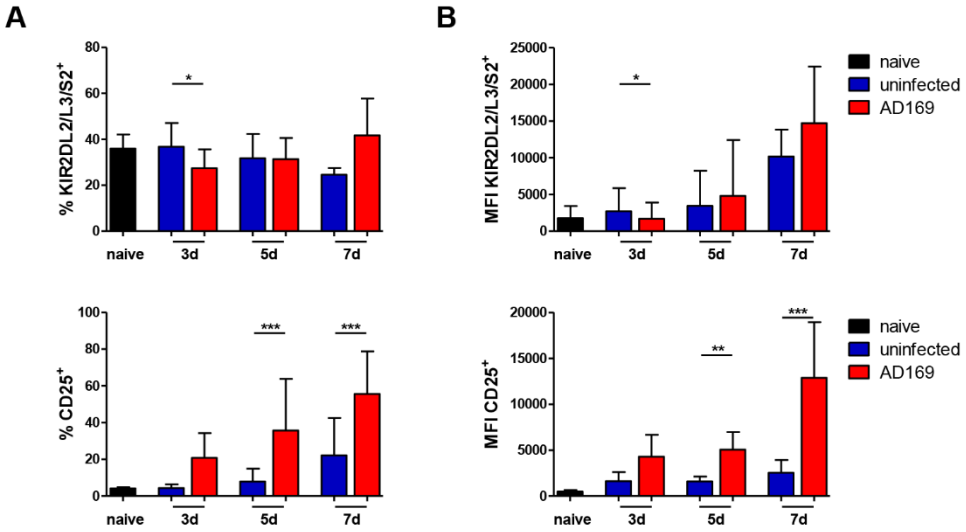


Figure 6.22: Kinetics of KIR and CD25 expression in HCMV infection. PBMCs were co-cultured with HCMV-infected or uninfected MRC-5 fibroblasts and analyzed by flow cytometry at the indicated time points. (A) Percentage positive population and (B) median fluorescence intensity (MFI) of indicated receptor expression at day 3, 5 and 7 p.i. Statistical analysis was performed with a two-way ANOVA followed by Bonferroni's multiple comparison test (n = 7 - 9, mean + SD). NK cells were defined by gating on 7-AAD– CD3– CD19– CD14– CD56+ lymphocytes.

Since stimulation of NK cells with IL-12/15/18 induced regulation of various NK receptors (Figure 6.1), we analyzed NK receptor expression in the HCMV co-culture system. In line with our observations upon IL-12/15/18 stimulation (Figure 6.1), CD16 and NKp80 expression was significantly downregulated upon HCMV infection (Figure 6.23 A & B), whereas expression density of DNAM-I was increased (Figure 6.23 B). Furthermore, the co-stimulatory TNFRSF members Ox40 and 4-1BB were highly upregulated as reflected by the

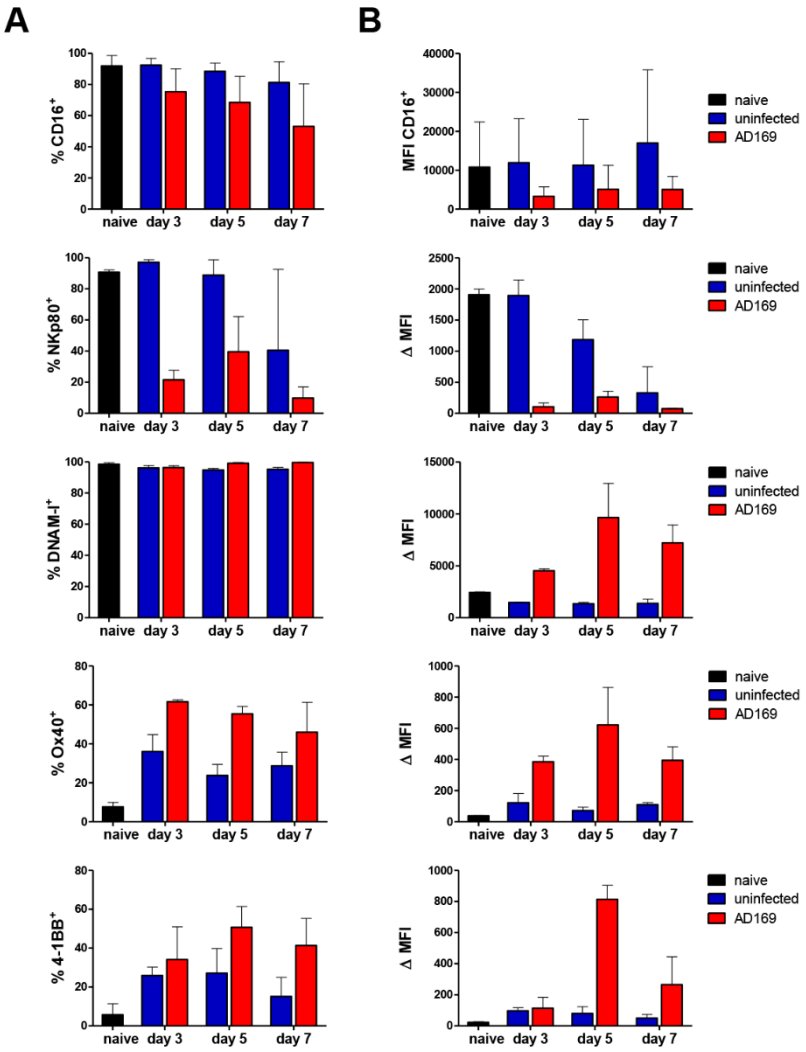


Figure 6.23: Regulation of NK cell receptor expression in an HCMV infection system. PBMCs were co-cultured with HCMV-infected or uninfected MRC-5 fibroblasts and analyzed by flow cytometry at the indicated time points. (A) Percentage receptor positive NK cells and (B) median fluorescence intensity (MFI) of the positive subpopulation or the isotype-corrected median fluorescence intensity (Δ MFI) of the indicated receptors. Statistical analysis was performed with a two-way ANOVA followed by Bonferroni's multiple comparison test (n = 2; mean + SD). NK cells were defined by gating on 7-AAD– CD3– CD19– CD14– CD56+ lymphocytes.

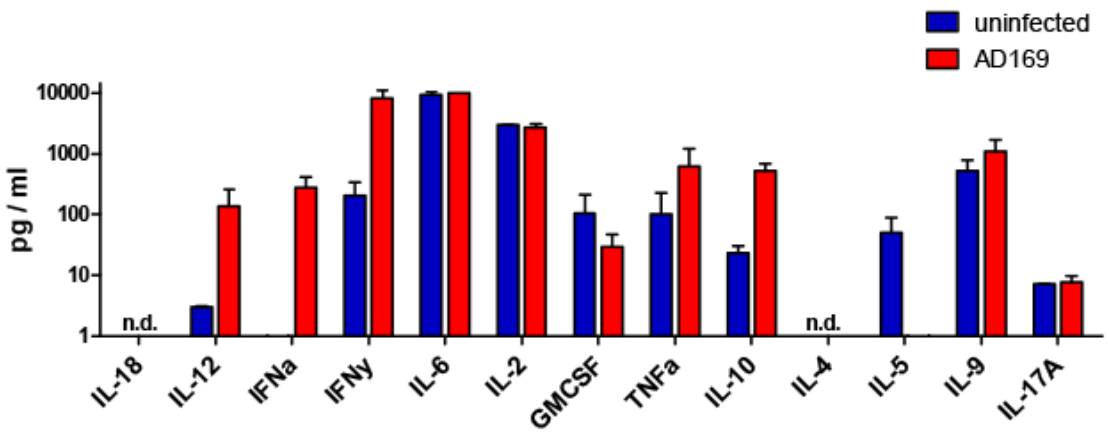


Figure 6.24: Cytokine profile of HCMV co-culture system. PBMCs were co-cultured with AD169-infected or uninfected MRC-5 fibroblasts for 3 days and supernatants were analyzed for secreted cytokines. MACSplex analysis of co-culture supernatants harvested 3 days p.i. MACSplex analysis was performed by M. Granzin. IL-18 was detected by ELISA (n = 2; mean + SD; n.d. not detectable).

percentage of receptor positive cells (Figure 6.23 A), as well as by their expression density levels (Figure 6.23 B).

6.4.2. IL-12–induced CD25 expression drives NK proliferation in HCMV infection

It was previously shown by our laboratory that IL-12 is produced by monocytes in HCMV-infected co-cultures and drives expansion of NKG2C$^+$ NK subsets upon infection [556]. IL-12 was detected in infected co-cultures starting after 48 h and IL-12 levels were increasing up to 72 h [556]. Indeed, MACSplex analysis of co-culture supernatants 3 days p.i. indicated enhanced concentrations of IL-12, IFN-α, IFN-γ and IL-10 in infected compared to uninfected co-cultures (Figure 6.24). Although not differentially produced, multiple cytokines such as IL-2, IL-6, IL-9, TNF-α and GMCSF were present in the co-culture system. IL-18 could not be detected in the co-culture supernatants by ELISA 3 days p.i. (Figure 6.24). Since we observed that IL-12 alone induced a partial KIR downregulation on purified NK cells (Figure 6.12), we investigated the role of IL-12 in KIR regulation upon HCMV infection. We neutralized IL-12 in the co-cultures by adding a blocking antibody and analyzed KIR expression 3 days p.i. . IL-12 neutralization had no effect on KIR2DL2/L3 or KIR2DL1 downregulation in HCMV-infected co-cultures, neither on KIR expression

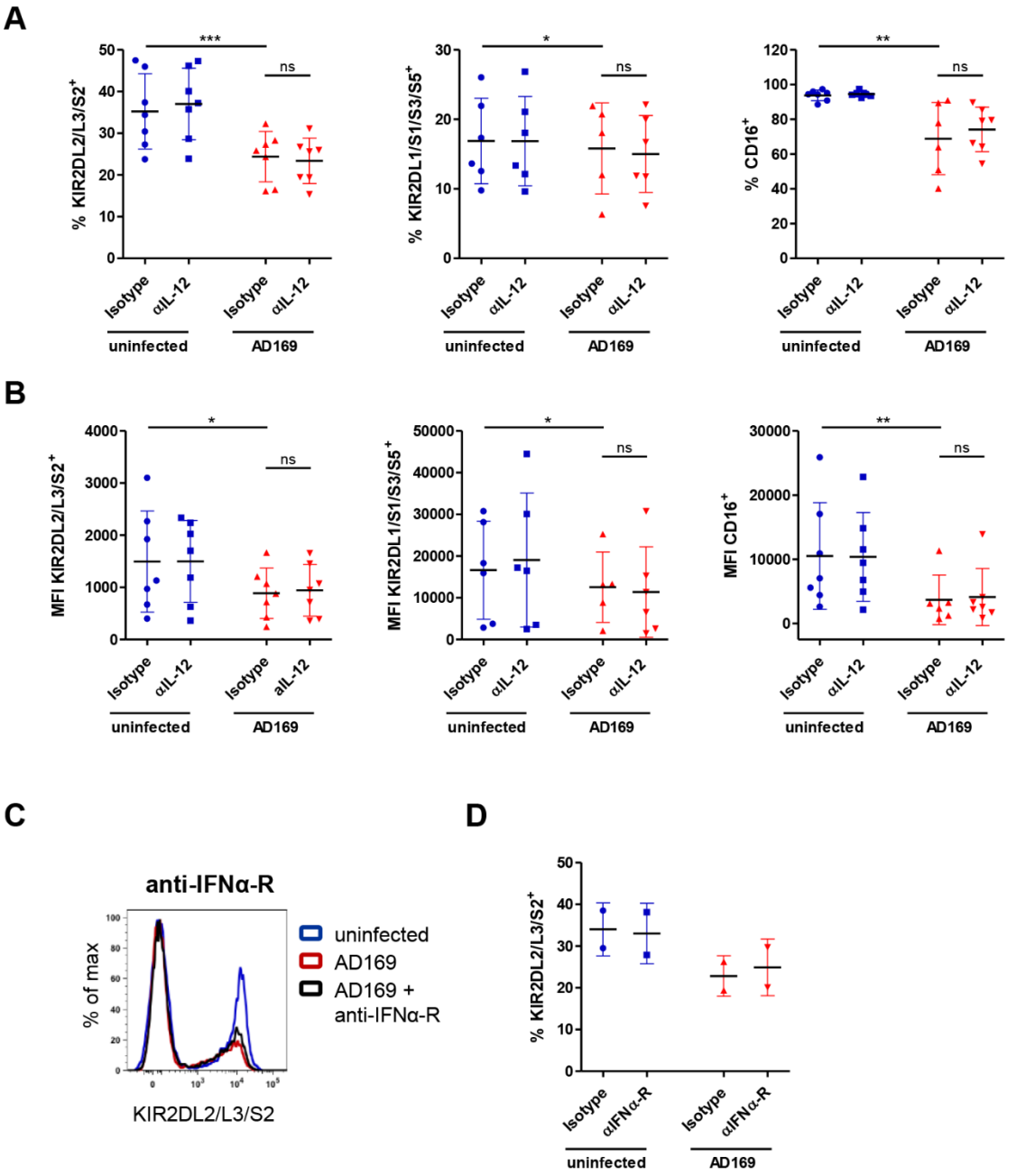


Figure 6.25: IL-12 neutralization and IFN-αR blockade in HCMV co-cultures. PBMCs were co-cultured with AD169-infected or uninfected MRC-5 fibroblasts in the presence of indicated neutralizing antibodies or isotype control. (A) Percentage receptor positive NK cells and (B) median fluorescence intensity (MFI) of the positive subpopulation of co-cultures with IL-12 neutralizing antibody at day 3 p.i. (n = 6 - 7; mean ± SD). Each symbol represents one donor. (C) Representative histogram and (D) summary of co-cultures in the presence of IFN-α receptor blocking antibody (n = 2; mean ± SD). Each symbol represents one donor. Statistical analysis was performed with a two-way ANOVA followed by Bonferroni's multiple comparison test. NK cells were defined by gating on 7-AAD– CD3– CD19– CD14– CD56+ lymphocytes.

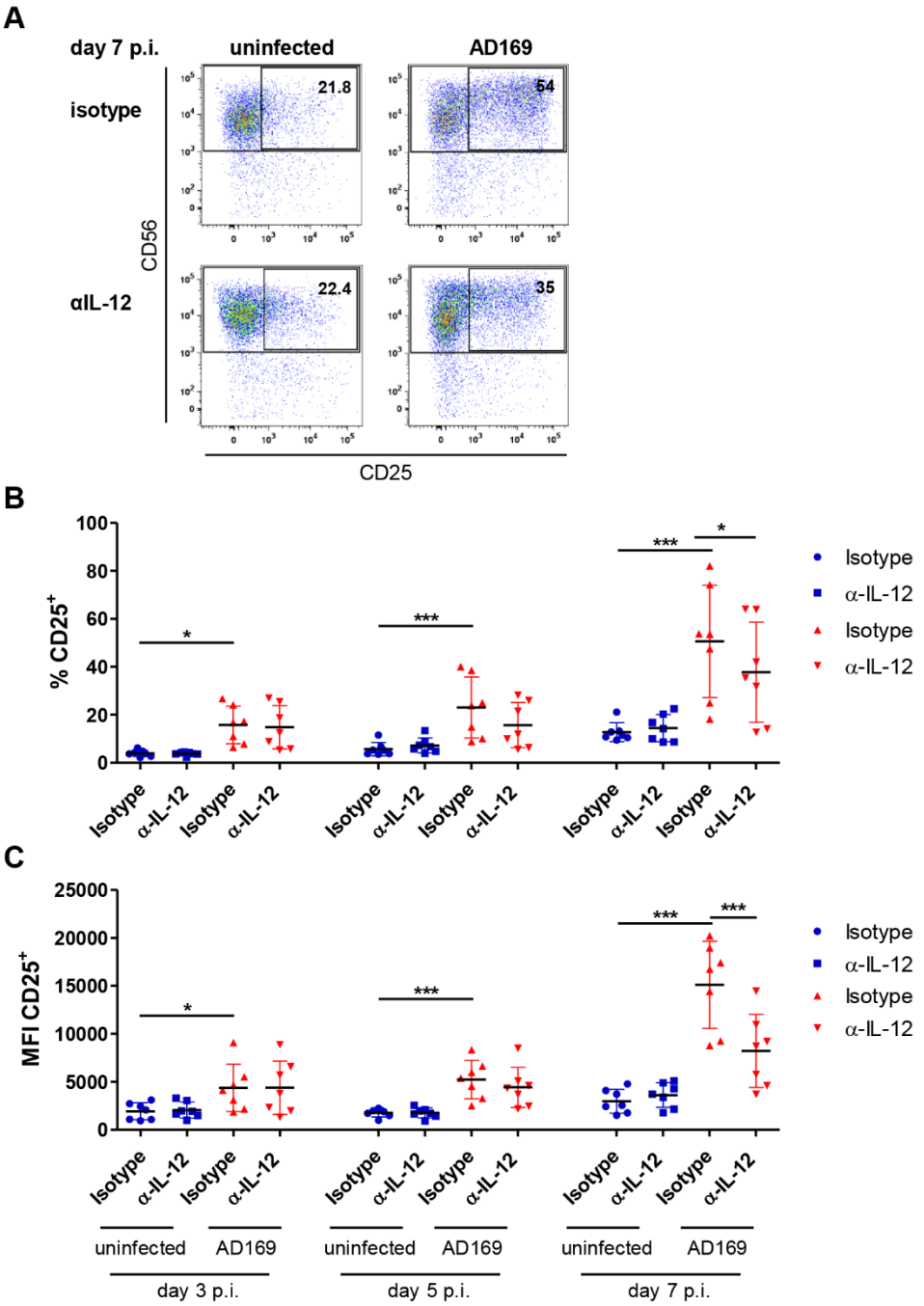


Figure 6.26: Upregulation of CD25 on NK cells in HCMV-infected co-cultures is partially IL-12 dependent. PBMCs were co-cultured with AD169-infected or uninfected MRC-5 fibroblasts in the presence of neutralizing antibody against IL-12 or isotype control. (A) Representative dot plots of CD25 expression on NK cells at day 7 p.i. Numbers indicate the percentage of CD25 positive cells among all CD3– CD56+ NK cells. (B) Statistical summary of percentage and (C) median fluorescence intensity (MFI) of CD25 positive NK cells at day 3, 5 and 7 p.i. (n = 7; mean ± SD). Each symbol represents one donor. Statistical analysis was performed with a two-way ANOVA followed by Bonferroni's multiple comparison test. NK cells were defined by gating on 7-AAD– CD3– CD19– CD14– CD56+ lymphocytes.

intensities (Figure 6.25 B) nor with regards to percentage of KIR expressing cells (Figure 6.25 A). Moreover, CD16 downregulation was not prevented by neutralization of IL-12 although it was described to involve IL-12–induced shedding of CD16 by matrix-metalloproteinases (MMPs) shedding [711,716]. This could indicate insufficient neutralization of IL-12 during the co-culture period or contribution of multiple cytokines. Since IFN-α was

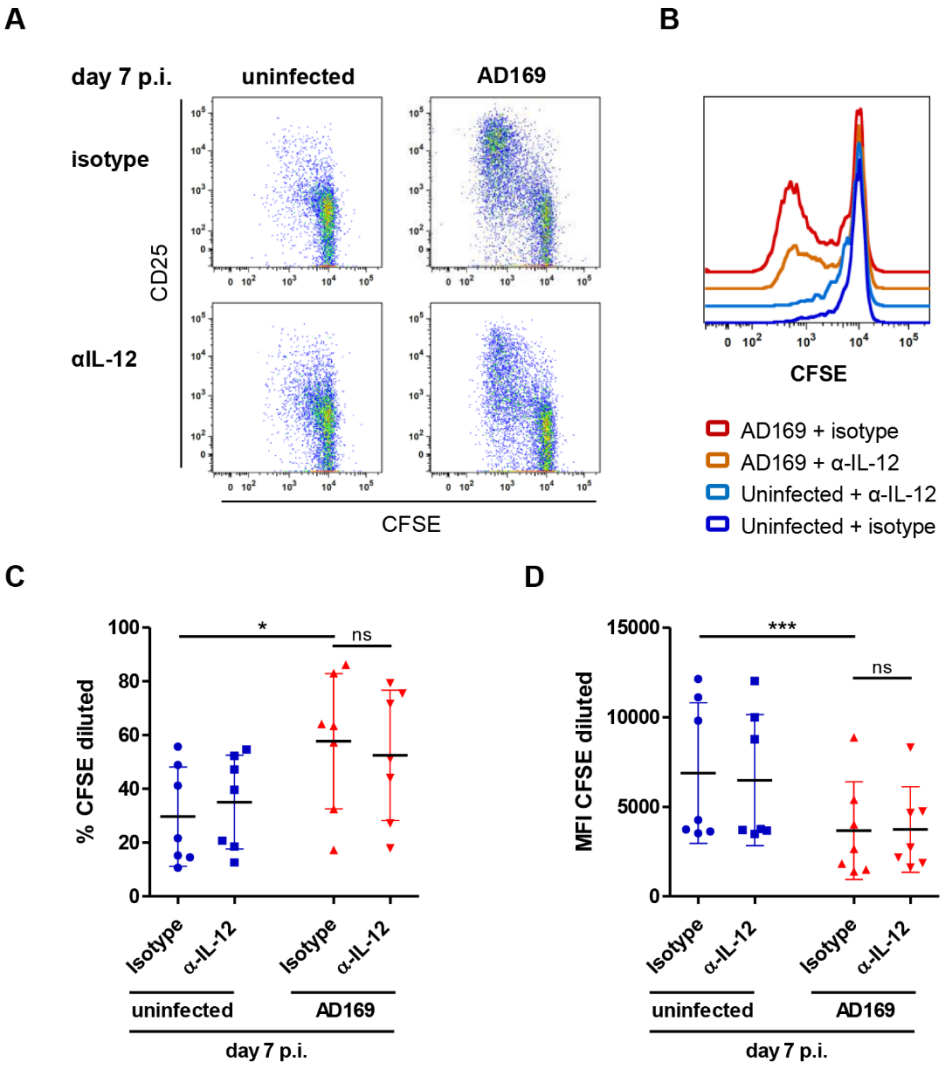


Figure 6.27: HCMV-induced proliferation of CD25+ NK cells is n not IL-12 dependent. PBMCs were labeled with the proliferation dye CFSE and co-cultured with AD169-infected or uninfected MRC-5 fibroblasts in the presence of IL-12 neutralizing antibody or isotype control. (A) Representative dot plots co-gating CFSE dilution and CD25 expression on NK cells at day 7 p.i. (B) Histogram overlays of CFSE dilution gated on CD3– CD56+ NK cells. (C) Summary of percentage and (D) median fluorescence intensity (MFI) of NK cells that showed CFSE dilution at day 7 p.i. (n = 7; mean ± SD). Each symbol represents one donor. Statistical analysis was performed with a two-way ANOVA followed by Bonferroni's multiple comparison test. NK cells were defined by gating on 7-AAD– CD3– CD19– CD14– CD56+ lymphocytes.

almost exclusively present in HCMV-infected supernatants, we blocked the IFN-α receptor on PBMCs in the co-culture system with a blocking antibody. Blockade of IFN-αR showed no effect on HCMV-induced downregulation of KIR2DL2/L3 at day 3 p.i. (Figure 6.25 C & D). Thus, neither IL-12 nor IFN-α alone were sufficient to drive KIR downregulation in the HCMV model system. In contrast, induction of CD25 on NK cells in HCMV-infected co-cultures was most pronounced at day 7 p.i. (Figure 6.26 A) and was partially dependent on IL-12 (Figure 6.26 A - C). Both, percentage of CD25 positive NK cells (Figure 6.26 B) as well as surface expression density (Figure 6.26 C) were reduced in HCMV-infected cultures when IL-12 was neutralized, whereas uninfected cultures remained unaffected.

CD25 represents the IL-2 high affinity receptor α-chain and therefore contributes to IL-2–dependent proliferation of NK cells. Since IL-2 is present in both infected and uninfected co-cultures (Figure 6.24 B), we investigated the impact of CD25 and IL-12 neutralization on NK proliferation. PBMCs were labeled with the fluorescent cell division tracker dye CFSE and analyzed 7 days p.i. by flow cytometry. CFSE dilution correlated with high CD25 expression (Figure 6.27 A) at day 7 p.i. and significantly more NK cells had proliferated in HCMV-infected cultures compared to uninfected (Figure 6.27 A - C). Furthermore, NK cells in infected co-cultures exhibited stronger CFSE dilution demonstrated by lower CFSE intensities (MFI) (Figure 6.27 D), indicating that more cells had undergone a higher number of cell divisions. Neutralization of IL-12 correlated with reduced CD25 expression (Figure 6.27 A) but showed only a modest reduction of CFSE dilution and proliferation (Figure 6.27 A - D). Although IL-12 neutralization significantly reduced CD25 expression (Figure 6.26), the high affinity IL-2Rα was still highly present on NK cells upon infection, which might be sufficient to react to low IL-2 concentrations and to induce strong proliferation.

Taken together, we could show downregulation of KIR expression upon HCMV infection 3 days p.i., which was restored during longer co-culture periods and not solely dependent on IL-12 or IFN-α alone. CD25 upregulation was dependent on IL-12 and high CD25 expression levels were associated with NK proliferation in HCMV infected co-cultures.

7. DISCUSSION

Parts of this section have been previously published in their original or modified form in Ewen et al. 2018 Eur. J. Immunol. [710] and in Rölle, Pollmann et al. 2014 J. Clin. Invest. [556]. The text has been originally written by myself.

Cancer immunotherapy based on NK cells is a promising field in the treatment of hematological malignancies [608,614,659]. In cancer patients, NK cell anti-tumor efficacy is often attenuated through inhibitory signals mediated by the interaction of inhibitory KIRs on NK cells with HLA-I molecules on autologous tumor cells. Therefore, several approaches are currently applied in NK cell-based immunotherapy to circumvent KIR-mediated inhibition and to unleash NK cell anti-tumor activity.

For instance, adoptive transfer of haploidentical KIR/ligand-mismatched NK cells has been shown to greatly improve the clinical outcome of leukemia patients, since they are not inhibited by KIR/self–HLA-I interactions [606,658,659]. However, adoptive transfer of allogeneic cells is limited by the selection of a matching donor, low persistence of transferred NK cells in the adoptive host and the risk of graft-vs-host disease (GvHD) [645,674,675,720]. Therefore, it is of overall importance to develop new strategies to improve the anti-cancer potency of NK cells against HLA-I–expressing tumors in autologous transfusions.

Here, we reveal a novel mechanism how NK cells can be transiently released from KIR-mediated self-inhibition. Stimulation of mature peripheral blood NK cells with the cytokines IL-12/15/18 resulted in transient, yet robust downregulation of mRNA and surface expression of inhibitory KIR2DL2/L3, KIR2DL1 and KIR3DL1 molecules. Additionally, we observed KIR downregulation on NK cells in an HCMV infection model, representing a physiological setting with high concentrations of pro-inflammatory cytokines. IL-12/15/18–stimulated NK cells experienced less inhibition upon KIR2DL2/L3 engagement and gained high responsiveness against HLA-I–bearing lymphomas, which may be translated into HLA-I matched settings of immunotherapy. Taken together, our study reports a novel mechanism of KIR downregulation on mature peripheral blood NK

cells by pro-inflammatory cytokines, generating potent effector cells for autologous NK cell infusions as treatment of HLA-I–expressing tumors.

7.1. Phenotype of cytokine-stimulated NK cells

Short overnight pre-activation with IL-12/15/18 has been demonstrated to induce NK cells with high proliferative capacity and potent effector functions [288,289,333,334,526]. These cells exhibit sustained functionality both after adoptive transfer *in vivo* and after a subsequent re-culture period in IL-2 or IL-15, implicating the generation memory-like features. So far, no comprehensive phenotypical and functional characterization of IL-12/15/18–stimulated NK cells have been performed after the initial cytokine exposure. To dissect the improved functionality of IL-12/15/18–activated NK cells, we conducted a systematic analysis of activating, co-stimulatory and inhibitory receptors on pbNK cells at different time points after IL-15 or IL-12/15/18 stimulation. Cytokine stimulation induced changes in the expression of many NK receptors (Figure 6.1), either reflected by the percentage of receptor expressing cells or by receptor surface expression density per cell (indicated by MFI).

Expression densities of most activating NK receptors were enhanced upon IL-15 or IL-12/15/18 stimulation (Figure 6.1 A), whereas expression of some other receptors, such as NKp46, was only marginally changed. In general, exposure of NK cells to single or multiple combinations of IL-12, IL-15 and IL-18 has been described to enhance expression of activating NK receptors [271,721,722]. In contrast, the activating receptors NKp80 and CD16 (FcγRIIIA) exhibit profound downregulation upon IL-12/15/18 stimulation. Reduction of CD16 expression on NK cells by cytokines has been shown to involve matrix-metalloproteinase (MMP)–mediated shedding of the receptor from the cell surface [711,716]. NKp80 downregulation and simultaneous upregulation of the NKp80L AICL has been implicated in controlling overreaction of highly activated NK cells by fratricide [156].

Expression levels of CD25 were substantially increased upon IL-12/15/18 stimulation compared to IL-15 (Figure 6.1). CD25 is expressed at low levels on $CD56^{bright}$ cells and not present on $CD56^{dim}$ NK cells [286,287], but various stimuli have been described to induce CD25 expression on NK cells e.g. by activating receptor stimulation or by cytokine treatments [99,271,556,723,724]. IL-12 and IL-18 have been demonstrated to increase CD25 levels

and the combination of IL-12/15/18 is particularly potent in promoting CD25 expression [271,288–290].

CD25 represents the high affinity IL-2Rα-chain that constitutes the IL-2Rαβγ, facilitating high responsiveness to picomolar concentrations of IL-2 [284,285]. Our laboratory and others have demonstrated that elevated CD25 expression levels on IL-12/15/18–stimulated NK cells were associated with high proliferative capacity and higher cell recoveries after *in vitro* culture in low dose IL-2 and in an *in vivo* xenograft mouse model [288,569]. This is especially interesting in therapeutic setting of adoptive NK cell transfer, since administration of high concentrations of IL-2 is involved in toxic side effects and expansion of $CD25^+$ regulatory T cells [302,303,643,725].

The TNFRSF members 4-1BB (CD137) and Ox40 (CD134) are absent on naïve NK cells but are induced by both IL-15 and IL-12/15/18, whereas the latter even further elevates their surface density levels (Figure 6.1 C). Although Ox40 is intensely studied as a co-stimulatory receptor in T cells [216,217,221], little is known about the functionality of Ox40 in NK cells. Our laboratory recently described an important role for Ox40-Ox40L interaction between $CD14^+$ monocyte-derived cells and NK cells, leading to NK cell activation and proliferation in hepatitis C virus (HCV) infection [291]. There, Pollmann et al. discovered induction of Ox40 expression on NK cells in HCV-infected co-cultures with PBMCs, which correlated with CD25 upregulation and enhanced proliferation of NK cells. 4-1BB expression has been reported to be induced on NK cells by IL-2, IL-15 and by triggering of CD16, and functions as a potent co-stimulatory receptor in CD16-mediated lysis [99,214,726]. Thus, CD25, 4-1BB and Ox40 could be considered as activation markers on NK cells.

7.2. IL-12/15/18–induced downregulation of inhibitory KIRs

In addition to increased activating receptors, we observed downregulation of inhibitory KIR2DL2/L3, KIR2DL1 and KIR3DL1 molecules upon IL-12/15/18 activation (Figure 6.2), which constitute the three most important inhibitory KIRs in tumor immunity. Stimulation with IL-12/15/18, but not with IL-15 alone, resulted in a significant decrease in the percentage of KIR positive NK cells and in an even more profound reduction of surface expression densities (MFI). KIR downregulation was detected on the entire population of

mature peripheral blood (pb)NK cells (Figure 6.2) as well as on sorted CD56^{dim} NK cells (Figure 6.5) and sorted KIR2DL2/L3 positive subsets (Figure 6.7).

Previous studies that analyzed NK cells pre-activated for 16 h with IL-12/15/18, focused on the memory-like properties of these cells after a subsequent re-culture period in IL-2 or IL-15 for 4 - 7 days [288,289,334,526]. However, no differential KIR expression was detected in these reports after 16 h of cytokine stimulation or upon IL-2 or IL-15 re-culture. Our kinetics studies revealed that KIR downregulation was first observed starting 36 h after exposure to IL-12/15/18 with most pronounced reduction after 48 h and 60 h (Figure 6.3). In contrast, KIR expression on IL-12/15/18 and IL-15–activated NK cells was comparable after 16 h, the time point investigated in previous studies [288,289,334,526]. Moreover, KIR downregulation was transient and could be restored upon re-culture in IL-2 and IL-15 after 5 days (Figure 6.9 and Figure 6.10) explaining why other reports did not detect differential KIR expression upon 7 days of re-culture.

7.3. Possible mechanism of cytokine-regulated KIR expression

While the acquisition of KIRs during development is well understood [463,464], little is known about the regulation of already expressed KIR molecules on mature CD56^{dim}KIR^{+} NK cells.

In our study, downregulation of KIR2DL2/3 expression was not accompanied by reduced proliferation or increased apoptosis of the KIR positive subset and a concomitant outgrowth of CD56^{bright}KIR^{-} cells or the sorted KIR2DL2/L3 negative population (Figure 6.8). Rather, we demonstrated that KIR regulation occurred in the CD56^{dim}KIR^{+} subset by sorting and separate stimulation of CD56^{dim} and CD56^{bright} NK cells (Figure 6.5).

In general, surface protein expression can be modulated by multiple processes such as post-translational and post-transcriptional mechanisms. Post-translational mechanisms could involve receptor shedding, internalization or retention. However, cytokine-induced KIR downregulation did not involve matrix-metalloproteinase (MMP)–mediated shedding from the surface, as treatment with the MMP inhibitor Batimastat during cytokine exposure did not influence KIR expression (Figure 6.14 C). Additionally, unaltered expression of NKp46 and upregulation of other receptors such as CD25, DNAM-I, Ox40 and 4-1BB (Figure 6.1) indicated that IL-12/15/18 activation did not result in a general abrogation of protein expression as observed in apoptotic cells.

Importantly, we could demonstrate that decreased KIR expression was transiently regulated at mRNA level upon IL-12/15/18 stimulation (Figure 6.11), mimicking the modulation observed in KIR protein expression. The reduction of *KIR* mRNA as well as the protein expression was reversible upon IL-2 or IL-15 re-culture, suggesting the contribution of transient mechanisms. In accordance with our results, we additionally observed decreased mRNA levels of *KIR2DL1, KIR3DL1, KIR3DL3, KIR2DL4 and KIR2DL5A* (Figure 6.11 B) upon analysis of publicly available microarray data [712] comparing naïve with IL-2/12/18–stimulated NK cells. Moreover, regulation of KIR expression at a post-transcriptional level could involve an influence of IL-12 and IL-18 on *KIR* mRNA stability or alternative splicing thus reducing the detectable mRNA concentrations.

Overall, we could demonstrate that KIR molecules are regulated at a transcriptional or post-transcriptional level rather than at post-translational levels upon cytokine stimulation in our setting.

7.3.1. KIR regulation by cytokines

While IL-12/15/18 decreased KIR expression, we found that stimulation with IL-15 alone increased KIR levels compared to naïve NK cells (Figure 6.7). We observed enhanced KIR surface expression density, as detected by enhanced MFI values, as well as increased frequencies of KIR expressing cells. Additionally, re-culture in IL-2 or low-dose IL-15 after cytokine stimulation restored and even further increased KIR levels (Figure 6.9 and Figure 6.10), indicating that IL-2 and IL-15 are capable of regulating KIR expression on mature NK cells. IL-15 is crucial for NK cell development and homeostasis and has been described to drive maturation of hematopoietic progenitor cells into mature $CD56^{+}KIR^{+}$ NK cells *in vitro* [55,727]. Although IL-2 and IL-15 execute similar functions in NK cell biology due to the shared β- and γ-chain of their receptor, IL-15 signaling is non-redundant and indispensable for NK cell development [294,307,311]. Several studies have investigated the potential implication of IL-15 and IL-2 in driving KIR expression in NK cells. For mouse Ly49 receptors (the mouse inhibitory MHC-I binding receptor equivalent of the human KIR family) regulation by cytokines has been confirmed in mature NK cells [728]. *In vitro* stimulation of human $CD56^{bright}KIR^{-}$ pbNK cells with IL-15 or IL-2 has been shown to drive their maturation into $CD56^{dim}$ NK cells and to induce *de novo* KIR expression [66,729]. Furthermore, Romagnani and colleagues demonstrated that stimulation of mature

CD56^{dim}KIR^{-} NK cells with IL-2 or IL-15 without accessory cells was sufficient to induce *de novo* KIR expression [66,510]. Moreover, IL-2 and IL-15 were shown to be capable of increasing KIR frequencies and surface expression intensities on KIR-expressing NK cells including KIR2DL2/L3 and KIR2DL1 molecules [729–732]. Together, these reports substantiate the role of cytokines, especially of IL-2 and IL-15, in driving *de novo* KIR acquisition and in increasing KIR expression in mature pbNK cells. To our knowledge, cytokine-induced downregulation of KIR expression has not been reported to date. Therefore, the here described transient downregulation of KIR expression on mature CD56^{dim}KIR^{+} NK cells by IL-12/15/18 might represent a novel mechanism of KIR regulation.

7.3.2. KIR regulation by epigenetic mechanisms

How *de novo* KIR expression is initiated during development is well studied and has been shown to involve multiple promoters and epigenetic mechanisms. The clonally restricted KIR expression patterns are fixed by DNA methylation and stably maintained during cell division [465,467,468]. As hematopoietic progenitor cells commit to the NK cell linage, histone acetylation and opening of chromatin is a prerequisite for promoter accessibility at the KIR locus [466].

Once a KIR allele is demethylated and the promoters are active, its expression is fixed and passed on during multiple cell divisions. How expression levels of an already expressed KIR genes are regulated remains poorly defined. However, the epigenetic mechanisms determining KIR expression during development are not fixed events but are reversible and can be influenced in mature NK cells. Treatment of mature NK cells with the demethylation reagent 5-aza-2'-deoxycytidine (5Aza-dC, a DNA methyltransferase inhibitor) was capable of inducing *de novo* expression of previously untranscribed KIR alleles and *in vitro* methylation of a KIR promoter construct was shown to repress its transcriptional activity vice versa [465,467].

To our knowledge, only upregulation of KIR expression by IL-15 and IL-2 has been reported [66,510,729–732]. Here, we describe for the first time the transient downregulation of KIR mRNA and protein expression on mature CD56^{dim}KIR^{+} pbNK cells by IL-12/15/18 stimulation. Intriguingly, we observed that IL-12/15/18 stimulation not only abrogated IL-15–mediated upregulation but further reduced KIR frequencies and surface expression intensities on KIR positive NK cells. Treatment of CD56^{dim} or complete pbNK cells with IL-2

or IL-15 increased the frequencies of KIR2DL2/L3 and KIR2DL1 expressing cells (Figure 6.9 and Figure 6.10), implicating *de novo* KIR gene transcription in NK cell clones that previously exhibited silencing of this particular KIR allele. Vice versa, activation with IL-12/15/18 resulted in decreased frequencies of KIR expressing cells indicating silencing of KIR2DL2/L3 and KIR2DL1 loci in individual NK cell clones. Additionally, the intensity of KIR surface expression levels (as measured by elevated MFI levels) on sorted KIR2DL2/L3 positive NK cells was enhanced by IL-15 and reduced by IL-12/15/18, respectively (Figure 6.7), suggesting that the transcriptional activity of KIR promoters might have been altered.

Activation with IL-12/15/18 might modulate KIR gene regulation via direct or indirect mechanisms by interfering with the effects mediated by IL-15 or IL-2. IL-15 responsiveness in $CD56^{bright}$ cells is associated with transcription from the distal promoter and initiation of proximal promoter activity leading to DNA demethylation and chromatin opening [464,471,472]. Moreover, epigenetic remodeling and DNA demethylation of the KIR3DL1 promoter has been described upon IL-2 stimulation of $CD56^{dim}$ NK cells, resulting in *de novo* acquisition of KIR3DL1 surface expression [510]. IL-12/18 signaling might impede the recruitment of DNA demethylating enzymes, modulate their functional activity or might interfere with interaction partners necessary for complex formation. Moreover, IL-12 and IL-18 might be directly involved in in epigenetic remodeling of the KIR locus through recruitment of DNA methyltransferases or other repressors such as histone modifying enzymes leading to re-silencing of the KIR promoter region.

Epigenetic remodeling by pro-inflammatory cytokines has been reported for other genes in different models. Pre-activation of NK cell with IL-12/15/18 and subsequent transfer into mice has been demonstrated to induce demethylation of the *IFN-γ* locus [569]. Furthermore, a recent publication indicated a general role of IL-12 and STAT-4 signaling in epigenetic remodeling of the *IRF8* locus during MCMV infection [540]. These data provide proof of principle that pro-inflammatory cytokines can induce epigenetic modifications that drive NK cell adaptation.

However, whether alterations of histone modifications would be involved in KIR downregulation in mature NK cells is open to question, because active as well as repressed KIR alleles exhibit the same high degree of histone acetylation and deacetylase inhibitors were unable to induce expression of silenced KIR genes [465,466]. Preliminary data from our laboratory indicated that IL-12/15/18 stimulation of NK cells in combination with

methyltransferase inhibitor treatment (e.g. 5Aza-dC) did not affect KIR downregulation. Analysis of a publicly available microarray data set [712], which compared naïve with IL-2/12/18–stimulated NK cells, did not reveal targets involved in epigenetic regulation. However, IL-12 and IL-18 might indirectly affect epigenetic enzymes and a complex interplay of individual epigenetic mechanisms might contribute to cytokine-mediated KIR downregulation.

7.3.3. Transcription factors and promoter elements involved in KIR regulation

Three kinds of general KIR promoter types have been described corresponding to their KIR expression characteristics: One unique for KIR3DL3 which is undetectable in pbNK cells, one for KIR2DL4 which is transcribed early in all NK cells and one promoter type common for all clonally distributed *KIR* genes such as KIR2DL2/L3, KIR2DL1 and KIR3DL1 [462,733]. These three types all contain a distal promoter (Pro-D), a bidirectional proximal promoter switch (Pro-S), intergenic elements and an intermediate promoter element (Pro-1), but their characteristics of regulation and transcription factor binding might be different for KIR2DL4, KIR3DL3 and the other clonally restricted KIRs according to their expression patterns [462,464,469,733]. In this study, we observed cytokine-induced changes of inhibitory KIR2DL2/L3, KIR2DL1 and KIR3DL1 expression, which belong to the third group of clonally distributed KIR genes and share the same promoter type. A gradual model describes how these different promoters orchestrate KIR expression during different developmental stages (see Figure 3.5) [464,469].

The probabilistic and bidirectional activity of the proximal promoter switch (Pro-S) accounts for the stochastic KIR expression during the transition of $CD56^{bright}$ to $CD56^{dim}$ NK cells. It is active in both the forward and reverse direction and only high ratios of sense vs. antisense transcripts result in functional KIR gene and protein expression [473–475]. The Pro-S element harbors multiple transcription factor binding sites and polymorphisms and the balance of transcription factor affinities determine whether either the forward or reverse activity is predominantly active [449,473]. Several transcription factor binding sites have been described to be present in the proximal promoter region such as CREB, AML/Runx, YY-1, STAT-5, AP-1/4, Sp-1 and Ets-1 [462,473,733–735]. A high functional redundancy exists between

these transcription factor binding sites and mutation of several sites was necessary to inactivate the KIR promoter [734].

The described STAT-5 binding site within the proximal promoter might account for the IL-15–mediated increase in KIR expression observed in our study. However, a direct correlation between STAT-5 binding and enhanced KIR expression has yet to be experimentally verified. A recent publication reported that another STAT family member, STAT-4 involved in IL-12 signaling, was capable of inducing epigenetic remodeling of the *IRF8* gene in MCMV infection [540]. They further suggested a cooperative function of IL-12 and STAT-4 together with IL-2 or IL-15 signaling in inducing epigenetic modifications. However, a STAT-4 binding site has not been detected in one of the KIR promoter elements.

AML/Runx binding sites have been described for all promoter types but controversial reports indicate activating as well as inhibitory regulatory functions of AML/Runx proteins in KIR transcription [462,734,736–738]. AML/Runx was shown to be involved in demethylation and establishment of an open chromatin confirmation of the proximal promoter (Pro-S) and to additionally possess either activating or repressive functionality depending on the association with different cofactors [462,736,739]. AML/Runx proteins might represent possible candidates for mediating KIR downregulation observed in our study. Future investigations could explore whether expression or functions of AML/Runx proteins are influenced by IL-12/15/18 stimulation.

A recently described intermediate promoter element (Pro-1) adds further complexity to KIR gene regulation and Pro-1 regions have been detected in all KIR loci [477,478]. It has been proposed that Pro-1 represents the main regulatory element controlling KIR transcription in mature $CD56^{dim}KIR^{+}$ NK cells while the bidirectional switch (Pro-S) primary functions in establishing the stochastic and clonal KIR expression during development. Li et al. identified several transcription factor binding sites located in the Pro-1 element, such as YY-1, Oct-1, Ets-1, AP-1 and C/EBP, which might be involved in controlling and maintaining KIR gene expression in mature NK cells [478]. Binding of the transcription factors YY-1, ETs-1 and AP-1 has also been described for the proximal promoter [462,473,733–735]. It would be highly relevant to analyze the Pro-1 element for binding sites of potentially repressive transcription factors downstream of IL-12 or IL-18 signaling. Future investigations of the Pro-1 element in cytokine-stimulated NK cells could help to uncover the molecular mechanisms involved in IL-12/15/18–mediated KIR downregulation.

Until now, only upregulation of KIR expression by IL-15 and IL-2 has been reported [66,510,729–732]. In our study, we observed that IL-12/15/18 stimulation not only abrogated IL-15–mediated upregulation but further decreased KIR frequencies and surface expression intensity on mature $CD56^{dim}KIR^{+}$ NK cells. However, to our knowledge no negative influence of IL-12 or IL-18 signaling on binding of the above-mentioned transcription factors has been described that would indicate for the observed KIR downregulation. Due to the high functional redundancy of the transcription factor binding sites in the KIR Pro-S and Pro1 region, candidates may be hard to identify and IL-12/18 signaling would need to interfere with several transcription factors simultaneously to reduce KIR expression. The mechanism of KIR downregulation upon IL-12/15/18 stimulation might be complex and involve multiple layers and interaction partners. IL-12/18 might interfere with IL-15 signaling, impeding the recruitment of activating transcription factors to the KIR promoter region. IL-12/18 signaling components might modulate the functional activity of transcription factors by inducing posttranslational changes within the protein structure or through inhibition of protein complex formation by segregating necessary interaction partners. Additionally, IL-12/18 might directly act on the KIR promoter through recruitment of inhibitory transcription factors, repressors or epigenetic complexes leading to silencing of KIR gene expression. Moreover, increasing the antisense-to-sense ratio of proximal transcripts through preferential reverse promoter activity might represent a potential mechanism how IL-12/18 stimulation reduces KIR expression. It would be intriguing to analyze whether IL-12/18 stimulation selectively promotes the reverse transcriptional activity of the bidirectional proximal promoter (Pro-S), since increased antisense transcripts have been shown to result in reduction of KIR expression [449,475].

In addition to the Pro-S and Pro-1 element, also other promoter elements, such as the distal promoter, may be affected by cytokine stimulation. Manipulation of the Pro-D might negatively influence the accessibility of the Pro-S or Pro-1 region or result in re-methylation of the entire KIR loci. The different layers of complexity of KIR gene regulation and the high redundancy of transcription factor binding sites might also account for our observation of only partial downregulation and not complete abrogation of KIR expression.

Additional hypotheses of cytokine-mediated KIR regulation involve more indirect mechanisms and crosstalk to other pathways as for instance the Notch signaling pathway. A recent publication by Felices and colleagues reported a contribution of the Notch

pathway not only for early stages of NK cell development but also for functional maturation and terminal differentiation of pbNK cells [740]. Notch signaling partially contributed to *de novo* acquisition of KIR expression in KIR negative $CD56^{dim}$ and $CD56^{bright}$ NK cells and was enhanced upon IL-15 stimulation. IL-15–induced KIR expression is facilitated through functional Notch signaling by releasing Notch from its inhibitor cis-DLL1, which was also shown to restrict KIR expression in $CD56^{bright}$ NK cells. In addition, IL-15 and Notch potently enhanced expression of the transcription factor c-Myc, which was demonstrated in a previous publication to be involved in the initiation of *de novo* KIR transcription in $CD56^{+}KIR^{-}$ pbNK cells [472]. Direct binding of c-Myc to the KIR distal promoter was able to drive full transcriptional activity resulting in opening of the proximal promoter element, which subsequently allowed KIR gene expression [464,472]. Together these studies present a mechanism of IL-15–induced KIR expression in $CD56^{+}$ NK cells through releasing Notch from DLL1-mediated inhibition leading to downstream c-Myc activation, which in turn binds to the distal KIR promoter resulting in initiation of KIR transcription.

IL-15 mediated activation of the distal promoter is in line with our observations that IL-15 stimulation enhanced the frequency of KIR expressing cells, indicating *de novo* KIR expression on formerly KIR negative clones. Stimulation with a combination of IL-15 together with IL-12 and IL-18 abrogated IL-15–mediated KIR expression and resulted in decreased frequencies of KIR expressing cells. Our data indicate that pro-inflammatory cytokines, such as IL-12 and IL-18, might impede the functions of IL-15 involved in KIR expression. The mechanism of IL-12/18–mediated KIR downregulation might involve interference with Notch signaling or c-Myc binding. C-Myc is a helix-loop-helix leucine zipper transcription factor that needs to form a dimer with Max proteins to be able to bind to regulatory DNA elements [741]. IL-12 and IL-18 might diminish c-Myc activity by interfering with c-Myc interaction partners or with IL-15–mediated induction of c-Myc expression, resulting in reduced distal promoter activity and lower KIR expression. Moreover, IL-12/18 signaling might interfere with the Notch pathway by inhibiting Notch inducers such as miRNA-181, or by promoting negative Notch regulators such as cis-DLL1 or another notch inhibitor NLK (nemo-like kinase) [740,742,743]. Stimulation with IL-12/15/18 might also directly target Notch pathway components or impair the formation of the ternary protein complex necessary for Notch-mediated gene expression [743]. Future studies could investigate possible interaction partners of IL-12 and IL-18 signaling with the Notch

and c-Myc pathway to unravel the complex mechanism of KIR downregulation by IL-12/15/18.

7.4. Improved functionality of IL-12/15/18–activated NK cells

Reduced KIR2DL2/L3 expression on IL-12/15/18–activated NK cells suggested that the sensitivity of NK cells to KIR2DL2/L3-based inhibitory signals might be affected. Indeed, IL-12/15/18–stimulated NK cells experienced less KIR-mediated inhibition upon KIR2DL2/L3 engagement in redirected lysis assays resulting in increased cytotoxicity (Figure 6.15). Moreover, downregulation of KIR2DL2/L3 expression on IL-12/15/18-activated NK cells translated into enhanced killing of lymphoma cells bearing the cognate HLA-Cw03 ligand (Figure 6.18). Blocking the interaction of KIR2DL2/L3 and HLA-Cw03 with an anti-KIR2DL2/L3 antibody confirmed the contribution of the KIR/HLA-I axis on the superior functionality of IL-12/15/18–stimulated NK cells against 221_Cw03 cells.

Importantly, in absence of KIR engagement both cytokine stimulations elicited the same degree of CD16-dependent cytotoxicity as illustrated in comparable levels of specific lysis for KIR negative subsets (Figure 6.15 B - C) and when only CD16 alone was triggered (Figure 6.14 A). Furthermore, in absence of cognate HLA-I on target cells, NK cells stimulated with IL-15 or IL-12/15/18 possessed the same cytotoxic potential against 221 cells (Figure 6.18 A – B, left). In parallel, KIR2DL2/L3 negative subsets exhibited comparable killing capabilities independent of KIR–HLA-I interactions against 221 and 221Cw03 cells (Figure 6.18). These observations indicate that regulation of KIR expression and its interaction with the cognate HLA-Cw03 ligand account for the superior functionality of IL-12/15/18 stimulated cells, which is not mediated via upregulation of activating pathways. Of note, KIR2DL2/L3 negative subsets exerted equally potent cytotoxicity and showed full functional capacity. Both KIR2DL2/L3 positive and negative sorted populations co-expressed additional self–HLA-I receptors such as NKG2A, KIR2DL1 or KIR3DL1 (Figure 6.6 C) and were most likely educated and functional. Together, these observations indicate that IL-12/15/18–mediated downregulation of inhibitory KIR receptors endowed NK cells with high responsiveness against HLA-I–expressing tumor cells, which may be translated into autologous settings of immunotherapy.

Autologous NK cell infusions against HLA-I positive malignancies are limited through impaired anti-tumor efficacy of autologous NK cells receiving inhibitory signals via KIR receptors recognizing self–HLA-I on cancer cells. To restore and improve NK cell activity, several studies attempted daily IL-2 administration, albeit with limited success due to toxic adverse effects and recruitment of $CD25^+$ regulatory T cells [641,643–645]. Moreover, autologous infusion of *ex vivo* IL-2–expanded NK cells showed only limited clinical efficacy [640]. Hence, novel approaches are needed to preserve NK cells activation and to improve their anti-cancer efficacy for treatments using autologous cell transfer.

KIR-mediated inhibition can be bypassed by selection of a haploidentical donor with KIR-ligand mismatch, lacking one or more cognate HLA-I ligands for donor inhibitory KIRs. This KIR ligand incompatibility model results in potent donor NK cells not inhibited by HLA-I on tumor cells [658,659]. This principle of NK cell alloreactivity in the GvH direction according to the 'missing self' hypothesis has been identified as the basis of successful allogeneic hematopoietic stem cell transplantations (HSCT) in leukemia therapy [606]. However, the adoptive transfer of allogenic NK cells alone or as HSCT requires the availability of a matching donor and might be hampered by short persistence of the transferred NK cells in the patient [645]. Moreover, it might bear the risk of promoting severe GvHD, probably exerted by contaminating alloreactive T cells as reported in recent studies [666,674–678].

Here, we present an alternative strategy of transiently and safely releasing autologous NK cells from KIR-mediated inhibition to harness their full anti-tumor potential of future immunotherapy protocols. Our data describes robust KIR downregulation on mature NK effector cells upon pre-activation with IL-12/15/18, resulting in less KIR-mediated inhibition and increased lysis of cognate HLA-I–expressing targets (Figure 6.18). Accordingly, reduced KIR expression may render autologous NK cells less sensitive to inhibition by self–HLA-I on tumor cells, exploiting the concept of 'missing-self' recognition in HLA-I matched settings. Short *ex vivo* stimulation of NK cells with IL-12/15/18 and subsequent infusion into the patient might translate into superior effector functions against HLA-I–expressing tumors and substantially improve autologous NK cells transfer. Our results imply that the transient resistance to self-inhibition might contribute to superior anti-cancer efficacy of NK cells even in autologous settings.

Furthermore, *ex vivo* activation with the combination of IL-12, IL-15 and IL-18 before adoptive transfer might be of particular interest as it has been demonstrated to prime for the generation of NK cells with memory-like properties [673]. IL-12/15/18 pre-activated NK

cells exhibited high proliferative capacity and pronounced anti-cancer responsiveness *in vivo* and *in vitro* [288,334,526,568,569]. The safety and feasibility of adoptive transfer of IL-12/15/18–activated NK cells has been recently reported in a phase-I clinical trial with AML patients [526]. In this trial, allogeneic NK cells were stimulated for 16 h with IL-12/15/18 and infused into the patient. Haploidentical donor NK cells exhibited substantial proliferation and long persistence in the recipient. Importantly, upon *ex vivo* re-stimulation 7 days post-infusion, IL-12/15/18–pre-activated NK cells showed potent anti-leukemia responses, correlating with improved survival. Mouse models employing allo-HCT of IL-12/15/18–pre-activated NK cells even suggested a protective role against GvHD while their GvL effect was preserved [744,745]. Accordingly, adoptive transfer of IL-12/15/18–activated cells has been demonstrated to be safe, feasible and to improve survival in AML patients.

The long persistence and sustained effector functions of IL-12/15/18–stimulated NK cells have been assigned to their memory-like features [288,289,334,526]. We and others have shown, that activation of NK cells with IL-12/15/18 induced profound expression of the high affinity IL-2R α-chain (CD25) (Figure 6.1)and [288,289], facilitating strong expansion and persistence in response to low-dose IL-2 after adoptive transfer in mice [288,569]. For adoptive transfer of memory-like NK cells, picomolar concentrations of IL-2 might be sufficient or exogeneous cytokine support might no longer be necessary at all to preserve NK cell activity and persistence *in vivo*. Our group could show that sustained proliferation of NK cells was assisted by IL-2 produced by autologous $CD4^+$ T cells upon adoptive transfer of IL-12/15/18 pre-activated NK cells in mice [288,569]. In contrast to allogeneic infusions, adoptive transfer of autologous memory-like NK cells might further increase their persistence in the host through intensive homeostatic proliferation assisted by autologous $CD4^+$ T cells in an MHC-I compatible setting.

The design of the clinical study employing adoptive transfer of cytokine-induced NK cell comprised overnight activation of allogeneic NK cells for 16 h and direct infusion into the patient. To address the question whether prolonged pre-activation for 48 h, as used here, instead of 16 h would influence memory-like NK cell differentiation, we pre-activated NK cells for 16 h or 48 h and re-cultured them subsequently in IL-2 or IL-15 for 5 days. As compared to 16 h stimulation of NK cells, pre-activation for 48 h resulted in similarly enhanced IFN-γ production of IL-12/15/18–stimulated NK cells upon re-stimulation with

K562 (Figure 6.19 and Figure 6.20). Thus, also longer IL-12/15/18 stimulation of NK cells was capable of inducing memory formation.

In addition to the generation of NK cells with sustained functionality and memory-like properties, we observed pronounced downregulation of inhibitory KIRs 48 h after stimulation with IL-12/15/18. Reduced KIR levels correlated with enhanced cytotoxicity against tumor cells expressing cognate HLA-I ligands. Therefore, we hypothesize that prolonged pre-activation of NK cells for two days could additionally enhance their early effector functions towards self–HLA-I–expressing tumors and could be exploited in autologous settings of NK cell immunotherapy. The transient release of KIR-mediated inhibition could combine high cytotoxicity against autologous tumor cells at early time points directly after adoptive transfer into the patient with high IFN-γ secretion, long persistence and sustained anti-tumor activity late after infusion. Thereby, besides the memory-like functionality of cytokine-induced NK cells, the here uncovered transient resistance to self-inhibition due to KIR downregulation could improve the benefits of autologous NK cell transfer.

7.5. KIR downregulation on NK cells in HCMV infection

Mouse and human cytomegalovirus (MCMV and HCMV) infection have been implicated in the formation of adaptive NK cells with memory-like properties [24,718]. A strong pro-inflammatory cytokine milieu is created during these viral infections and in particular IL-12 has been shown to be involved in NK cell subset expansion [556]. Therefore, we employed an *in vitro* model of HCMV-infected fibroblasts co-cultured with PBMCs to investigate the impact of the produced cytokines on KIR expression.

In addition to IL-12/15/18 stimulation of purified NK cells, we observed downregulation of KIR expression on NK cells in HCMV infection (Figure 6.21). In accordance with our observations of KIR downregulation after 48 h to 60 h of cytokine treatment, the percentage of KIR expressing cells and the surface densities were reduced 3 days p.i. compared to uninfected co-cultures. Decreased KIR expression did not originate from increased proliferation and preferential outgrowth of the KIR negative population as no proliferation of NK cells was detectable 3 days p.i. (data not shown). Furthermore, KIR downregulation was transient and KIR expression levels were restored and even further

increased in both uninfected and infected co-cultures after 7 days (Figure 6.22). Increased KIR expression at later times in HCMV infection might be attributed to IL-2 produced by T cells in the co-cultures [556], since IL-2 was reported to induce KIR expression [66,510,729–732].

A strong pro-inflammatory cytokine milieu (e.g. IL-12, IFN-α, IFN-γ) is created in HCMV infected co-cultures mimicking viral infections *in vivo* (Figure 6.24). However, IL-18 was not detected in infected or uninfected co-cultures 3 days p.i. . Individual blocking of IL-12 and the IFN-αR was not sufficient to prevent KIR downregulation (Figure 6.25), revealing that neither IL-12 nor IFN-α alone were responsible for driving KIR downregulation in HCMV infection. Possibly, the combination of several pro-inflammatory cytokines is needed to reduce KIR expression as observed in our *in vitro* stimulation with IL-12/15/18 (Figure 6.12). Potential mechanisms could also involve additional cellular factors and interaction with other activated immune cells present in the PBMC co-cultures. Taken together, downregulation of inhibitory KIR receptors in HCMV infection suggests a general and physiological mechanism of reducing inhibition of NK cells and improving their functionality in pro-inflammatory settings as viral infections or cancer.

In addition to KIR regulation, we observed substantial upregulation of CD25 on NK cells in HCMV-infected co-cultures (Figure 6.21 B) resembling CD25 induction by IL-12/15/18 (Figure 6.1 B). Indeed, increased expression of CD25 was partially IL-12 dependent with highest expression detectable 7 days p.i. (Figure 6.26). CD25 represents the high affinity IL-2Rα-chain that constitutes the IL-2Rαβγ, facilitating high responsiveness to picomolar concentrations of IL-2 and therefore contributes to IL-2–dependent proliferation of NK cells. Although IL-12 neutralization significantly decreased CD25 expression, proliferation was only moderately affected (Figure 6.27), since also reduced CD25 surface expression might be sufficient for increased sensitivity to IL-2 and induction of strong proliferation. Rölle and colleagues from our group recently demonstrated that the NKG2C/HLA-E axis and IL-12 produced by inflammatory monocytes are critical for the expansion of NKG2C$^+$ NK cells in response to HCMV infection [556]. We therefore envision a scenario in which IL-12 induces high CD25 expression on NK cells, thereby permitting strong proliferation and contributing to expansion of the NKG2C$^+$ subset. Moreover, upregulation of CD25 on NK cells has been recently reported by our group to play an important role in in hepatitis C virus (HCV) infection [291]. CD25 expression and enhanced proliferation correlated with Ox40 induction and enhanced IFN-γ production, marking a NK cell subset with superior functionality. Interestingly, we could also detect high levels of Ox40 and 4-1BB (another

TNFRSF member) in HCMV infected co-cultures (Figure 6.23). Thus, high CD25 expression induced upon viral infection might serve as a marker of activated and highly functional NK cells.

7.6. Therapeutic implications

NK cell-based immunotherapy is a promising treatment approach for different types of hematological malignancies. However, one major obstacle in effective autologous NK cell therapy represents the impaired NK cell activation through interaction of self–HLA-I on tumor cells with inhibitory KIR receptors. The success of haploidentical NK cell transfer provided evidence that KIR/ligand-mismatched donor NK cells can elicit potent anti-leukemia effector functions if they are not inhibited by self–HLA-I [606,658,659]. However, not all patients are eligible for HSCT and adoptive allogeneic NK cell transfer still bears the risk of GvHD [645,674,675]. Therefore, new treatment strategies are needed to unleash NK cell anti-tumor efficacy in HLA-I matched settings.

An alternative approach could involve transfer of autologous NK cells in combination with modulation of inhibitory KIR molecules to reduce the threshold of NK cell activation. Indeed, checkpoint blockade of inhibitory pan-KIR2D receptors with the antagonistic antibody Lirilumab (1-7F9/IPH2101/IPH2102) is currently explored in clinical trials to increase NK cell responsiveness across self–HLA-I barriers [693,694].The safety and efficacy of KIR blockade has been reported for the treatment of patients with AML or multiple myeloma [700–705]. Additionally, clinical investigations of KIR checkpoint blockade have been recently extended for various other hematological malignancies and for solid tumors [706]. However, a recent study indicated contraction and functional detuning of KIR2D positive NK cells upon single therapy with Lirilumab in smoldering multiple myeloma patients [707]. In addition, constant KIR engagement bears the risk of inducing hyporesponsiveness and anergy in NK cells, similar to processes observed in NK cell education [479,480]. Therefore, combination of anti-KIR therapy with other approaches are currently considered, for instance together with anti–CTLA-4 or anti–PD-1 blockade [682,708] or with ADCC inducing therapeutic antibodies [697,698,709].

We here present a novel strategy of transiently and safely releasing NK cells from KIR-mediated self-inhibition to improve autologous NK cells transfer. Transient modulation of

inhibitory KIR receptors may be well suited to improve the anti-tumor efficacy of autologous NK cells in adoptive transfer. Our data describes robust KIR downregulation by IL-12/15/18 on mature NK effector cells, resulting in less KIR-mediated inhibition and increased lysis of cognate HLA-I–expressing targets (Figure 6.15 and Figure 6.18). Accordingly, reduced KIR expression may render autologous NK cells less sensitive to inhibition by self–HLA-I on tumor cells, exploiting the concept of 'missing-self' recognition in HLA-I matched settings. Moreover, the transient nature of KIR regulation on NK cells might provide safe application regarding autoimmunity against healthy tissue, since inhibitory KIR expression is most likely restored after the initial NK cell effector phase in patients. Transient KIR modulation and the recovery of KIR expression might also be beneficial in preventing functional detuning and anergic hyporesponsiveness of transferred NK cells. We envisage that the transient release from KIR-mediated inhibition could combine advantages of safety and alloreactivity in autologous NK cell transfer together with the sustained anti-leukemia effectiveness and memory-like properties that have been recently demonstrated for IL-12/15/18–activated NK cells in a clinical trial [526]. Our study implies that transient modulation of the KIR/HLA-I axis by pro-inflammatory cytokines could be of high functional importance for immunotherapy protocols especially for autologous NK cell infusions.

In general, harnessing the adaptive features of memory-like NK cells is currently considered for application in immunotherapy [23,673,746,747]. Adoptive transfer of allogeneic NK cells pre-activated with IL-12/15/18 has been recently employed in a clinical trial of AML patients and could induce remission in a subset of patients [526]. Moreover, clinical studies have suggested anti-leukemia properties for HCMV-induced adaptive NKG2C$^+$ NK cells [545–547]. Compared to conventional NK cells, FcεRIγ-deficient adaptive NK cells elicited superior ADCC in response to antibody-coated target cells [564]. Furthermore, adaptive NK cells have been shown to exhibit lower TIGIT expression and to be therefore less susceptible to MDSC-mediated inhibition in the tumor bed [748]. Recently, a protocol for successful *ex vivo* expansion of these adaptive NKG2C$^+$ NK cells has been reported, resulting in effector cells with high anti-tumor potential for the application in cancer immunotherapy [549].

We hypothesize that the advantages of their memory-like properties together with the here uncovered reduced self-inhibition of IL-12/15/18–activated NK cells make them suitable targets for adaptive autologous cell transfer. In addition, IL-12/15/18–stimulated NK cells

could be employed in combinatorial strategies of cancer immunotherapy. Thereby, autologous NK cells with memory-like properties and high effector functions against HLA-I–expressing tumors could be harnessed to improve other therapies currently tested in the clinics. Adoptive transfer allows the combination of *ex vivo* activation of NK cells with other treatment options that have been shown to enhance NK cell ani-tumor efficacy, for instance with therapeutic antibodies or immunomodulatory drugs. The concept of combining NK cells released from self-inhibition together with the immunomodulatory drug lenalidomide is currently explored in clinical trials with the pan-KIR2D blocking antibody Lirilumab [698,701,704].

Therapeutic antibodies and antibody-derived constructs can specifically redirect immune cells to tumor-restricted antigens expressed on cancer cells and can elicit potent NK cells effector function through the induction of ADCC [100,101]. The anti-tumor efficacy of therapeutic antibodies or BiKEs (bispecific killer engagers) can be enhanced by administration of cytokines that stimulate and expand NK cells *in vivo* [109,673,684]. However, *in vivo* administration of cytokines might involve toxic adverse effects and expansion of regulatory T cells [641,643–645]. *Ex vivo* cytokine-induced memory-like NK cells have been shown to potently exert ADCC in response to therapeutic antibodies such as rituximab [512]. line with this, we observed high capacity of CD16-mediated cytotoxicity of NK cells stimulated with IL-12/15/18 in redirected lysis assays (Figure 6.15). Of note, cytokine stimulation resulted in shedding of CD16 by matrix metalloproteinases (MMP) [711] but MMPi treatment restored CD16 surface expression (Figure 6.14). Sustaining the expression of CD16 and preventing MMP-mediated shedding is currently explored for ADCC protocols in the clinics by administration of broad MMPi or more selective ADAM17 inhibitors [749,750]. Additionally, the reduced KIR expression on IL-12/15/18–activated NK cells observed in our study translated in reduced KIR-mediated inhibition upon KIR co-triggering and upon engagement with cognate HLA-I ligands (Figure 6.15 and Figure 6.18). We therefore envisage that KIR downregulation by IL-12/15/18 might greatly improve ADCC against HLA-I–expressing tumor targets by unleashing NK cells from self-inhibition. Indeed, the combination of therapeutic antibodies together with the anti-KIR blocking antibody Lirilumab have been reported to enhance NK cell-mediated ADCC *in vitro* against multiple myeloma and lymphoma cells [697,698,709]. Autologous transfer of NK cells that are transiently and safely released from KIR-mediated self-inhibition might therefore improve the clinical efficacy of therapeutic or bispecific antibodies in the treatment of HLA-I positive cancers.

Furthermore, adoptive transfer of memory-like NK cells together with antibody therapy could exploit the sustained functionality of long lived effector cells with potent CD16-dependent activation and specific redirection towards tumor cells. Recently, our group has described the implication of CD16 engagement with therapeutic antibodies in priming NK cells with sustained effector functions, resembling memory-like NK cells with adaptive features [99]. Upon a re-culture period in IL-2, CD16-primed NK cells showed extensive proliferation due to high CD25 expression and exhibited enhanced IFN-γ production and cytotoxicity against various tumor targets. CD16-priming might further sustain the anti-tumor functionality of IL-12/15/18–stimulated NK cells, which might result in superior anti-cancer efficacy against HLA-I–expressing tumors. Therefore, adoptive transfer of cytokine-induced NK cells together with the application of therapeutic antibodies or bispecific constructs targeting CD16 might represent a promising strategy for HLA-I matched settings in tumor therapy.

8. BIBLIOGRAPHY

1. Orange, J. S. Natural killer cell deficiency. *J. Allergy Clin. Immunol.* **132,** 515–525 (2013).
2. Murphy, K. & Weaver, C. *Janeway's Immunobiology, 9th edition*. (CRC Press, 2016).
3. Kawai, T. & Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* **11,** 373–384 (2010).
4. Medzhitov, R. Recognition of microorganisms and activation of the immune response. *Nature* **449,** 819–826 (2007).
5. Gasteiger, G. & Rudensky, A. Y. Interactions between innate and adaptive lymphocytes. *Nat. Rev. Immunol.* **14,** 631–639 (2014).
6. Iwasaki, A. & Medzhitov, R. Control of adaptive immunity by the innate immune system. *Nat. Immunol.* **16,** 343–353 (2015).
7. McBlane, J. F. *et al.* Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. *Cell* **83,** 387–395 (1995).
8. Jung, D., Giallourakis, C., Mostoslavsky, R. & Alt, F. W. Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. *Annu. Rev. Immunol.* **24,** 541–70 (2006).
9. Hamon, M. A. & Quintin, J. Innate immune memory in mammals. *Semin. Immunol.* **28,** 351–358 (2016).
10. Lanier, L. L. Shades of grey--the blurring view of innate and adaptive immunity. *Nat. Rev. Immunol.* **13,** 73–4 (2013).
11. Lau, C. M. & Sun, J. C. The widening spectrum of immunological memory. *Curr. Opin. Immunol.* **54,** 42–49 (2018).
12. Nimmerjahn, F. & Ravetch, J. V. Fcγ receptors as regulators of immune responses. *Nat. Rev. Immunol.* **8,** 34–47 (2008).
13. Zhu, J. & Paul, W. E. CD4 T cells: fates, functions, and faults. *Blood* **112,** 1557–69 (2008).
14. Vantourout, P. & Hayday, A. Six-of-the-best: unique contributions of γδ T cells to immunology. *Nat. Rev. Immunol.* **13,** 88–100 (2013).
15. Berzins, S. P. & Ritchie, D. S. Natural killer T cells: drivers or passengers in preventing human disease? *Nat. Rev. Immunol.* **14,** 640–646 (2014).
16. Herberman, R. B., Nunn, M. E. & Lavrin, D. H. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity. *Int. J. Cancer* **16,** 216–229 (1975).
17. Kiessling, R., Klein, E. & Wigzell, H. „Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur. J. Immunol.* **5,** 112–117 (1975).
18. Kiessling, R., Petranyi, G., Klein, G. & Wigzell, H. Genetic variation of in vitro cytolytic activity and in vivo rejection potential of non-immunized semi-syngeneic mice against a mouse lymphoma line. *Int. J. Cancer* **15,** 933–940 (1975).
19. Kärre, K., Ljunggren, H. G., Piontek, G. & Kiessling, R. Selective rejection of H–2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* **319,** 675–678 (1986).
20. Moretta, A., Bottino, C., Mingari, M. C., Biassoni, R. & Moretta, L. What is a natural killer cell? *Nat Immunol* **3,** 6–8. (2002).
21. Kondo, M., Weissman, I. L. & Akashi, K. Identification of Clonogenic Common Lymphoid Progenitors in Mouse Bone Marrow. *Cell* **91,** 661–672 (1997).
22. Karo, J. M., Schatz, D. G. & Sun, J. C. The RAG recombinase dictates functional heterogeneity and cellular fitness in natural killer cells. *Cell* **159,** 94–107 (2014).
23. Cerwenka, A. & Lanier, L. L. Natural killer cell memory in infection, inflammation and cancer. *Nat. Rev. Immunol.* **16,** 112–123 (2016).
24. Geary, C. D. & Sun, J. C. Memory responses of natural killer cells. *Semin. Immunol.* **31,** 11–19 (2017).
25. Holmes, T. D. & Bryceson, Y. T. Natural killer cell memory in context. *Semin. Immunol.* **28,** 368–376 (2016).
26. Caligiuri, M. A. Human natural killer cells. *Blood* **112,** 461–9 (2008).

27. Vivier, E., Tomasello, E., Baratin, M., Walzer, T. & Ugolini, S. Functions of natural killer cells. *Nat. Immunol.* **9,** 503–10 (2008).
28. Colucci, F., Caligiuri, M. A. & Di Santo, J. P. What does it take to make a natural killer? *Nat. Rev. Immunol.* **3,** 413–425 (2003).
29. Raulet, D. H. Interplay of natural killer cells and their receptors with the adaptive immune response. *Nat. Immunol.* **5,** 996–1002 (2004).
30. Moffett-King, A. Natural killer cells and pregnancy. *Nat. Rev. Immunol.* **2,** 656–663 (2002).
31. Yu, Y. Y. L., Kumar, V. & Bennett, M. Murine Natural Killer Cells and Marrow Graft Rejection. *Annu. Rev. Immunol.* **10,** 189–213 (1992).
32. Cerwenka, A. & Lanier, L. L. Natural killer cells, viruses and cancer. *Nat. Rev. Immunol.* **1,** 41–49 (2001).
33. Lanier, L. L. Evolutionary struggles between NK cells and viruses. *Nat. Rev. Immunol.* **8,** 259–68 (2008).
34. Malmberg, K.-J. *et al.* Natural killer cell-mediated immunosurveillance of human cancer. *Semin. Immunol.* **31,** 20–29 (2017).
35. McKenzie, A. N. J., Spits, H. & Eberl, G. Innate Lymphoid Cells in Inflammation and Immunity. *Immunity* **41,** 366–374 (2014).
36. Spits, H. & Di Santo, J. P. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. *Nat. Immunol.* **12,** 21–27 (2011).
37. Bernink, J. H., Mjösberg, J. & Spits, H. Human ILC1: To Be or Not to Be. *Immunity* **46,** 756–757 (2017).
38. Artis, D. & Spits, H. The biology of innate lymphoid cells. *Nature* **517,** 293–301 (2015).
39. Spits, H. *et al.* Innate lymphoid cells--a proposal for uniform nomenclature. *Nat. Rev. Immunol.* **13,** 145–9 (2013).
40. Lanier, L. L., Testi, R., Bindl, J. & Phillips, J. H. Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. *J. Exp. Med.* **169,** 2233–8 (1989).
41. Sivori, S. *et al.* p46, a novel natural killer cell-specific surface molecule that mediates cell activation. *J. Exp. Med.* **186,** 1129–36 (1997).
42. Walzer, T., Jaeger, S., Chaix, J. & Vivier, E. Natural killer cells: from CD3−NKp46+ to post-genomics meta-analyses. *Curr. Opin. Immunol.* **19,** 365–372 (2007).
43. Walzer, T. *et al.* Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. *Proc. Natl. Acad. Sci. U. S. A.* **104,** 3384–9 (2007).
44. Björkström, N. K., Ljunggren, H.-G. & Sandberg, J. K. CD56 negative NK cells: origin, function, and role in chronic viral disease. *Trends Immunol.* **31,** 401–406 (2010).
45. Narni-Mancinelli, E. *et al.* Fate mapping analysis of lymphoid cells expressing the NKp46 cell surface receptor. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 18324–9 (2011).
46. Freud, A. G. G. *et al.* NKp80 Defines a Critical Step during Human Natural Killer Cell Development. *Cell Rep.* **16,** 379–391 (2016).
47. Vitale, M. *et al.* Identification of NKp80, a novel triggering molecule expressed by human NK cells. *Eur. J. Immunol.* **31,** 233–242 (2001).
48. Freud, A. G. *et al.* Human natural killer cell development. *Immunol. Rev.* **214,** 56–72 (2006).
49. Di Santo, J. P. NATURAL KILLER CELL DEVELOPMENTAL PATHWAYS: A Question of Balance. *Annu. Rev. Immunol.* **24,** 257–286 (2006).
50. Yu, J., Freud, A. G. & Caligiuri, M. A. Location and cellular stages of natural killer cell development. *Trends Immunol.* **34,** 573–82 (2013).
51. Freud, A. G., Yu, J. & Caligiuri, M. A. Human natural killer cell development in secondary lymphoid tissues. *Semin. Immunol.* **26,** 132–137 (2014).
52. Freud, A. G. *et al.* Evidence for discrete stages of human natural killer cell differentiation in vivo. *J. Exp. Med.* **203,** 1033–1043 (2006).
53. Huntington, N. D., Vosshenrich, C. A. J. J. & Di Santo, J. P. Developmental pathways that generate natural-killer-cell diversity in mice and humans. *Nat. Rev. Immunol.* **7,** 703–714 (2007).
54. Freud, A. G. *et al.* A Human CD34(+) Subset Resides in Lymph Nodes and Differentiates into CD56brightNatural Killer Cells. *Immunity* **22,** 295–304 (2005).
55. Mrozek, E. *et al.* Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells. *Blood* **87,** 2632–2640 (1996).
56. Becknell, B. & Caligiuri, M. A. Interleukin-2, interleukin-15, and their roles in human natural killer cells. *Adv. Immunol.* **86,** 209–239 (2005).
57. Cooper, M. A. *et al.* In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. *Blood* **100,** 3633–3638 (2002).

58. Huntington, N. D. *et al.* Interleukin 15-mediated survival of natural killer cells is determined by interactions among Bim, Noxa and Mcl-1. *Nat. Immunol.* **8,** 856–863 (2007).
59. Gascoyne, D. M. *et al.* The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development. *Nat. Immunol.* **10,** 1118–1124 (2009).
60. Aliahmad, P., de la Torre, B. & Kaye, J. Shared dependence on the DNA-binding factor TOX for the development of lymphoid tissue–inducer cell and NK cell lineages. *Nat. Immunol.* **11,** 945–952 (2010).
61. Kamizono, S. *et al.* Nfil3/E4bp4 is required for the development and maturation of NK cells in vivo. *J. Exp. Med.* **206,** 2977–2986 (2009).
62. Gordon, S. M. M. *et al.* The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation. *Immunity* **36,** 55–67 (2012).
63. Serafini, N., Vosshenrich, C. A. J. & Di Santo, J. P. Transcriptional regulation of innate lymphoid cell fate. *Nat. Rev. Immunol.* **15,** 415–428 (2015).
64. Bjorkstrom, N. K. *et al.* Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. *Blood* **116,** 3853–3864 (2010).
65. Béziat, V., Descours, B., Parizot, C., Debré, P. & Vieillard, V. NK Cell Terminal Differentiation: Correlated Stepwise Decrease of NKG2A and Acquisition of KIRs. *PLoS One* **5,** e11966 (2010).
66. Romagnani, C. *et al.* CD56brightCD16- Killer Ig-Like Receptor- NK Cells Display Longer Telomeres and Acquire Features of CD56dim NK Cells upon Activation. *J. Immunol.* **178,** 4947–4955 (2007).
67. Grzywacz, B. *et al.* Coordinated acquisition of inhibitory and activating receptors and functional properties by developing human natural killer cells. *Blood* **108,** 3824–3833 (2006).
68. Cooper, M. A., Fehniger, T. A. & Caligiuri, M. A. The biology of human natural killer-cell subsets. *Trends Immunol.* **22,** 633–640 (2001).
69. Freud, A. G., Mundy-Bosse, B. L., Yu, J. & Caligiuri, M. A. The Broad Spectrum of Human Natural Killer Cell Diversity. *Immunity* **47,** 820–833 (2017).
70. Cichocki, F. *et al.* in *Natural Killer Cells. Current Topics in Microbiology and Immunology* (eds. Vivier, E., Di Santo, J. & Moretta, A.) 63–93 (Springer International Publishing, 2015). doi:10.1007/82_2015_487
71. Fehniger, T. A. *et al.* CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. *Blood* **101,** 3052–7 (2003).
72. Cooper, M. A. *et al.* Human natural killer cells: A unique innate immunoregulatory role for the CD56brightsubset. *Blood* **97,** 3146–3151 (2001).
73. Jacobs, R. *et al.* CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. *Eur. J. Immunol.* **31,** 3121–3126 (2001).
74. Fehniger, T. A. *et al.* Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J. Immunol.* **162,** 4511–4520 (1999).
75. Fauriat, C., Long, E. O., Ljunggren, H.-G. G. & Bryceson, Y. T. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood* **115,** 2167–76 (2010).
76. Chan, A. *et al.* CD56bright human NK cells differentiate into CD56dim cells: role of contact with peripheral fibroblasts. *J. Immunol.* **179,** 89–94 (2007).
77. Huntington, N. D. *et al.* IL-15 trans-presentation promotes human NK cell development and differentiation in vivo. *J. Exp. Med.* **206,** 25–34 (2009).
78. Vago, L. *et al.* Temporal, quantitative, and functional characteristics of single-KIR-positive alloreactive natural killer cell recovery account for impaired graft-versus-leukemia activity after haploidentical hematopoietic stem cell transplantation. *Blood* **112,** 3488–99 (2008).
79. Shilling, H. G. Reconstitution of NK cell receptor repertoire following HLA-matched hematopoietic cell transplantation. *Blood* **101,** 3730–3740 (2003).
80. Jacobs, R. *et al.* CD16- CD56+ natural killer cells after bone marrow transplantation. *Blood* **79,** 3239–44 (1992).
81. Yu, J. *et al.* CD94 surface density identifies a functional intermediary between the CD56bright and CD56dim human NK-cell subsets. *Blood* **115,** 274–281 (2010).
82. Lopez-Vergès, S. *et al.* CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. *Blood* **116,** 3865–3874 (2010).
83. Long, E. O. *et al.* Controlling Natural Killer Cell Responses: Integration of Signals for Activation and Inhibition. *Annu. Rev. Immunol.* **31,** 227–258 (2013).
84. Bryceson, Y. T., March, M. E., Ljunggren, H.-G. & Long, E. O. Activation, coactivation, and costimulation of resting human natural killer cells. *Immunol. Rev.* **214,** 73–91 (2006).

85. Bryceson, Y. T. & Long, E. O. Line of attack: NK cell specificity and integration of signals. *Curr. Opin. Immunol.* **20,** 344–352 (2008).
86. Smyth, M. J. *et al.* Activation of NK cell cytotoxicity. *Mol. Immunol.* **42,** 501–510 (2005).
87. Wallin, R. P. A., Screpanti, V., Michaëlsson, J., Grandien, A. & Ljunggren, H.-G. Regulation of perforin-independent NK cell-mediated cytotoxicity. *Eur. J. Immunol.* **33,** 2727–2735 (2003).
88. Berke, G. The CTL's kiss of death. *Cell* **81,** 9–12 (1995).
89. Guicciardi, M. E. & Gores, G. J. Life and death by death receptors. *FASEB J.* **23,** 1625–1637 (2009).
90. Walczak, H. Death receptor-ligand systems in cancer, cell death, and inflammation. *Cold Spring Harb. Perspect. Biol.* **5,** a008698 (2013).
91. Voskoboinik, I., Whisstock, J. C. & Trapani, J. A. Perforin and granzymes: function, dysfunction and human pathology. *Nat. Rev. Immunol.* **15,** 388–400 (2015).
92. Orange, J. S. Formation and function of the lytic NK-cell immunological synapse. *Nat. Rev. Immunol.* **8,** 713–25 (2008).
93. Krzewski, K. & Coligan, J. E. Human NK cell lytic granules and regulation of their exocytosis. *Front. Immunol.* **3,** 335 (2012).
94. Lieberman, J. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat. Rev. Immunol.* **3,** 361–370 (2003).
95. Ewen, C. L., Kane, K. P. & Bleackley, R. C. A quarter century of granzymes. *Cell Death Differ.* **19,** 28–35 (2012).
96. Bryceson, Y. T. *et al.* Molecular Mechanisms of Natural Killer Cell Activation. *J. Innate Immun.* **3,** 216–226 (2011).
97. Bryceson, Y. T., March, M. E., Barber, D. F., Ljunggren, H.-G. & Long, E. O. Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. *J. Exp. Med.* **202,** 1001–1012 (2005).
98. Bryceson, Y. T., March, M. E., Ljunggren, H.-G. & Long, E. O. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* **107,** 159–66 (2006).
99. Pahl, J. H. W. *et al.* CD16A Activation of NK Cells Promotes NK Cell Proliferation and Memory-Like Cytotoxicity against Cancer Cells. *Cancer Immunol. Res.* **6,** 517–527 (2018).
100. Terme, M., Ullrich, E., Delahaye, N. F., Chaput, N. & Zitvogel, L. Natural killer cell-directed therapies: moving from unexpected results to successful strategies. *Nat. Immunol.* **9,** 486–94 (2008).
101. Ochoa, M. C. *et al.* Antibody-dependent cell cytotoxicity: immunotherapy strategies enhancing effector NK cells. *Immunol. Cell Biol.* **95,** 347–355 (2017).
102. Dunn, G. P., Koebel, C. M. & Schreiber, R. D. Interferons, immunity and cancer immunoediting. *Nat. Rev. Immunol.* **6,** 836–848 (2006).
103. Braumüller, H. *et al.* T-helper-1-cell cytokines drive cancer into senescence. *Nature* **494,** 361–365 (2013).
104. Schroder, K., Hertzog, P. J., Ravasi, T. & Hume, D. A. Interferon-γ: an overview of signals, mechanisms and functions. *J. Leukoc. Biol.* **75,** 163–189 (2004).
105. Reefman, E. *et al.* Cytokine secretion is distinct from secretion of cytotoxic granules in NK cells. *J. Immunol.* **184,** 4852–62 (2010).
106. Lanier, L. L. Up on the tightrope: natural killer cell activation and inhibition. *Nat. Immunol.* **9,** 495–502 (2008).
107. Schuster, I. S., Coudert, J. D., Andoniou, C. E. & Degli-Esposti, M. A. "Natural Regulators": NK Cells as Modulators of T Cell Immunity. *Front. Immunol.* **7,** 235 (2016).
108. Crouse, J., Xu, H. C., Lang, P. A. & Oxenius, A. NK cells regulating T cell responses: mechanisms and outcome. *Trends Immunol.* **36,** 49–58 (2015).
109. Romee, R. *et al.* Utilizing cytokines to function-enable human NK cells for the immunotherapy of cancer. *Sci.* **2014,** 205796 (2014).
110. Long, E. O. Regulation of immune responses through inhibitory receptors. *Annu. Rev. Immunol.* **17,** 875–904 (1999).
111. Lanier, L. L. NK cell recognition. *Annu. Rev. Immunol.* **23,** 225–74 (2005).
112. Moretta, A. *et al.* Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu. Rev. Immunol.* **19,** 197–223 (2001).
113. Raulet, D. H. & Guerra, N. Oncogenic stress sensed by the immune system: role of natural killer cell receptors. *Nat. Rev. Immunol.* **9,** 568–80 (2009).
114. Cerwenka, A. & Lanier, L. L. Ligands for natural killer cell receptors: redundancy or specificity. *Immunol. Rev.* **181,** 158–169 (2001).

115. Vivier, E. Natural Killer Cell Signaling Pathways. *Science (80-.).* **306,** 1517–1519 (2004).
116. Collins, K. L. & Baltimore, D. HIV's evasion of the cellular immune response. *Immunol. Rev.* **168,** 65–74 (1999).
117. Ljunggren, H. G. & Kärre, K. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. *J. Exp. Med.* **162,** 1745–59 (1985).
118. Ljunggren, H.-G. & Kärre, K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today* **11,** 237–244 (1990).
119. Bix, M. *et al.* Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice. *Nature* **349,** 329–331 (1991).
120. Raulet, D. H. Missing self recognition and self tolerance of natural killer (NK) cells. *Semin. Immunol.* **18,** 145–50 (2006).
121. Moretta, L. *et al.* Different checkpoints in human NK-cell activation. *Trends Immunol.* **25,** 670–6 (2004).
122. Cerwenka, A., Baron, J. L. & Lanier, L. L. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc. Natl. Acad. Sci.* **98,** 11521–11526 (2001).
123. Diefenbach, A., Jensen, E. R., Jamieson, A. M. & Raulet, D. H. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature* **413,** 165–71 (2001).
124. Arase, H., Mocarski, E. S., Campbell, A. E., Hill, A. B. & Lanier, L. L. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science* **296,** 1323–6 (2002).
125. Vitale, M., Cantoni, C., Pietra, G., Mingari, M. C. & Moretta, L. Effect of tumor cells and tumor microenvironment on NK-cell function. *Eur. J. Immunol.* **44,** 1582–1592 (2014).
126. De Maria, A. *et al.* The impaired NK cell cytolytic function in viremic HIV-1 infection is associated with a reduced surface expression of natural cytotoxicity receptors (NKp46, NKp30 and NKp44). *Eur. J. Immunol.* **33,** 2410–2418 (2003).
127. Pietra, G. *et al.* Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity. *Cancer Res.* **72,** 1407–15 (2012).
128. Watzl, C. & Long, E. O. in *Current Protocols in Immunology* **90:11.9B,** 11.9B.1-11.9B.17 (John Wiley & Sons, Inc., 2010).
129. Vitale, M. *et al.* NKp44, a Novel Triggering Surface Molecule Specifically Expressed by Activated Natural Killer Cells, Is Involved in Non–Major Histocompatibility Complex–restricted Tumor Cell Lysis. *J. Exp. Med.* **187,** 2065–2072 (1998).
130. Pende, D. *et al.* Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *J. Exp. Med.* **190,** 1505–16 (1999).
131. Kruse, P. H., Matta, J., Ugolini, S. & Vivier, E. Natural cytotoxicity receptors and their ligands. *Immunol. Cell Biol.* **92,** 221–229 (2014).
132. Pessino, A. *et al.* Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. *J. Exp. Med.* **188,** 953–60 (1998).
133. Cantoni, C. *et al.* NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily. *J Exp Med* **189,** 787–96 (1999).
134. Lanier, L. L. DAP10- and DAP12-associated receptors in innate immunity. *Immunol. Rev.* **227,** 150–60 (2009).
135. Lanier, L. L., Corliss, B. C., Wu, J., Leong, C. & Phillips, J. H. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature* **391,** 703–707 (1998).
136. Mandelboim, O. *et al.* Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* **409,** 1055–1060 (2001).
137. Mavoungou, E., Held, J., Mewono, L. & Kremsner, P. G. A Duffy Binding–Like Domain Is Involved in the NKp30-Mediated Recognition of *Plasmodium falciparum* –Parasitized Erythrocytes by Natural Killer Cells. *J. Infect. Dis.* **195,** 1521–1531 (2007).
138. Arnon, T. I. *et al.* Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus. *Nat. Immunol.* **6,** 515–523 (2005).
139. Bloushtain, N. *et al.* Membrane-associated heparan sulfate proteoglycans are involved in the recognition of cellular targets by NKp30 and NKp46. *J. Immunol.* **173,** 2392–401 (2004).
140. Hecht, M.-L. *et al.* Natural Cytotoxicity Receptors NKp30, NKp44 and NKp46 Bind to Different Heparan Sulfate/Heparin Sequences. *J. Proteome Res.* **8,** 712–720 (2009).
141. Hershkovitz, O. *et al.* Altered glycosylation of recombinant NKp30 hampers binding to heparan sulfate: a lesson for the use of recombinant immunoreceptors as an immunological tool.

Glycobiology **18,** 28–41 (2008).
142. Li, S. S. *et al.* Identification of the fungal ligand triggering cytotoxic PRR-mediated NK cell killing of Cryptococcus and Candida. *Nat. Commun.* **9,** 751 (2018).
143. Narni-Mancinelli, E. *et al.* Complement factor P is a ligand for the natural killer cell–activating receptor NKp46. *Sci. Immunol* **2,** (2017).
144. Lakshmikanth, T. *et al.* NCRs and DNAM-1 mediate NK cell recognition and lysis of human and mouse melanoma cell lines in vitro and in vivo. *J. Clin. Invest.* **119,** 1251–63 (2009).
145. Glasner, A. *et al.* Recognition and prevention of tumor metastasis by the NK receptor NKp46/NCR1. *J. Immunol.* **188,** 2509–15 (2012).
146. Glasner, A. *et al.* Increased NK cell immunity in a transgenic mouse model of NKp46 overexpression. *Sci. Rep.* **7,** 13090 (2017).
147. Glasner, A. *et al.* NKp46 Receptor-Mediated Interferon-γ Production by Natural Killer Cells Increases Fibronectin 1 to Alter Tumor Architecture and Control Metastasis. *Immunity* **48,** 107–119.e4 (2018).
148. Brandt, C. S. *et al.* The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. *J. Exp. Med.* **206,** 1495–503 (2009).
149. Pogge von Strandmann, E. *et al.* Human leukocyte antigen-B-associated transcript 3 is released from tumor cells and engages the NKp30 receptor on natural killer cells. *Immunity* **27,** 965–74 (2007).
150. Fiegler, N. *et al.* Downregulation of the activating NKp30 ligand B7-H6 by HDAC inhibitors impairs tumor cell recognition by NK cells. *Blood* **122,** 684–93 (2013).
151. Matta, J. *et al.* Induction of B7-H6, a ligand for the natural killer cell-activating receptor NKp30, in inflammatory conditions. *Blood* **122,** 394–404 (2013).
152. Barrow, A. D. *et al.* Natural Killer Cells Control Tumor Growth by Sensing a Growth Factor. *Cell* **172,** 534–548.e19 (2017).
153. Baychelier, F. *et al.* Identification of a cellular ligand for the natural cytotoxicity receptor NKp44. *Blood* **122,** 2935–2942 (2013).
154. Rosental, B. *et al.* Proliferating cell nuclear antigen is a novel inhibitory ligand for the natural cytotoxicity receptor NKp44. *J. Immunol.* **187,** 5693–702 (2011).
155. Welte, S., Kuttruff, S., Waldhauer, I. & Steinle, A. Mutual activation of natural killer cells and monocytes mediated by NKp80-AICL interaction. *Nat. Immunol.* **7,** 1334–1342 (2006).
156. Klimosch, S. N., Bartel, Y., Wiemann, S. & Steinle, A. Genetically coupled receptor–ligand pair NKp80-AICL enables autonomous control of human NK cell responses. *Blood* **122,** (2013).
157. Borrego, F., Masilamani, M., Marusina, A. I., Tang, X. & Coligan, J. E. The CD94/NKG2 Family of Receptors: From Molecules and Cells to Clinical Relevance. *Immunol. Res.* **35,** 263–278 (2006).
158. Yokoyama, W. M. & Plougastel, B. F. M. Immune functions encoded by the natural killer gene complex. *Nat. Rev. Immunol.* **3,** 304–16 (2003).
159. López-Botet, M., Llano, M., Navarro, F. & Bellon, T. NK cell recognition of non-classical HLA class I molecules. *Semin. Immunol.* **12,** 109–119 (2000).
160. Lazetic, S., Chang, C., Houchins, J. P., Lanier, L. L. & Phillips, J. H. Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. *J. Immunol.* **157,** 4741–5 (1996).
161. Lanier, L. L., Corliss, B., Wu, J. & Phillips, J. H. Association of DAP12 with activating CD94/NKG2C NK cell receptors. *Immunity* **8,** 693–701 (1998).
162. Braud, V. M. *et al.* HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* **391,** 795–799 (1998).
163. Vance, R. E., Jamieson, A. M. & Raulet, D. H. Recognition of the class Ib molecule Qa-1(b) by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells. *J. Exp. Med.* **190,** 1801–12 (1999).
164. Lopez-Vergès, S. *et al.* Expansion of a unique CD57^{+}NKG2Chi natural killer cell subset during acute human cytomegalovirus infection. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 14725–32 (2011).
165. Rölle, A. Deciphering the biology of NKG2C+ Natural Killer Cells. *Oncotarget* **6,** 19930–1 (2015).
166. Della Chiesa, M., Sivori, S., Carlomagno, S., Moretta, L. & Moretta, A. Activating KIRs and NKG2C in Viral Infections: Toward NK Cell Memory? *Front. Immunol.* **6,** 573 (2015).
167. Bauer, S. *et al.* Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* **285,** 727–9 (1999).
168. Jamieson, A. M. *et al.* The role of the NKG2D immunoreceptor in immune cell activation and natural killing. *Immunity* **17,** 19–29 (2002).
169. Wu, J. *et al.* An activating immunoreceptor complex formed by NKG2D and DAP10. *Science* **285,**

730–2 (1999).
170. Wu, J., Cherwinski, H., Spies, T., Phillips, J. H. & Lanier, L. L. DAP10 and DAP12 form distinct, but functionally cooperative, receptor complexes in natural killer cells. *J. Exp. Med.* **192,** 1059–68 (2000).
171. Billadeau, D. D., Upshaw, J. L., Schoon, R. A., Dick, C. J. & Leibson, P. J. NKG2D-DAP10 triggers human NK cell–mediated killing via a Syk-independent regulatory pathway. *Nat. Immunol.* **4,** 557–564 (2003).
172. Diefenbach, A. *et al.* Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. *Nat. Immunol.* **3,** 1142–1149 (2002).
173. Nausch, N. & Cerwenka, A. NKG2D ligands in tumor immunity. *Oncogene* **27,** 5944–58 (2008).
174. Raulet, D. H., Gasser, S., Gowen, B. G., Deng, W. & Jung, H. Regulation of ligands for the NKG2D activating receptor. *Annu. Rev. Immunol.* **31,** 413–41 (2013).
175. Cosman, D. *et al.* ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* **14,** 123–33 (2001).
176. Groh, V. *et al.* Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 6879–84 (1999).
177. Cerwenka, A. *et al.* Retinoic Acid Early Inducible Genes Define a Ligand Family for the Activating NKG2D Receptor in Mice. *Immunity* **12,** 721–727 (2000).
178. Carayannopoulos, L. N., Naidenko, O. V, Fremont, D. H. & Yokoyama, W. M. Cutting edge: murine UL16-binding protein-like transcript 1: a newly described transcript encoding a high-affinity ligand for murine NKG2D. *J. Immunol.* **169,** 4079–83 (2002).
179. Gasser, S., Orsulic, S., Brown, E. J. & Raulet, D. H. The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature* **436,** 1186–1190 (2005).
180. Stern-Ginossar, N. *et al.* Human microRNAs regulate stress-induced immune responses mediated by the receptor NKG2D. *Nat. Immunol.* **9,** 1065–1073 (2008).
181. Textor, S. *et al.* Human NK cells are alerted to induction of p53 in cancer cells by upregulation of the NKG2D ligands ULBP1 and ULBP2. *Cancer Res.* **71,** 5998–6009 (2011).
182. Groh, V. *et al.* Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. *Proc. Natl. Acad. Sci.* **93,** (1996).
183. Groh, V., Wu, J., Yee, C. & Spies, T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature* **419,** 734–8 (2002).
184. Castriconi, R. *et al.* Transforming growth factor beta 1 inhibits expression of NKp30 and NKG2D receptors: consequences for the NK-mediated killing of dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 4120–5 (2003).
185. Lanier, L. L. NKG2D Receptor and Its Ligands in Host Defense. *Cancer Immunol. Res.* **3,** 575–582 (2015).
186. Zhang, J., Basher, F. & Wu, J. D. NKG2D Ligands in Tumor Immunity: Two Sides of a Coin. *Front. Immunol.* **6,** 97 (2015).
187. Ferrari de Andrade, L. *et al.* Antibody-mediated inhibition of MICA and MICB shedding promotes NK cell–driven tumor immunity. *Science (80-.).* **359,** 1537–1542 (2018).
188. Guerra, N. *et al.* NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity* **28,** 571–80 (2008).
189. Pende, D. *et al.* Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. *Cancer Res.* **62,** 6178–86 (2002).
190. Smyth, M. J. *et al.* NKG2D function protects the host from tumor initiation. *J. Exp. Med.* **202,** 583–8 (2005).
191. Bruhns, P. Properties of mouse and human IgG receptors and their contribution to disease models. *Blood* **119,** 5640–9 (2012).
192. Bonnema, J. D., Karnitz, L. M., Schoon, R. A., Abraham, R. T. & Leibson, P. J. Fc receptor stimulation of phosphatidylinositol 3-kinase in natural killer cells is associated with protein kinase C-independent granule release and cell-mediated cytotoxicity. *J. Exp. Med.* **180,** 1427–35 (1994).
193. Perussia, B., Starr, S., Abraham, S., Fanning, V. & Trinchieri, G. Human natural killer cells analyzed by B73.1, a monoclonal antibody blocking Fc receptor functions. I. Characterization of the lymphocyte subset reactive with B73.1. *J. Immunol.* **130,** 2133–2141 (1983).
194. Perussia, B. *et al.* The Fc receptor for IgG on human natural killer cells: phenotypic, functional, and comparative studies with monoclonal antibodies. *J. Immunol.* **133,** 180–9 (1984).
195. Shibuya, K. *et al.* Physical and Functional Association of LFA-1 with DNAM-1 Adhesion Molecule. *Immunity* **11,** 615–623 (1999).

196. Shibuya, A., Lanier, L. L. & Phillips, J. H. Protein Kinase C Is Involved in the Regulation of Both Signaling and Adhesion Mediated by DNAX Accessory Molecule-1 Receptor. *J. Immunol.* **161,** (1998).
197. Shibuya, A. *et al.* DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes. *Immunity* **4,** 573–81 (1996).
198. Seth, S. *et al.* Heterogeneous expression of the adhesion receptor CD226 on murine NK and T cells and its function in NK-mediated killing of immature dendritic cells. *J. Leukoc. Biol.* **86,** 91–101 (2009).
199. Martinet, L. & Smyth, M. J. Balancing natural killer cell activation through paired receptors. *Nat. Rev. Immunol.* **15,** 243–254 (2015).
200. Boles, K. S. *et al.* A novel molecular interaction for the adhesion of follicular CD4 T cells to follicular DC. *Eur. J. Immunol.* **39,** 695–703 (2009).
201. Mendelsohn, C. L., Wimmer, E. & Racaniello, V. R. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. *Cell* **56,** 855–65 (1989).
202. Bottino, C. *et al.* Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *J. Exp. Med.* **198,** 557–67 (2003).
203. Chan, C. J. *et al.* The receptors CD96 and CD226 oppose each other in the regulation of natural killer cell functions. *Nat. Immunol.* **15,** 431–438 (2014).
204. Soriani, A. *et al.* ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype. *Blood* **113,** 3503–3511 (2009).
205. Iguchi-Manaka, A. *et al.* Accelerated tumor growth in mice deficient in DNAM-1 receptor. *J. Exp. Med.* **205,** 2959–64 (2008).
206. Reymond, N. *et al.* DNAM-1 and PVR regulate monocyte migration through endothelial junctions. *J. Exp. Med.* **199,** 1331–41 (2004).
207. Locksley, R. M., Killeen, N. & Lenardo, M. J. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104,** 487–501 (2001).
208. Ware, C. F. The TNF receptor super family in immune regulation. *Immunol. Rev.* **244,** 5–8 (2011).
209. Aggarwal, B. B. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat. Rev. Immunol.* **3,** 745–756 (2003).
210. Chen, G. & Goeddel, D. V. TNF-R1 signaling: a beautiful pathway. *Science* **296,** 1634–5 (2002).
211. Lin, W. *et al.* Fc-dependent expression of CD137 on human NK cells: insights into "agonistic" effects of anti-CD137 monoclonal antibodies. *Blood* **112,** 699–707 (2008).
212. Vujanovic, N. L. Role of TNF superfamily ligands in innate immunity. *Immunol. Res.* **50,** 159–174 (2011).
213. Watts, T. H. Tnf/Tnfr Family Members in Costimulation of T Cell Responses. *Annu. Rev. Immunol.* **23,** 23–68 (2005).
214. Barao, I. The TNF receptor-ligands 4-1BB-4-1BBL and GITR-GITRL in NK cell responses. *Front. Immunol.* **3,** 402 (2013).
215. Ward-Kavanagh, L. K., Lin, W. W., Šedý, J. R. & Ware, C. F. The TNF Receptor Superfamily in Co-stimulating and Co-inhibitory Responses. *Immunity* **44,** 1005–1019 (2016).
216. Croft, M. The role of TNF superfamily members in T-cell function and diseases. *Nat. Rev. Immunol.* **9,** 271–285 (2009).
217. Croft, M. Control of Immunity by the TNFR-Related Molecule OX40 (CD134). *Annu. Rev. Immunol.* **28,** 57–78 (2010).
218. Zingoni, A. *et al.* Cross-talk between activated human NK cells and CD4+ T cells via OX40-OX40 ligand interactions. *J. Immunol.* **173,** 3716–24 (2004).
219. Kim, M.-Y. *et al.* CD4(+)CD3(-) accessory cells costimulate primed CD4 T cells through OX40 and CD30 at sites where T cells collaborate with B cells. *Immunity* **18,** 643–54 (2003).
220. Wang, C., Lin, G. H. Y., McPherson, A. J. & Watts, T. H. Immune regulation by 4-1BB and 4-1BBL: complexities and challenges. *Immunol. Rev.* **229,** 192–215 (2009).
221. Croft, M. *et al.* TNF superfamily in inflammatory disease: Translating basic insights. *Trends Immunol.* **33,** 144–152 (2012).
222. Sanmamed, M. F. *et al.* Agonists of Co-stimulation in Cancer Immunotherapy Directed Against CD137, OX40, GITR, CD27, CD28, and ICOS. *Semin. Oncol.* **42,** 640–55 (2015).
223. Chester, C., Sanmamed, M. F., Wang, J. & Melero, I. Immunotherapy targeting 4-1BB: mechanistic rationale, clinical results, and future strategies. *Blood* **131,** 49–57 (2018).
224. Buqué, A. *et al.* Trial Watch: Immunomodulatory monoclonal antibodies for oncological

indications. *Oncoimmunology* **4,** e1008814 (2015).
225. Buchan, S. L., Rogel, A. & Al-Shamkhani, A. The immunobiology of CD27 and OX40 and their potential as targets for cancer immunotherapy. *Blood* **131,** 39–48 (2018).
226. Vinay, D. S. & Kwon, B. S. Immunotherapy of cancer with 4-1BB. *Mol. Cancer Ther.* **11,** 1062–70 (2012).
227. Ravetch, J. V & Lanier, L. L. Immune inhibitory receptors. *Science* **290,** 84–9 (2000).
228. Natarajan, K., Dimasi, N., Wang, J., Mariuzza, R. A. & Margulies, D. H. Structure and function of natural killer cell receptors: multiple molecular solutions to self, nonself discrimination. *Annu. Rev. Immunol.* **20,** 853–85 (2002).
229. Valés-Gómez, M., Reyburn, H. T., Erskine, R. A., López-Botet, M. & Strominger, J. L. Kinetics and peptide dependency of the binding of the inhibitory NK receptor CD94/NKG2-A and the activating receptor CD94/NKG2-C to HLA-E. *EMBO J.* **18,** 4250–60 (1999).
230. Valés-Gómez, M., Reyburn, H. T., Erskine, R. A. & Strominger, J. Differential binding to HLA-C of p50-activating and p58-inhibitory natural killer cell receptors. *Proc. Natl. Acad. Sci.* **95,** 14326–14331 (1998).
231. Valés-Gómez, M., Reyburn, H. T., Mandelboim, M. & Strominger, J. L. Kinetics of interaction of HLA-C ligands with natural killer cell inhibitory receptors. *Immunity* **9,** 337–344 (1998).
232. Levi-Schaffer, F. & Mandelboim, O. Inhibitory and Coactivating Receptors Recognising the Same Ligand: Immune Homeostasis Exploited by Pathogens and Tumours. *Trends Immunol.* **39,** 112–122 (2018).
233. Long, E. O. *et al.* Inhibition of natural killer cell activation signals by killer cell immunoglobulin-like receptors (CD158). *Immunol. Rev.* **181,** 223–233 (2001).
234. Stebbins, C. C. *et al.* Vav1 Dephosphorylation by the Tyrosine Phosphatase SHP-1 as a Mechanism for Inhibition of Cellular Cytotoxicity. *Mol. Cell. Biol.* **23,** 6291–6299 (2003).
235. Peterson, M. E. & Long, E. O. Inhibitory Receptor Signaling via Tyrosine Phosphorylation of the Adaptor Crk. *Immunity* **29,** 578–588 (2008).
236. Mesecke, S., Urlaub, D., Busch, H., Eils, R. & Watzl, C. Integration of activating and inhibitory receptor signaling by regulated phosphorylation of Vav1 in immune cells. *Sci. Signal.* **4,** ra36 (2011).
237. Burshtyn, D. N. *et al.* Recruitment of tyrosine phosphatase HCP by the killer cell inhibitory receptor. *Immunity* **4,** 77–85 (1996).
238. Binstadt, B. A. *et al.* Sequential Involvement of Lck and SHP-1 with MHC-Recognizing Receptors on NK Cells Inhibits FcR-Initiated Tyrosine Kinase Activation. *Immunity* **5,** 629–638 (1996).
239. Aldrich, C. J. *et al.* Identification of a Tap-dependent leader peptide recognized by alloreactive T cells specific for a class Ib antigen. *Cell* **79,** 649–58 (1994).
240. Brooks, A. G., Posch, P. E., Scorzelli, C. J., Borrego, F. & Coligan, J. E. NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. *J. Exp. Med.* **185,** 795–800 (1997).
241. Carretero, M. *et al.* Specific engagement of the CD94/NKG2-A killer inhibitory receptor by the HLA-E class Ib molecule induces SHP-1 phosphatase recruitment to tyrosine-phosphorylated NKG2-A: evidence for receptor function in heterologous transfectants. *Eur. J. Immunol.* **28,** 1280–1291 (1998).
242. Le Dréan, E. *et al.* Inhibition of antigen-induced T cell response and antibody-induced NK cell cytotoxicity by NKG2A: association of NKG2A with SHP-1 and SHP-2 protein-tyrosine phosphatases. *Eur. J. Immunol.* **28,** 264–276 (1998).
243. Long, E. O. Negative signaling by inhibitory receptors: the NK cell paradigm. *Immunol. Rev.* **224,** 70–84 (2008).
244. Lee, N., Goodlett, D. R., Ishitani, A., Marquardt, H. & Geraghty, D. E. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J. Immunol.* **160,** 4951–60 (1998).
245. Borrego, F., Ulbrecht, M., Weiss, E. H., Coligan, J. E. & Brooks, A. G. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J. Exp. Med.* **187,** 813–8 (1998).
246. Braud, V., Yvonne Jones, E. & McMichael, A. The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. *Eur. J. Immunol.* **27,** 1164–1169 (1997).
247. Levin, S. D. *et al.* Vstm3 is a member of the CD28 family and an important modulator of T-cell function. *Eur. J. Immunol.* **41,** 902–915 (2011).
248. Yu, X. *et al.* The surface protein TIGIT suppresses T cell activation by promoting the generation of

mature immunoregulatory dendritic cells. *Nat. Immunol.* **10,** 48–57 (2009).
249. Stanietsky, N. *et al.* The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 17858–63 (2009).
250. Wu, H. *et al.* Follicular regulatory T cells repress cytokine production by follicular helper T cells and optimize IgG responses in mice. *Eur. J. Immunol.* **46,** 1152–1161 (2016).
251. Joller, N. *et al.* Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. *J. Immunol.* **186,** 1338–42 (2011).
252. Joller, N. *et al.* Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. *Immunity* **40,** 569–81 (2014).
253. Stanietsky, N. *et al.* Mouse TIGIT inhibits NK-cell cytotoxicity upon interaction with PVR. *Eur. J. Immunol.* **43,** 2138–2150 (2013).
254. Li, M. *et al.* T-cell immunoglobulin and ITIM domain (TIGIT) receptor/poliovirus receptor (PVR) ligand engagement suppresses interferon-γ production of natural killer cells via β-arrestin 2-mediated negative signaling. *J. Biol. Chem.* **289,** 17647–57 (2014).
255. Liu, S. *et al.* Recruitment of Grb2 and SHIP1 by the ITT-like motif of TIGIT suppresses granule polarization and cytotoxicity of NK cells. *Cell Death Differ.* **20,** 456–464 (2013).
256. Masson, D. *et al.* Overexpression of the CD155 gene in human colorectal carcinoma. *Gut* **49,** 236–40 (2001).
257. Chauvin, J.-M. *et al.* TIGIT and PD-1 impair tumor antigen–specific CD8+ T cells in melanoma patients. *J. Clin. Invest.* **125,** 2046–2058 (2015).
258. Dougall, W. C., Kurtulus, S., Smyth, M. J. & Anderson, A. C. TIGIT and CD96: new checkpoint receptor targets for cancer immunotherapy. *Immunol. Rev.* **276,** 112–120 (2017).
259. Anderson, A. C., Joller, N. & Kuchroo, V. K. Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation. *Immunity* **44,** 989–1004 (2016).
260. Johnston, R. J. *et al.* The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function. *Cancer Cell* **26,** 923–937 (2014).
261. Stengel, K. F. *et al.* Structure of TIGIT immunoreceptor bound to poliovirus receptor reveals a cell-cell adhesion and signaling mechanism that requires cis-trans receptor clustering. *Proc. Natl. Acad. Sci. U. S. A.* **109,** 5399–404 (2012).
262. Kurtulus, S. *et al.* TIGIT predominantly regulates the immune response via regulatory T cells. *J. Clin. Invest.* **125,** 4053–4062 (2015).
263. Blake, S. J. *et al.* Suppression of Metastases Using a New Lymphocyte Checkpoint Target for Cancer Immunotherapy. *Cancer Discov.* **6,** 446–59 (2016).
264. Chan, C. J., Andrews, D. M. & Smyth, M. J. Receptors that interact with nectin and nectin-like proteins in the immunosurveillance and immunotherapy of cancer. *Curr. Opin. Immunol.* **24,** 246–251 (2012).
265. Chan, C. J. *et al.* DNAM-1/CD155 interactions promote cytokine and NK cell-mediated suppression of poorly immunogenic melanoma metastases. *J. Immunol.* **184,** 902–11 (2010).
266. Gilfillan, S. *et al.* DNAM-1 promotes activation of cytotoxic lymphocytes by nonprofessional antigen-presenting cells and tumors. *J. Exp. Med.* **205,** 2965–73 (2008).
267. Sarhan, D. *et al.* Adaptive NK Cells with Low TIGIT Expression Are Inherently Resistant to Myeloid-Derived Suppressor Cells. *Cancer Res.* **76,** 5696–5706 (2016).
268. Wu, Y., Tian, Z. & Wei, H. Developmental and Functional Control of Natural Killer Cells by Cytokines. *Front. Immunol.* **8,** 930 (2017).
269. Müller, L., Aigner, P. & Stoiber, D. Type I Interferons and Natural Killer Cell Regulation in Cancer. *Front. Immunol.* **8,** 304 (2017).
270. Strengell, M. *et al.* IL-21 in synergy with IL-15 or IL-18 enhances IFN-gamma production in human NK and T cells. *J. Immunol.* **170,** 5464–9 (2003).
271. Nielsen, C. M., Wolf, A.-S., Goodier, M. R. & Riley, E. M. Synergy between Common γ Chain Family Cytokines and IL-18 Potentiates Innate and Adaptive Pathways of NK Cell Activation. *Front. Immunol.* **7,** 101 (2016).
272. Meazza, R., Azzarone, B., Orengo, A. M. & Ferrini, S. Role of Common-Gamma Chain Cytokines in NK Cell Development and Function: Perspectives for Immunotherapy. *J. Biomed. Biotechnol.* **2011,** 1–16 (2011).
273. Rochman, Y., Spolski, R. & Leonard, W. J. New insights into the regulation of T cells by γc family cytokines. *Nat. Rev. Immunol.* **9,** 480–490 (2009).
274. Kovanen, P. E. & Leonard, W. J. Cytokines and immunodeficiency diseases: critical roles of the gammac-dependent cytokines interleukins 2, 4, 7, 9, 15, and 21, and their signaling pathways. *Immunol. Rev.* **202,** 67–83 (2004).

275. Ma, A., Koka, R. & Burkett, P. DIVERSE FUNCTIONS OF IL-2, IL-15, AND IL-7 IN LYMPHOID HOMEOSTASIS. *Annu. Rev. Immunol.* **24,** 657–679 (2006).
276. Takeshita, T. *et al.* Cloning of the gamma chain of the human IL-2 receptor. *Science (80-.).* **257,** 379–382 (1992).
277. Kondo, M. *et al.* Sharing of the interleukin-2 (IL-2) receptor gamma chain between receptors for IL-2 and IL-4. *Science* **262,** 1874–7 (1993).
278. Ghoreschi, K., Laurence, A. & O'Shea, J. J. Janus kinases in immune cell signaling. *Immunol. Rev.* **228,** 273–287 (2009).
279. de Totero, D. *et al.* The opposite effects of IL-15 and IL-21 on CLL B cells correlate with differential activation of the JAK/STAT and ERK1/2 pathways. *Blood* **111,** 517–24 (2008).
280. Giri, J. G. *et al.* Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO J.* **13,** 2822–30 (1994).
281. Grabstein, K. H. *et al.* Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. *Science* **264,** 965–8 (1994).
282. Waldmann, T. A. The shared and contrasting roles of IL2 and IL15 in the life and death of normal and neoplastic lymphocytes: implications for cancer therapy. *Cancer Immunol. Res.* **3,** 219–27 (2015).
283. Waldmann, T. A. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nat. Rev. Immunol.* **6,** 595–601 (2006).
284. Teshigawara, K., Wang, H. M., Kato, K. & Smith, K. A. Interleukin 2 high-affinity receptor expression requires two distinct binding proteins. *J. Exp. Med.* **165,** 223–38 (1987).
285. Dukovich, M. *et al.* A second human interleukin-2 binding protein that may be a component of high-affinity interleukin-2 receptors. *Nature* **327,** 518–522 (1987).
286. Caligiuri, M. A. *et al.* Functional consequences of interleukin 2 receptor expression on resting human lymphocytes. Identification of a novel natural killer cell subset with high affinity receptors. **171,** 1509–26 (1990).
287. Nagler, A., Lanier, L. L. & Phillips, J. H. Constitutive expression of high affinity interleukin 2 receptors on human CD16-natural killer cells in vivo. *J. Exp. Med.* **171,** 1527–33 (1990).
288. Ni, J., Miller, M., Stojanovic, A., Garbi, N. & Cerwenka, A. Sustained effector function of IL-12/15/18–preactivated NK cells against established tumors. *J. Exp. Med.* **209,** 2351–2365 (2012).
289. Leong, J. W. *et al.* Preactivation with IL-12, IL-15, and IL-18 Induces CD25 and a Functional High-Affinity IL-2 Receptor on Human Cytokine-Induced Memory-like Natural Killer Cells. *Biol. Blood Marrow Transplant.* **20,** 463–473 (2014).
290. Lee, S.-H., Fragoso, M. F. & Biron, C. a. Cutting edge: a novel mechanism bridging innate and adaptive immunity: IL-12 induction of CD25 to form high-affinity IL-2 receptors on NK cells. *J. Immunol.* **189,** 2712–6 (2012).
291. Pollmann, J. *et al.* Hepatitis C virus-induced natural killer cell proliferation involves monocyte-derived cells and the OX40/OX40L axis. *J. Hepatol.* **68,** 421–430 (2018).
292. Stonier, S. W. & Schluns, K. S. Trans-presentation: A novel mechanism regulating IL-15 delivery and responses. *Immunol. Lett.* **127,** 85–92 (2010).
293. Giri, J. G. *et al.* Identification and cloning of a novel IL-15 binding protein that is structurally related to the alpha chain of the IL-2 receptor. *EMBO J.* **14,** 3654–63 (1995).
294. Lodolce, J. P. *et al.* IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* **9,** 669–76 (1998).
295. Lucas, M., Schachterle, W., Oberle, K., Aichele, P. & Diefenbach, A. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. *Immunity* **26,** 503–17 (2007).
296. Miyazaki, T. *et al.* Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits. *Science* **266,** 1045–7 (1994).
297. Marçais, A. *et al.* Regulation of Mouse NK Cell Development and Function by Cytokines. *Front. Immunol.* **4,** 450 (2013).
298. Ali, A. K., Nandagopal, N. & Lee, S.-H. IL-15-PI3K-AKT-mTOR: A Critical Pathway in the Life Journey of Natural Killer Cells. *Front. Immunol.* **6,** 355 (2015).
299. Rautela, J. & Huntington, N. D. IL-15 signaling in NK cell cancer immunotherapy. *Curr. Opin. Immunol.* **44,** 1–6 (2017).
300. Nandagopal, N., Ali, A. K., Komal, A. K. & Lee, S.-H. The Critical Role of IL-15â€“PI3Kâ€“mTOR Pathway in Natural Killer Cell Effector Functions. *Front. Immunol.* **5,** (2014).
301. Mao, Y. *et al.* IL-15 activates mTOR and primes stress-activated gene expression leading to prolonged antitumor capacity of NK cells. *Blood* **128,** 1475–89 (2016).
302. Gasteiger, G., Hemmers, S., Bos, P. D., Sun, J. C. & Rudensky, A. Y. IL-2–dependent adaptive control

of NK cell homeostasis. *J. Exp. Med.* **210,** 1179–1187 (2013).
303. Gasteiger, G. *et al.* IL-2–dependent tuning of NK cell sensitivity for target cells is controlled by regulatory T cells. *J. Exp. Med.* **210,** 1167–78 (2013).
304. Sitrin, J., Ring, A., Garcia, K. C., Benoist, C. & Mathis, D. Regulatory T cells control NK cells in an insulitic lesion by depriving them of IL-2. *J. Exp. Med.* **210,** 1153–1165 (2013).
305. Martin, J. F., Perry, J. S. A., Jakhete, N. R., Wang, X. & Bielekova, B. An IL-2 paradox: blocking CD25 on T cells induces IL-2-driven activation of CD56(bright) NK cells. *J. Immunol.* **185,** 1311–20 (2010).
306. Waldmann, T. A., Dubois, S. & Tagaya, Y. Contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for immunotherapy. *Immunity* **14,** 105–10 (2001).
307. Kennedy, M. K. *et al.* Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J. Exp. Med.* **191,** 771–80 (2000).
308. Ring, A. M. *et al.* Mechanistic and structural insight into the functional dichotomy between IL-2 and IL-15. *Nat. Immunol.* **13,** 1187–1195 (2012).
309. Schorle, H., Holtschke, T., Hünig, T., Schimpl, A. & Horak, I. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature* **352,** 621–624 (1991).
310. Newman, K. C. & Riley, E. M. Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. *Nat. Rev. Immunol.* **7,** 279–291 (2007).
311. Eckelhart, E. *et al.* A novel Ncr1-Cre mouse reveals the essential role of STAT5 for NK-cell survival and development. *Blood* **117,** 1565–73 (2011).
312. Yu, H. *et al.* Flt3 Ligand Promotes the Generation of a Distinct CD34+Human Natural Killer Cell Progenitor That Responds to Interleukin-15. *Blood* **92,** (1998).
313. Kobayashi, M. *et al.* Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.* **170,** 827–45 (1989).
314. Stern, A. S. *et al.* Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6808–12 (1990).
315. Jalah, R. *et al.* The p40 subunit of interleukin (IL)-12 promotes stabilization and export of the p35 subunit: implications for improved IL-12 cytokine production. *J. Biol. Chem.* **288,** 6763–76 (2013).
316. Hsieh, C. S. *et al.* Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science* **260,** 547–9 (1993).
317. Macatonia, S. E. *et al.* Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J. Immunol.* **154,** 5071–9 (1995).
318. Cella, M. *et al.* Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J. Exp. Med.* **184,** 747–52 (1996).
319. D'Andrea, A. *et al.* Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. *J. Exp. Med.* **176,** 1387–98 (1992).
320. Presky, D. H. *et al.* A functional interleukin 12 receptor complex is composed of two beta-type cytokine receptor subunits. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14002–7 (1996).
321. Thierfelder, W. E. *et al.* Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* **382,** 171–174 (1996).
322. Robertson, M. J. *et al.* Response of human natural killer (NK) cells to NK cell stimulatory factor (NKSF): cytolytic activity and proliferation of NK cells are differentially regulated by NKSF. *J. Exp. Med.* **175,** 779–88 (1992).
323. Desai, B. B. *et al.* IL-12 receptor. II. Distribution and regulation of receptor expression. *J. Immunol.* **148,** 3125–32 (1992).
324. Zundler, S. & Neurath, M. F. Interleukin-12: Functional activities and implications for disease. *Cytokine Growth Factor Rev.* **26,** 559–568 (2015).
325. Jacobson, N. G. *et al.* Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4. *J. Exp. Med.* **181,** 1755–62 (1995).
326. Bacon, C. M. *et al.* Interleukin 12 (IL-12) induces tyrosine phosphorylation of JAK2 and TYK2: differential use of Janus family tyrosine kinases by IL-2 and IL-12. *J. Exp. Med.* **181,** 399–404 (1995).
327. Kaplan, M. H., Sun, Y.-L., Hoey, T. & Grusby, M. J. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* **382,** 174–177 (1996).
328. Chan, S. H. *et al.* Induction of interferon gamma production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J. Exp. Med.* **173,**

869–79 (1991).
329. Chan, S. H., Kobayashi, M., Santoli, D., Perussia, B. & Trinchieri, G. Mechanisms of IFN-gamma induction by natural killer cell stimulatory factor (NKSF/IL-12). Role of transcription and mRNA stability in the synergistic interaction between NKSF and IL-2. *J. Immunol.* **148,** 92–8 (1992).
330. Parihar, R., Dierksheide, J., Hu, Y. & Carson, W. E. IL-12 enhances the natural killer cell cytokine response to Ab-coated tumor cells. *J. Clin. Invest.* **110,** 983–92 (2002).
331. Walker, W., Aste-Amezaga, M., Kastelein, R. A., Trinchieri, G. & Hunter, C. A. IL-18 and CD28 use distinct molecular mechanisms to enhance NK cell production of IL-12-induced IFN-gamma. *J. Immunol.* **162,** 5894–901 (1999).
332. Wang, K. S., Frank, D. A. & Ritz, J. Interleukin-2 enhances the response of natural killer cells to interleukin-12 through up-regulation of the interleukin-12 receptor and STAT4. *Blood* **95,** (2000).
333. Cooper, M. a *et al.* Cytokine-induced memory-like natural killer cells. *Proc. Natl. Acad. Sci. USA* **106,** 1915–1919 (2009).
334. Romee, R. *et al.* Cytokine activation induces human memory-like NK cells. *Blood* **120,** 4751–4760 (2012).
335. Nakahira, M. *et al.* An absolute requirement for STAT4 and a role for IFN-gamma as an amplifying factor in IL-12 induction of the functional IL-18 receptor complex. *J. Immunol.* **167,** 1306–12 (2001).
336. Chang, J. T., Segal, B. M., Nakanishi, K., Okamura, H. & Shevach, E. M. The costimulatory effect of IL-18 on the induction of antigen-specific IFN-γ production by resting T cells is IL-12 dependent and is mediated by up-regulation of the IL-12 receptor β2 subunit. *Eur. J. Immunol.* **30,** 1113–1119 (2000).
337. Sareneva, T., Julkunen, I. & Matikainen, S. IFN-alpha and IL-12 induce IL-18 receptor gene expression in human NK and T cells. *J. Immunol.* **165,** 1933–8 (2000).
338. Yoshimoto, T. *et al.* IL-12 Up-Regulates IL-18 Receptor Expression on T Cells, Th1 Cells, and B Cells: Synergism with IL-18 for IFN-γ Production. *J. Immunol.* **161,** (1998).
339. Manetti, R. *et al.* Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J. Exp. Med.* **177,** 1199–204 (1993).
340. Rogge, L. *et al.* Selective expression of an interleukin-12 receptor component by human T helper 1 cells. *J. Exp. Med.* **185,** 825–31 (1997).
341. Mattner, F. *et al.* Genetically resistant mice lacking interleukin-12 are susceptible to infection withLeishmania major and mount a polarized Th2 cell response. *Eur. J. Immunol.* **26,** 1553–1559 (1996).
342. Trinchieri, G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* **3,** 133–146 (2003).
343. Okamura, H. *et al.* Cloning of a new cytokine that induces IFN-γ production by T cells. *Nature* **378,** 88–91 (1995).
344. Ghayur, T. *et al.* Caspase-1 processes IFN-γ-inducing factor and regulates LPS-induced IFN- γ production. *Nature* **386,** 619–623 (1997).
345. Gu, Y. *et al.* Activation of interferon-gamma inducing factor mediated by interleukin-1beta converting enzyme. *Science* **275,** 206–9 (1997).
346. CSERNOK, E., ERNST, M., SCHMITT, W., BAINTON, D. F. & GROSS, W. L. Activated neutrophils express proteinase 3 on their plasma membrane in vitro and in vivo. *Clin. Exp. Immunol.* **95,** 244–250 (2008).
347. Pizarro, T. T. *et al.* IL-18, a Novel Immunoregulatory Cytokine, Is Up-Regulated in Crohn's Disease: Expression and Localization in Intestinal Mucosal Cells. *J. Immunol.* **162,** (1999).
348. Kaplanski, G. Interleukin-18: Biological properties and role in disease pathogenesis. *Immunol. Rev.* **281,** 138–153 (2018).
349. Dinarello, C. A., Novick, D., Kim, S. & Kaplanski, G. Interleukin-18 and IL-18 Binding Protein. *Front. Immunol.* **4,** 289 (2013).
350. Kanai, T. *et al.* Macrophage-derived IL-18–mediated intestinal inflammation in the murine model of Crohn's disease. *Gastroenterology* **121,** 875–888 (2001).
351. Torigoe, K. *et al.* Purification and characterization of the human interleukin-18 receptor. *J. Biol. Chem.* **272,** 25737–42 (1997).
352. Hoshino, K. *et al.* Cutting Edge: Generation of IL-18 Receptor-Deficient Mice: Evidence for IL-1 Receptor-Related Protein as an Essential IL-18 Binding Receptor. *J. Immunol.* **162,** (1999).
353. Thomassen, E., Bird, T. a, Renshaw, B. R., Kennedy, M. K. & Sims, J. E. Binding of interleukin-18 to the interleukin-1 receptor homologous receptor IL-1Rrp1 leads to activation of signaling

pathways similar to those used by interleukin-1. *J. Interf. Cytokine Res.* **18,** 1077–88 (1998).
354. Kalina, U. *et al.* IL-18 activates STAT3 in the natural killer cell line 92, augments cytotoxic activity, and mediates IFN-gamma production by the stress kinase p38 and by the extracellular regulated kinases p44erk-1 and p42erk-21. *J. Immunol.* **165,** 1307–13 (2000).
355. Alboni, S. *et al.* Interleukin 18 activates MAPKs and STAT3 but not NF-κB in hippocampal HT-22 cells. *Brain. Behav. Immun.* **40,** 85–94 (2014).
356. Wyman, T. H. *et al.* Physiological levels of interleukin-18 stimulate multiple neutrophil functions through p38 MAP kinase activation. *J. Leukoc. Biol.* **72,** 401–409 (2002).
357. Nakamura, S. *et al.* Expression and responsiveness of human interleukin-18 receptor (IL-18R) on hematopoietic cell lines. *Leukemia* **14,** 1052–1059 (2000).
358. Gerdes, N. *et al.* Expression of interleukin (IL)-18 and functional IL-18 receptor on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for atherogenesis. *J. Exp. Med.* **195,** 245–57 (2002).
359. Gutzmer, R. *et al.* Human dendritic cells express the IL-18R and are chemoattracted to IL-18. *J. Immunol.* **171,** 6363–71 (2003).
360. Möller, B. *et al.* Expression of interleukin-18 receptor in fibroblast-like synoviocytes. *Arthritis Res.* **4,** 139 (2002).
361. Novick, D. *et al.* Interleukin-18 binding protein: a novel modulator of the Th1 cytokine response. *Immunity* **10,** 127–36 (1999).
362. Tsutsui, H. *et al.* IFN-gamma-inducing factor up-regulates Fas ligand-mediated cytotoxic activity of murine natural killer cell clones. *J. Immunol.* **157,** 3967–73 (1996).
363. French, A. R., Holroyd, E. B., Yang, L., Kim, S. & Yokoyama, W. M. IL-18 acts synergistically with IL-15 in stimulating natural killer cell proliferation. *Cytokine* **35,** 229–234 (2006).
364. Hyodo, Y. *et al.* IL-18 up-regulates perforin-mediated NK activity without increasing perforin messenger RNA expression by binding to constitutively expressed IL-18 receptor. *J. Immunol.* **162,** 1662–8 (1999).
365. Takeda, K. *et al.* Defective NK cell activity and Th1 response in IL-18-deficient mice. *Immunity* **8,** 383–90 (1998).
366. Chaix, J. *et al.* Cutting edge: Priming of NK cells by IL-18. *J. Immunol.* **181,** 1627–31 (2008).
367. Smeltz, R. B., Chen, J., Ehrhardt, R. & Shevach, E. M. Role of IFN-gamma in Th1 differentiation: IFN-gamma regulates IL-18R alpha expression by preventing the negative effects of IL-4 and by inducing/maintaining IL-12 receptor beta 2 expression. *J. Immunol.* **168,** 6165–72 (2002).
368. Hoshino, T. *et al.* Cutting edge: IL-18-transgenic mice: in vivo evidence of a broad role for IL-18 in modulating immune function. *J. Immunol.* **166,** 7014–8 (2001).
369. Nakanishi, K., Yoshimoto, T., Tsutsui, H. & Okamura, H. Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu. *Cytokine Growth Factor Rev.* **12,** 53–72 (2001).
370. Yoshimoto, T. *et al.* IL-18 induction of IgE: dependence on CD4+ T cells, IL-4and STAT6. *Nat. Immunol.* **1,** 132–137 (2000).
371. Weaver, C. T., Harrington, L. E., Mangan, P. R., Gavrieli, M. & Murphy, K. M. Th17: An Effector CD4 T Cell Lineage with Regulatory T Cell Ties. *Immunity* **24,** 677–688 (2006).
372. Moretta, A. *et al.* A novel surface antigen expressed by a subset of human CD3- CD16+ natural killer cells. Role in cell activation and regulation of cytolytic function. *J. Exp. Med.* **171,** 695–714 (1990).
373. Moretta, A. *et al.* Identification of four subsets of human CD3-CD16+ natural killer (NK) cells by the expression of clonally distributed functional surface molecules: correlation between subset assignment of NK clones and ability to mediate specific alloantigen recognition. *J. Exp. Med.* **172,** 1589–98 (1990).
374. Colonna, M., Borsellino, G., Falco, M., Ferrara, G. B. & Strominger, J. L. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 12000–4 (1993).
375. Gumperz, J. E., Litwin, V., Phillips, J. H., Lanier, L. L. & Parham, P. The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. *J. Exp. Med.* **181,** 1133–1144 (1995).
376. Moretta, A. *et al.* P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. *J. Exp. Med.* **178,** 597–604 (1993).
377. Storkus, W. J. *et al.* Class I-induced resistance to natural killing: identification of nonpermissive

residues in HLA-A2. *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5989–92 (1991).
378. Colonna, M. & Samaridis, J. Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science* **268,** 405–8 (1995).
379. Seillet, C. *et al.* Deciphering the Innate Lymphoid Cell Transcriptional Program. *Cell Rep.* **17,** 436–447 (2016).
380. D'Andrea, A. *et al.* Molecular cloning of NKB1. A natural killer cell receptor for HLA-B allotypes. *J. Immunol.* **155,** 2306–10 (1995).
381. Wagtmann, N., Rajagopalan, S., Winter, C. C., Peruui, M. & Long, E. O. Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. *Immunity* **3,** 801–809 (1995).
382. Wagtmann, N. *et al.* Molecular clones of the p58 NK cell receptor reveal immunoglobulin-related molecules with diversity in both the extra- and intracellular domains. *Immunity* **2,** 439–449 (1995).
383. Colonna, M. *et al.* Alloantigen recognition by two human natural killer cell clones is associated with HLA-C or a closely linked gene. *Proc. Natl. Acad. Sci. USA* **89,** 7983–7985 (1992).
384. Deng, L. & Mariuzza, R. A. Structural basis for recognition of MHC and MHC-like ligands by natural killer cell receptors. *Semin. Immunol.* **18,** 159–166 (2006).
385. Rahim, M. M. A. & Makrigiannis, A. P. Ly49 receptors: evolution, genetic diversity, and impact on immunity. *Immunol. Rev.* **267,** 137–147 (2015).
386. Guethlein, L. A., Norman, P. J., Hilton, H. H. G. H. G. & Parham, P. Co-evolution of MHC class I and variable NK cell receptors in placental mammals. *Immunol. Rev.* **267,** 259–82 (2015).
387. Parham, P. & Guethlein, L. A. Genetics of Natural Killer Cells in Human Health , Disease , and Survival. *Annu. Rev. Immunol.* **36,** 1–30 (2018).
388. Parham, P. MHC class I molecules and kirs in human history, health and survival. *Nat. Rev. Immunol.* **5,** 201–214 (2005).
389. Hilton, H. G. & Parham, P. Missing or altered self: human NK cell receptors that recognize HLA-C. *Immunogenetics* **69,** (2017).
390. Bashirova, A. A., Martin, M. P., McVicar, D. W. & Carrington, M. The Killer Immunoglobulin-Like Receptor Gene Cluster: Tuning the Genome for Defense. *Annu. Rev. Genomics Hum. Genet.* **7,** 277–300 (2006).
391. Khakoo, S. I. & Carrington, M. KIR and disease: A model system or system of models? *Immunological Reviews* **214,** 186–201 (2006).
392. Björkström, N. K. *et al.* CD8 T cells express randomly selected KIRs with distinct specificities compared with NK cells. *Blood* **120,** 3455–65 (2012).
393. Vivier, E. & Anfossi, N. Inhibitory NK-cell receptors on T cells: witness of the past, actors of the future. *Nat. Rev. Immunol.* **4,** 190–198 (2004).
394. Ferrini, S. *et al.* T cell clones expressing the natural killer cell-related p58 receptor molecule display heterogeneity in phenotypic properties and p58 function. *Eur. J. Immunol.* **24,** 2294–2298 (1994).
395. Bakker, A. B., Phillips, J. H., Figdor, C. G. & Lanier, L. L. Killer cell inhibitory receptors for MHC class I molecules regulate lysis of melanoma cells mediated by NK cells, gamma delta T cells, and antigen-specific CTL. *J. Immunol.* **160,** 5239–45 (1998).
396. Snyder, M. R., Weyand, C. M. & Goronzy, J. J. The double life of NK receptors: stimulation or co-stimulation? *Trends Immunol.* **25,** 25–32 (2004).
397. Uhrberg, M. *et al.* The repertoire of killer cell Ig-like receptor and CD94:NKG2A receptors in T cells: clones sharing identical alpha beta TCR rearrangement express highly diverse killer cell Ig-like receptor patterns. *J. Immunol.* **166,** 3923–32 (2001).
398. Ugolini, S. *et al.* Involvement of inhibitory NKRs in the survival of a subset of memory-phenotype CD8+ T cells. *Nat. Immunol.* **2,** 430–435 (2001).
399. Vely, F. *et al.* Regulation of inhibitory and activating killer-cell Ig-like receptor expression occurs in T cells after termination of TCR rearrangements. *J. Immunol.* **166,** 2487–94 (2001).
400. Young, N. T., Uhrberg, M., Phillips, J. H., Lanier, L. L. & Parham, P. Differential expression of leukocyte receptor complex-encoded Ig-like receptors correlates with the transition from effector to memory CTL. *J. Immunol.* **166,** 3933–41 (2001).
401. Marsh, S. G. E. *et al.* Killer-cell immunoglobulin-like receptor (KIR) nomenclature report, 2002. *Tissue Antigens* **62,** 79–86 (2003).
402. Rajalingam, R. in *Methods in Molecular Biology* (eds. Christiansen, F. T. & Tait, B. D.) **882,** 391–414 (Humana Press, 2012).
403. Faure, M. & Long, E. O. KIR2DL4 (CD158d), an NK cell-activating receptor with inhibitory

potential. *J. Immunol.* **168,** 6208–6214 (2002).
404. Rajagopalan, S., Fu, J. & Long, E. O. Cutting Edge: Induction of IFN-γ Production but Not Cytotoxicity by the Killer Cell Ig-Like Receptor KIR2DL4 (CD158d) in Resting NK Cells. *J. Immunol.* **167,** (2001).
405. Kikuchi-Maki, A., Catina, T. L. & Campbell, K. S. Cutting Edge: KIR2DL4 Transduces Signals into Human NK Cells through Association with the Fc Receptor γ Protein. *J. Immunol.* **174,** (2005).
406. Vilches, C. & Parham, P. KIR: Diverse, Rapidly Evolving Receptors of Innate and Adaptive Immunity. *Annu. Rev. Immunol.* **20,** 217–251 (2002).
407. Saunders, P. M. *et al.* A bird's eye view of NK cell receptor interactions with their MHC class I ligands. *Immunol. Rev.* **267,** 148–66 (2015).
408. Béziat, V., Hilton, H. G., Norman, P. J. & Traherne, J. A. Deciphering the killer-cell immunoglobulin-like receptor system at super-resolution for natural killer and T-cell biology. *Immunology* **150,** 248–264 (2017).
409. Boyington, J. C., Motyka, S. A., Schuck, P., Brooks, A. G. & Sun, P. D. Crystal structure of an NK cell immunoglobulin-like receptor in complex with its class I MHC ligand. *Nature* **405,** 537–543 (2000).
410. Boyington, J. C. & Sun, P. D. A structural perspective on MHC class I recognition by killer cell immunoglobulin-like receptors. *Mol. Immunol.* **38,** 1007–1021 (2002).
411. Winter, C. C. & Long, E. A single amino acid in the p58 killer cell inhibitory receptor controls the ability of natural killer cells to discriminate between the two groups of HLA-C allotypes. *J. Immunol.* **142,** 142–144 (1997).
412. Colonna, M. *et al.* A Common Inhibitory Receptor for Major Histocompatibility Complex Class I Molecules on Human Lymphoid and Myelomonocytic Cells. *J. Exp. Med.* **186,** 1809–1818 (1997).
413. Colonna, M. Specificity and function of immunoglobulin superfamily NK cell inhibitory and stimulatory receptors. *Immunological Reviews* **155,** 127–133 (1997).
414. Moesta, A. K. *et al.* Synergistic Polymorphism at Two Positions Distal to the Ligand-Binding Site Makes KIR2DL2 a Stronger Receptor for HLA-C Than KIR2DL3. *J. Immunol.* **180,** 3969–3979 (2008).
415. Winter, C. C., Gumperz, J. E., Parham, P., Long, E. O. & Wagtmann, N. Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. *J. Immunol.* **161,** 571–7 (1998).
416. Litwin, V., Gumperz, J., Parham, P., Phillips, J. H. & Lanier, L. L. NKB1: a natural killer cell receptor involved in the recognition of polymorphic HLA-B molecules. *J. Exp. Med.* **180,** 537–43 (1994).
417. Cella, M., Longo, A., Ferrara, G. B., Strominger, J. L. & Colonna, M. NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80. *J. Exp. Med.* **180,** (1994).
418. Cassidy, S. A., Cheent, K. S. & Khakoo, S. I. Effects of Peptide on NK cell-mediated MHC I recognition. *Front. Immunol.* **5,** 133 (2014).
419. Das, J. & Khakoo, S. I. NK cells: tuned by peptide? *Immunol. Rev.* **267,** 214–27 (2015).
420. Hansasuta, P. *et al.* Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific. *Eur. J. Immunol.* **34,** 1673–1679 (2004).
421. Pende, D. *et al.* The natural killer cell receptor specific for HLA-A allotypes: a novel member of the p58/p70 family of inhibitory receptors that is characterized by three immunoglobulin-like domains and is expressed as a 140-kD disulphide-linked dimer. *J. Exp. Med.* **184,** (1996).
422. Fadda, L. *et al.* Common HIV-1 Peptide Variants Mediate Differential Binding of KIR3DL1 to HLA-Bw4 Molecules. *J. Virol.* **85,** 5970–5974 (2011).
423. Malnati, M. *et al.* Peptide specificity in the recognition of MHC class I by natural killer cell clones. *Science (80-.).* **267,** (1995).
424. Peruzzi, M., Wagtmann, N. & Long, E. O. A p70 killer cell inhibitory receptor specific for several HLA-B allotypes discriminates among peptides bound to HLA-B*2705. *J. Exp. Med.* **184,** 1585–1590 (1996).
425. Das, J. & Khakoo, S. I. NK cells: Tuned by peptide? *Immunol. Rev.* **267,** 214–227 (2015).
426. Stewart, C. A. *et al.* Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors. *Proc. Natl. Acad. Sci.* **102,** 13224–13229 (2005).
427. Chapel, A. *et al.* Peptide-specific engagement of the activating NK cell receptor KIR2DS1. *Sci. Rep.* **7,** 2414 (2017).
428. Liu, J., Xiao, Z., Ko, H. L., Shen, M. & Ren, E. C. Activating killer cell immunoglobulin-like receptor 2DS2 binds to HLA-A*11. *Proc. Natl. Acad. Sci.* **111,** 2662–2667 (2014).
429. Naiyer, M. M. *et al.* KIR2DS2 recognizes conserved peptides derived from viral helicases in the context of HLA-C. *Sci. Immunol.* **2,** eaal5296 (2017).

430. Thiruchelvam-Kyle, L. *et al.* The Activating Human NK Cell Receptor KIR2DS2 Recognizes a β 2 -Microglobulin–Independent Ligand on Cancer Cells. *J. Immunol.* **198,** 2556–2567 (2017).
431. Graef, T. *et al.* KIR2DS4 is a product of gene conversion with KIR3DL2 that introduced specificity for HLA-A * 11 while diminishing avidity for HLA-C. *J. Exp. Med.* **206,** 2557–2572 (2009).
432. Carr, W. H. *et al.* Cutting Edge: KIR3DS1, a Gene Implicated in Resistance to Progression to AIDS, Encodes a DAP12-Associated Receptor Expressed on NK Cells That Triggers NK Cell Activation. *J. Immunol.* **178,** 647–51 (2007).
433. O'Connor, G. M. *et al.* Peptide-Dependent Recognition of HLA-B*57:01 by KIR3DS1. *J. Virol.* **89,** 5213–5221 (2015).
434. Burian, A. *et al.* HLA-F and MHC-I Open Conformers Bind Natural Killer Cell Ig-Like Receptor KIR3DS1. *PLoS One* **11,** e0163297 (2016).
435. Garcia-Beltran, W. F. *et al.* Open conformers of HLA-F are high-affinity ligands of the activating NK-cell receptor KIR3DS1. *Nat. Immunol.* **17,** 1067–1074 (2016).
436. Carrillo-Bustamante, P., de Boer, R. J. & Keşmir, C. Specificity of inhibitory KIRs enables NK cells to detect changes in an altered peptide environment. *Immunogenetics* **70,** 87–97 (2018).
437. Rajagopalan, S. & Long, E. O. A Human Histocompatibility Leukocyte Antigen (HLA)-G-specific Receptor Expressed on All Natural Killer Cells. *J. Exp. Med.* **189,** 1093–1100 (1999).
438. Rajagopalan, S. & Long, E. O. KIR2DL4 (CD158d): An activation receptor for HLA-G. *Front. Immunol.* **3,** 258 (2012).
439. Ashkar, A. A., Di Santo, J. P. & Croy, B. A. Interferon γ Contributes to Initiation of Uterine Vascular Modification, Decidual Integrity, and Uterine Natural Killer Cell Maturation during Normal Murine Pregnancy. *J. Exp. Med.* **192,** 259–270 (2000).
440. Trowsdale, J. *et al.* The genomic context of natural killer receptor extended gene families. *Immunol. Rev.* **181,** 20–38 (2001).
441. Colucci, F. & Traherne, J. Killer-cell immunoglobulin-like receptors on the cusp of modern immunogenetics. *Immunology* **152,** 556–561 (2017).
442. Valiante, N. M. *et al.* Functionally and Structurally Distinct NK Cell Receptor Repertoires in the Peripheral Blood of Two Human Donors. *Immunity* **7,** 739–751 (1997).
443. Uhrberg, M. *et al.* Human Diversity in Killer Cell Inhibitory Receptor Genes. *Immunity* **7,** 753–763 (1997).
444. Manser, A. R., Weinhold, S. & Uhrberg, M. Human KIR repertoires: shaped by genetic diversity and evolution. *Immunol. Rev.* **267,** 178–196 (2015).
445. Andersson, S., Fauriat, C., Malmberg, J.-A. A., Ljunggren, H.-G. G. & Malmberg, K.-J. J. KIR acquisition probabilities are independent of self-HLA class I ligands and increase with cellular KIR expression. *Blood* **114,** 95–104 (2009).
446. Gardiner, C. M. Killer cell immunoglobulin-like receptors on NK cells: the how, where and why. *Int. J. Immunogenet.* **35,** 1–8 (2008).
447. O'Connor, G. M. *et al.* Functional polymorphism of the KIR3DL1/S1 receptor on human NK cells. *J. Immunol.* **178,** 235–41 (2007).
448. Béziat, V. *et al.* Influence of KIR gene copy number on natural killer cell education. *Blood* **121,** 4703–4708 (2015).
449. Li, H., Pascal, V., Martin, M. P., Carrington, M. & Anderson, S. K. Genetic Control of Variegated KIR Gene Expression: Polymorphisms of the Bi-Directional KIR3DL1 Promoter Are Associated with Distinct Frequencies of Gene Expression. *PLoS Genet.* **4,** e1000254 (2008).
450. Raulet, D. H., Vance, R. E. & McMahon, C. W. Regulation of the natural killer cell receptor repertoire. *Annu Rev Immunol* **19,** 291–330 (2001).
451. Fahlen, L., Lendahl, U. & Sentman, C. L. MHC Class I-Ly49 Interactions Shape the Ly49 Repertoire on Murine NK Cells. *J. Immunol.* **166,** 6585–6592 (2001).
452. Held, W., Dorfman, J. R., Wu, M.-F. & Raulet, D. H. Major histocompatibility complex class I-dependent skewing of the natural killer cell Ly49 receptor reportoire. *Eur. J. Immunol.* **26,** 2286–2292 (1996).
453. Johansson, S. *et al.* Natural killer cell education in mice with single or multiple major histocompatibility complex class I molecules. *J. Exp. Med.* **201,** 1145–1155 (2005).
454. Schönberg, K., Sribar, M., Enczmann, J., Fischer, J. C. & Uhrberg, M. Analyses of HLA-C-specific KIR repertoires in donors with group A and B haplotypes suggest a ligand-instructed model of NK cell receptor acquisition. *Blood* **117,** 98–107 (2011).
455. Shilling, H. G. *et al.* Genetic Control of Human NK Cell Repertoire. *J. Immunol.* **169,** 239–247 (2002).
456. Yawata, M. *et al.* Roles for HLA and KIR polymorphisms in natural killer cell repertoire selection

and modulation of effector function. *J. Exp. Med.* **203,** 633–645 (2006).
457. van Bergen, J. *et al.* HLA Reduces Killer Cell Ig-like Receptor Expression Level and Frequency in a Humanized Mouse Model. *J. Immunol.* **190,** 2880–2885 (2013).
458. Gumperz, B. J. E., Valiante, N. M., Parham, P., Lanier, L. L. & Tyan, D. Heterogeneous phenotypes of expression of the NKB1 natural killer cell class I receptor among individuals of different human histocompatibility leukocyte antigens types appear genetically regulated, but not linked to major histocompatibililty complex hapl. *J. Exp. Med.* **183,** 1817–1827 (1996).
459. Frohn, C., Schlenke, P. & Kirchner, H. The repertoire of HLA-Cw-specific NK cell receptors CD158 a/b (EB6 and GL183) in individuals with different HLA phenotypes. *Immunology* **92,** 567–570 (1997).
460. Hsu, K. C., Chida, S., Geraghty, D. E. & Dupont, B. The killer cell immunoglobulin-like receptor (KIR) genomic region: gene-order, haplotypes and allelic polymorphism. *Immunol Rev* **190,** (2002).
461. Stewart, C. A., Van Bergen, J. & Trowsdale, J. Different and divergent regulation of the KIR2DL4 and KIR3DL1 promoters. *J. Immunol.* **170,** 6073–6081 (2003).
462. Trompeter, H.-I. *et al.* Three Structurally and Functionally Divergent Kinds of Promoters Regulate Expression of Clonally Distributed Killer Cell Ig-Like Receptors (KIR), of KIR2DL4, and of KIR3DL3. *J. Immunol.* **174,** 4135–4143 (2005).
463. Pascal, V., Stulberg, M. J. & Anderson, S. K. Regulation of class I major histocompatibility complex receptor expression in natural killer cells: one promoter is not enough! *Immunol. Rev.* **214,** 9–21 (2006).
464. Cichocki, F., Miller, J. S. & Anderson, S. K. Killer Immunoglobulin-Like Receptor Transcriptional Regulation: A Fascinating Dance of Multiple Promoters. *J. Innate Immun.* **3,** 242–248 (2011).
465. Chan, H.-W., Miller, J. S., Moore, M. B. & Lutz, C. T. Epigenetic Control of Highly Homologous Killer Ig-Like Receptor Gene Alleles. *J. Immunol.* **175,** 5966–5974 (2005).
466. Santourlidis, S., Graffmann, N., Christ, J. & Uhrberg, M. Lineage-Specific Transition of Histone Signatures in the Killer Cell Ig-Like Receptor Locus from Hematopoietic Progenitor to NK Cells. *J. Immunol.* **180,** 418–425 (2008).
467. Santourlidis, S. *et al.* Crucial Role of DNA Methylation in Determination of Clonally Distributed Killer Cell Ig-like Receptor Expression Patterns in NK Cells. *J. Immunol.* **169,** 4253–4261 (2002).
468. Chan, H.-W. *et al.* DNA Methylation Maintains Allele-specific KIR Gene Expression in Human Natural Killer Cells. *J. Exp. Med.* **197,** 245–255 (2003).
469. Uhrberg, M. Shaping the human NK cell repertoire: an epigenetic glance at KIR gene regulation. *Mol. Immunol.* **42,** 471–475 (2005).
470. Wright, P. W. *et al.* Identification of a KIR antisense lncRNA expressed by progenitor cells. *Genes Immun.* **14,** 427–33 (2013).
471. Stulberg, M. J. *et al.* Identification of distal KIR promoters and transcripts. *Genes Immun.* **8,** 124–130 (2007).
472. Cichocki, F. *et al.* The transcription factor c-Myc enhances KIR gene transcription through direct binding to an upstream distal promoter element. *Blood* **113,** 3245–3253 (2009).
473. Davies, G. E. *et al.* Identification of bidirectional promoters in the human KIR genes. *Genes Immun.* **8,** 245–53 (2007).
474. Anderson, S. K. Probabilistic Bidirectional Promoter Switches: Noncoding RNA Takes Control. *Mol. Ther. - Nucleic Acids* **3,** e191 (2014).
475. Cichocki, F. *et al.* Cutting Edge: KIR Antisense Transcripts Are Processed into a 28-Base PIWI-Like RNA in Human NK Cells. *J. Immunol.* **185,** 2009–2012 (2010).
476. Freund, J. *et al.* Activating Receptor Signals Drive Receptor Diversity in Developing Natural Killer Cells. *PLOS Biol.* **14,** e1002526 (2016).
477. Wright, P. W. *et al.* Characterization of a weakly expressed KIR2DL1 variant reveals a novel upstream promoter that controls KIR expression. *Genes Immun.* **15,** 440–448 (2014).
478. Li, H., Wright, P. W., McCullen, M. & Anderson, S. K. Characterization of KIR intermediate promoters reveals four promoter types associated with distinct expression patterns of KIR subtypes. *Genes Immun.* **17,** 66–74 (2016).
479. Boudreau, J. E. & Hsu, K. C. Natural Killer Cell Education and the Response to Infection and Cancer Therapy: Stay Tuned. *Trends Immunol.* **39,** 222–239 (2018).
480. Höglund, P. & Brodin, P. Current perspectives of natural killer cell education by MHC class I molecules. *Nat. Rev. Immunol.* **10,** 724–734 (2010).
481. Kim, S. *et al.* Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* **436,** 709–13 (2005).
482. Anfossi, N. *et al.* Human NK cell education by inhibitory receptors for MHC class I. *Immunity* **25,**

331–42 (2006).
483. Fernandez, N. C. *et al.* A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood* **105,** 4416–23 (2005).
484. Zimmer, J. *et al.* Activity and phenotype of natural killer cells in peptide transporter (TAP)-deficient patients (type I bare lymphocyte syndrome). *J. Exp. Med.* **187,** 117–22 (1998).
485. Höglund, P. *et al.* Recognition of beta 2-microglobulin-negative (beta 2m-) T-cell blasts by natural killer cells from normal but not from beta 2m- mice: nonresponsiveness controlled by beta 2m-bone marrow in chimeric mice. *Proc. Natl. Acad. Sci. U. S. A.* **88,** 10332–6 (1991).
486. Wu, N. *et al.* A hematopoietic cell-driven mechanism involving SLAMF6 receptor, SAP adaptors and SHP-1 phosphatase regulates NK cell education. *Nat. Immunol.* **17,** 387–396 (2016).
487. Brodin, P., Lakshmikanth, T., Johansson, S., Karre, K. & Hoglund, P. The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. *Blood* **113,** 2434–2441 (2009).
488. He, Y. & Tian, Z. NK cell education via nonclassical MHC and non-MHC ligands. *Cell. Mol. Immunol.* **14,** 321–330 (2017).
489. Meazza, R. *et al.* Inhibitory 2B4 contributes to NK cell education and immunological derangements in XLP1 patients. *Eur. J. Immunol.* **47,** 1051–1061 (2017).
490. Chalifour, A. *et al.* A Role for cis Interaction between the Inhibitory Ly49A Receptor and MHC Class I for Natural Killer Cell Education. *Immunity* **30,** 337–347 (2009).
491. Bessoles, S. *et al.* Education of Murine NK Cells Requires Both cis and trans Recognition of MHC Class I Molecules. *J. Immunol.* **191,** 5044–5051 (2013).
492. Boudreau, J. E. *et al.* Cell-Extrinsic MHC Class I Molecule Engagement Augments Human NK Cell Education Programmed by Cell-Intrinsic MHC Class I. *Immunity* **45,** 280–291 (2016).
493. Held, W. & Mariuzza, R. A. Cis interactions of immunoreceptors with MHC and non-MHC ligands. *Nat. Rev. Immunol.* **8,** 269–78 (2008).
494. Doucey, M.-A. *et al.* Cis association of Ly49A with MHC class I restricts natural killer cell inhibition. *Nat. Immunol.* **5,** 328–336 (2004).
495. Yokoyama, W. M. & Kim, S. Licensing of natural killer cells by self-major histocompatibility complex class I. *Immunol. Rev.* **214,** 143–54 (2006).
496. Elliott, J. M. & Yokoyama, W. M. Unifying concepts of MHC-dependent natural killer cell education. *Trends Immunol.* **32,** 364–372 (2011).
497. Raulet, D. H. & Vance, R. E. Self-tolerance of natural killer cells. *Nat. Rev. Immunol.* **6,** 520–531 (2006).
498. Joncker, N. T., Fernandez, N. C., Treiner, E., Vivier, E. & Raulet, D. H. NK Cell Responsiveness Is Tuned Commensurate with the Number of Inhibitory Receptors for Self-MHC Class I: The Rheostat Model. *J. Immunol.* **182,** 4572–4580 (2009).
499. Yu, J. *et al.* Hierarchy of the human natural killer cell response is determined by class and quantity of inhibitory receptors for self-HLA-B and HLA-C ligands. *J. Immunol.* **179,** 5977–89 (2007).
500. Boudreau, J. E., Mulrooney, T. J., Le Luduec, J.-B., Barker, E. & Hsu, K. C. KIR3DL1 and HLA-B Density and Binding Calibrate NK Education and Response to HIV. *J. Immunol.* **196,** 3398–3410 (2016).
501. Kim, S. *et al.* HLA alleles determine differences in human natural killer cell responsiveness and potency. *Proc. Natl. Acad. Sci.* **105,** 3053–3058 (2008).
502. Brodin, P., Kärre, K. & Höglund, P. NK cell education: not an on-off switch but a tunable rheostat. *Trends Immunol.* **30,** 143–9 (2009).
503. Sun, J. C. Re-educating natural killer cells. *J. Exp. Med.* **207,** 2049–52 (2010).
504. Sun, J. C. & Lanier, L. L. Tolerance of NK cells encountering their viral ligand during development. *J. Exp. Med.* **205,** 1819–1828 (2008).
505. Tripathy, S. K. *et al.* Continuous engagement of a self-specific activation receptor induces NK cell tolerance. *J. Exp. Med.* **205,** 1829–1841 (2008).
506. Fauriat, C. *et al.* Education of human natural killer cells by activating killer cell immunoglobulin-like receptors. *Blood* **115,** 1166–1174 (2010).
507. Orr, M. T. & Lanier, L. L. Natural killer cell education and tolerance. *Cell* **142,** 847–56 (2010).
508. Elliott, J. M., Wahle, J. A. & Yokoyama, W. M. MHC class I–deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I–sufficient environment. *J. Exp. Med.* **207,** 2073–9 (2010).
509. Joncker, N. T., Shifrin, N., Delebecque, F. & Raulet, D. H. Mature natural killer cells reset their responsiveness when exposed to an altered MHC environment. *J. Exp. Med.* **207,** 2065–2072 (2010).
510. Juelke, K., Killig, M., Thiel, A., Dong, J. & Romagnani, C. Education of hyporesponsive NK cells by

cytokines. *Eur. J. Immunol.* **39,** 2548–2555 (2009).
511. Ardolino, M. *et al.* Cytokine therapy reverses NK cell anergy in MHC-deficient tumors. *J. Clin. Invest.* **124,** 4781–4794 (2014).
512. Wagner, J. A. *et al.* Cytokine-Induced Memory-Like Differentiation Enhances Unlicensed Natural Killer Cell Antileukemia and FcγRIIIa-Triggered Responses. *Biol. Blood Marrow Transplant.* **23,** 398–404 (2017).
513. Marçais, A. *et al.* High mTOR activity is a hallmark of reactive natural killer cells and amplifies early signaling through activating receptors. *Elife* **6,** e26423 (2017).
514. Sun, J. C. & Lanier, L. L. Cutting Edge: Viral Infection Breaks NK Cell Tolerance to 'Missing Self'. *J. Immunol.* **181,** 7453–7457 (2008).
515. Zamora, A. E. *et al.* Licensing delineates helper and effector NK cell subsets during viral infection. *JCI Insight* **2,** 415–422 (2017).
516. Orr, M. T. & Lanier, L. L. Natural killer cell licensing during viral infection. *Adv. Exp. Med. Biol.* **780,** 37–44 (2011).
517. Alvarez, M., Sun, K. & Murphy, W. J. Mouse host unlicensed NK cells promote donor allogeneic bone marrow engraftment. *Blood* **127,** 1202–1205 (2016).
518. Orr, M. T., Murphy, W. J. & Lanier, L. L. 'Unlicensed' natural killer cells dominate the response to cytomegalovirus infection. *Nat. Immunol.* **11,** 321–7 (2010).
519. Tarek, N. *et al.* Unlicensed NK cells target neuroblastoma following anti-GD2 antibody treatment. *J. Clin. Invest.* **122,** 3260–3270 (2012).
520. Du, J. *et al.* CALGB 150905 (Alliance): Rituximab Broadens the Antilymphoma Response by Activating Unlicensed NK Cells. *Cancer Immunol. Res.* **2,** 878–889 (2014).
521. Erbe, A. K. *et al.* Neuroblastoma Patients' KIR and KIR-Ligand Genotypes Influence Clinical Outcome for Dinutuximab-based Immunotherapy: A Report from the Children's Oncology Group. *Clin. Cancer Res.* **24,** 189–196 (2018).
522. Williams, M. A. & Bevan, M. J. Effector and Memory CTL Differentiation. *Annu. Rev. Immunol.* **25,** 171–192 (2007).
523. Min-oo, G., Kamimura, Y., Hendricks, D. W., Nabekura, T. & Lanier, L. L. Natural killer cells : walking three paths down memory lane. *Trends Immunol.* **34,** 251–258 (2013).
524. Rölle, A., Pollmann, J. & Cerwenka, A. Memory of infections: an emerging role for natural killer cells. *PLoS Pathog.* **9,** e1003548 (2013).
525. O'Leary, J. G., Goodarzi, M., Drayton, D. L. & von Andrian, U. H. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat. Immunol.* **7,** 507–16 (2006).
526. Romee, R. *et al.* Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. *Sci. Transl. Med.* **8,** 357ra123-357ra123 (2016).
527. Paust, S. *et al.* Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. *Nat. Immunol.* **11,** 1127–35 (2010).
528. Beaulieu, A. M., Madera, S. & Sun, J. C. Molecular Programming of Immunological Memory in Natural Killer Cells. *Adv. Exp. Med. Biol.* **850,** 81–91 (2015).
529. Sun, J. C., Beilke, J. N. & Lanier, L. L. Adaptive immune features of natural killer cells. *Nature* **457,** 557–61 (2009).
530. Sun, J. C., Beilke, J. N. & Lanier, L. L. Immune memory redefined: characterizing the longevity of natural killer cells. *Immunol. Rev.* **236,** 83–94 (2010).
531. Sun, J. C. *et al.* Proinflammatory cytokine signaling required for the generation of natural killer cell memory. *J. Exp. Med.* **209,** 947–54 (2012).
532. Madera, S. & Sun, J. C. Cutting Edge: Stage-Specific Requirement of IL-18 for Antiviral NK Cell Expansion. *J. Immunol.* **194,** 1408–1412 (2015).
533. Madera, S. *et al.* Type I IFN promotes NK cell expansion during viral infection by protecting NK cells against fratricide. *J. Exp. Med.* **213,** 225–233 (2016).
534. Nabekura, T. *et al.* Costimulatory Molecule DNAM-1 Is Essential for Optimal Differentiation of Memory Natural Killer Cells during Mouse Cytomegalovirus Infection. *Immunity* **40,** 225–234 (2014).
535. Zawislak, C. L. *et al.* Stage-specific regulation of natural killer cell homeostasis and response against viral infection by microRNA-155. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 6967–72 (2013).
536. Bezman, N. A. *et al.* Molecular definition of the identity and activation of natural killer cells. *Nat. Immunol.* **13,** 1000–1009 (2012).
537. Min-Oo, G., Bezman, N. A., Madera, S., Sun, J. C. & Lanier, L. L. Proapoptotic Bim regulates antigen-specific NK cell contraction and the generation of the memory NK cell pool after cytomegalovirus infection. *J. Exp. Med.* **211,** 1289–1296 (2014).

538. O'Sullivan, T. E. *et al.* BNIP3- and BNIP3L-Mediated Mitophagy Promotes the Generation of Natural Killer Cell Memory. *Immunity* **43,** 331–342 (2015).
539. Beaulieu, A. M., Zawislak, C. L., Nakayama, T. & Sun, J. C. The transcription factor Zbtb32 controls the proliferative burst of virus-specific natural killer cells responding to infection. *Nat. Immunol.* **15,** 546–53 (2014).
540. Adams, N. M. *et al.* Transcription Factor IRF8 Orchestrates the Adaptive Natural Killer Cell Response. *Immunity* **48,** 1172–1182.e6 (2018).
541. Gumá, M. *et al.* Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood* **104,** 3664–71 (2004).
542. Gumá, M. *et al.* Expansion of CD94/NKG2C+ NK cells in response to human cytomegalovirus-infected fibroblasts. *Blood* **107,** 3624–31 (2006).
543. Foley, B. *et al.* Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated NKG2C+ natural killer cells with potent function. *Blood* **119,** 2665–74 (2012).
544. Foley, B. *et al.* Human Cytomegalovirus (CMV)-Induced Memory-like NKG2C+ NK Cells Are Transplantable and Expand In Vivo in Response to Recipient CMV Antigen. *J. Immunol.* **189,** 5082–5088 (2012).
545. Cichocki, F. *et al.* CD56dimCD57+NKG2C+ NK cell expansion is associated with reduced leukemia relapse after reduced intensity HCT. *Leukemia* **30,** 456–63 (2016).
546. Elmaagacli, A. H. *et al.* Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients. *Blood* **118,** 1402–12 (2011).
547. Green, M. L. *et al.* CMV reactivation after allogeneic HCT and relapse risk: evidence for early protection in acute myeloid leukemia. *Blood* **122,** 1316–24 (2013).
548. Davis, Z. B. *et al.* Adaptive Natural Killer Cell and Killer Cell Immunoglobulin-Like Receptor-Expressing T Cell Responses are Induced by Cytomegalovirus and Are Associated with Protection against Cytomegalovirus Reactivation after Allogeneic Donor Hematopoietic Cell Transpla. *Biol. Blood Marrow Transplant.* **21,** 1653–1662 (2015).
549. Liu, L. L. *et al.* Ex Vivo Expanded Adaptive NK Cells Effectively Kill Primary Acute Lymphoblastic Leukemia Cells. *Cancer Immunol. Res.* **5,** 654–665 (2017).
550. Björkström, N. K. *et al.* Rapid expansion and long-term persistence of elevated NK cell numbers in humans infected with hantavirus. *J. Exp. Med.* **208,** 13–21 (2011).
551. Petitdemange, C. *et al.* Unconventional repertoire profile is imprinted during acute chikungunya infection for natural killer cells polarization toward cytotoxicity. *PLoS Pathog.* **7,** e1002268 (2011).
552. Béziat, V. *et al.* CMV drives clonal expansion of NKG2C + NK cells expressing self-specific KIRs in chronic hepatitis patients. *Eur. J. Immunol.* **42,** 447–457 (2012).
553. Brunetta, E. *et al.* Chronic HIV-1 viremia reverses NKG2A/NKG2C ratio on natural killer cells in patients with human cytomegalovirus co-infection. *AIDS* **24,** 27–34 (2010).
554. Gumá, M. *et al.* Human Cytomegalovirus Infection Is Associated with Increased Proportions of NK Cells That Express the CD94/NKG2C Receptor in Aviremic HIV-1–Positive Patients. *J. Infect. Dis.* **194,** 38–41 (2006).
555. Reeves, R. K. *et al.* Antigen-specific NK cell memory in rhesus macaques. *Nat. Immunol.* **16,** 927–932 (2015).
556. Rölle, A. *et al.* IL-12-producing monocytes and HLA-E control HCMV-driven NKG2C+ NK cell expansion. *J. Clin. Invest.* **124,** 5305–16 (2014).
557. Rölle, A. *et al.* CD2-CD58 interactions are pivotal for the activation and function of adaptive natural killer cells in human cytomegalovirus infection. *Eur. J. Immunol.* **46,** 2420–2425 (2016).
558. Béziat, V. *et al.* NK cell responses to cytomegalovirus infection lead to stable imprints in the human KIR repertoire and involve activating KIRs. *Blood* **121,** 2678–88 (2013).
559. Charoudeh, H. N. *et al.* Modulation of the natural killer cell KIR repertoire by cytomegalovirus infection. *Eur. J. Immunol.* **43,** 480–7 (2013).
560. Béziat, V. *et al.* NK cell responses to cytomegalovirus infection lead to stable imprints in the human KIR repertoire and involve activating KIRs. *Blood* **121,** 2678–88 (2013).
561. Della Chiesa, M. *et al.* Human cytomegalovirus infection promotes rapid maturation of NK cells expressing activating killer Ig-like receptor in patients transplanted with NKG2C-/- umbilical cord blood. *J. Immunol.* **192,** 1471–9 (2014).
562. Luetke-Eversloh, M. *et al.* Human cytomegalovirus drives epigenetic imprinting of the IFNG locus in NKG2Chi natural killer cells. *PLoS Pathog.* **10,** e1004441 (2014).

563. Hwang, I. *et al.* Identification of human NK cells that are deficient for signaling adaptor FcRγ and specialized for antibody-dependent immune functions. *Int. Immunol.* **24,** 793–802 (2012).
564. Zhang, T., Scott, J. M., Hwang, I. & Kim, S. Cutting edge: antibody-dependent memory-like NK cells distinguished by FcRγ deficiency. *J. Immunol.* **190,** 1402–6 (2013).
565. Lee, J. *et al.* Epigenetic Modification and Antibody-Dependent Expansion of Memory-like NK Cells in Human Cytomegalovirus-Infected Individuals. *Immunity* **42,** 431–442 (2015).
566. Schlums, H. *et al.* Cytomegalovirus infection drives adaptive epigenetic diversification of NK cells with altered signaling and effector function. *Immunity* **42,** 443–56 (2015).
567. Berrien-Elliott, M. M., Wagner, J. A. & Fehniger, T. A. Human Cytokine-Induced Memory-Like Natural Killer Cells. *J. Innate Immun.* **7,** 563–71 (2015).
568. Cooper, M. A. *et al.* Hidden talents of natural killers: NK cells in innate and adaptive immunity. *EMBO Rep.* **10,** 1103–10 (2009).
569. Ni, J. *et al.* Adoptively transferred natural killer cells maintain long-term antitumor activity by epigenetic imprinting and CD4(+) T cell help. *Oncoimmunology* **5,** e1219009 (2016).
570. Hanahan, D. & Weinberg, R. A. Hallmarks of Cancer: The Next Generation. *Cell* **144,** 646–674 (2011).
571. Cavallo, F., De Giovanni, C., Nanni, P., Forni, G. & Lollini, P.-L. 2011: the immune hallmarks of cancer. *Cancer Immunol. Immunother.* **60,** 319–326 (2011).
572. Davies, M. A. & Samuels, Y. Analysis of the genome to personalize therapy for melanoma. *Oncogene* **29,** 5545–5555 (2010).
573. Sherr, C. J. Principles of Tumor Suppression. *Cell* **116,** 235–246 (2004).
574. Croce, C. M. Oncogenes and Cancer. *N. Engl. J. Med.* **358,** 502–511 (2008).
575. Baylin, S. B. DNA methylation and gene silencing in cancer. *Nat. Clin. Pract. Oncol.* **2,** S4–S11 (2005).
576. Waris, G. & Ahsan, H. Reactive oxygen species: role in the development of cancer and various chronic conditions. *J. Carcinog.* **5,** 14 (2006).
577. Robey, I. F., Lien, A. D., Welsh, S. J., Baggett, B. K. & Gillies, R. J. Hypoxia-inducible factor-1alpha and the glycolytic phenotype in tumors. *Neoplasia* **7,** 324–30 (2005).
578. Vesely, M. D., Kershaw, M. H., Schreiber, R. D. & Smyth, M. J. Natural Innate and Adaptive Immunity to Cancer. *Annu. Rev. Immunol.* **29,** 235–271 (2011).
579. Dunn, G. P., Old, L. J. & Schreiber, R. D. The Three Es of Cancer Immunoediting. *Annu. Rev. Immunol.* **22,** 329–360 (2004).
580. Schreiber, R. D., Old, L. J. & Smyth, M. J. Cancer immunoediting: Integrating immunity's roles in cancer suppression and promotion. *Science* **331,** 1565–1570 (2011).
581. Coulie, P. G., Van den Eynde, B. J., van der Bruggen, P. & Boon, T. Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. *Nat. Rev. Cancer* **14,** 135–146 (2014).
582. Guillerey, C. & Smyth, M. J. in *Current Topics in Microbiology and Immunology* **395,** 115–145 (Springer, Cham, 2015).
583. Vignali, D. A. A., Collison, L. W. & Workman, C. J. How regulatory T cells work. *Nat. Rev. Immunol.* **8,** 523–532 (2008).
584. Ghiringhelli, F. *et al.* CD4 + CD25 + regulatory T cells inhibit natural killer cell functions in a transforming growth factor–β–dependent manner. *J. Exp. Med.* **202,** 1075–1085 (2005).
585. Dahlberg, C. I. M., Sarhan, D., Chrobok, M., Duru, A. D. & Alici, E. Natural Killer Cell-Based Therapies Targeting Cancer: Possible Strategies to Gain and Sustain Anti-Tumor Activity. *Front. Immunol.* **6,** 605 (2015).
586. Umansky, V., Blattner, C., Gebhardt, C. & Utikal, J. The Role of Myeloid-Derived Suppressor Cells (MDSC) in Cancer Progression. *Vaccines* **4,** 36 (2016).
587. Umansky, V. *et al.* Myeloid-derived suppressor cells and tumor escape from immune surveillance. *Semin. Immunopathol.* **39,** 295–305 (2017).
588. Schlecker, E. *et al.* Tumor-infiltrating monocytic myeloid-derived suppressor cells mediate CCR5-dependent recruitment of regulatory T cells favoring tumor growth. *J. Immunol.* **189,** 5602–11 (2012).
589. Marvel, D. & Gabrilovich, D. I. Myeloid-derived suppressor cells in the tumor microenvironment: expect the unexpected. *J. Clin. Invest.* **125,** 3356–3364 (2015).
590. Fleming, V. *et al.* Targeting Myeloid-Derived Suppressor Cells to Bypass Tumor-Induced Immunosuppression. *Front. Immunol.* **9,** 398 (2018).
591. Couzin-Frankel, J. Cancer Immunotherapy. *Science (80-.).* **342,** 1432 LP-1433 (2013).
592. Fang, F., Xiao, W. & Tian, Z. NK cell-based immunotherapy for cancer. *Semin. Immunol.* **31,** 37–54 (2017).

593. Imai, K., Matsuyama, S., Miyake, S., Suga, K. & Nakachi, K. Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet* **356,** 1795–1799 (2000).
594. Roder, J. C. *et al.* A new immunodeficiency disorder in humans involving NK cells. *Nature* **284,** 553–555 (1980).
595. Sullivan, J., Byron, K., Brewster, F. & Purtilo, D. Deficient natural killer cell activity in x-linked lymphoproliferative syndrome. *Science (80-.).* **210,** 543–545 (1980).
596. Hersey, P., Edwards, A., Honeyman, M. & McCarthy, W. H. Low natural-killer-cell activity in familial melanoma patients and their relatives. *Br. J. Cancer* **40,** 113–122 (1979).
597. Kim, S., Iizuka, K., Aguila, H. L., Weissman, I. L. & Yokoyama, W. M. In vivo natural killer cell activities revealed by natural killer cell-deficient mice. *Proc. Natl. Acad. Sci.* **97,** 2731–2736 (2000).
598. Stojanovic, A. & Cerwenka, A. Natural killer cells and solid tumors. *J. Innate Immun.* **3,** 355–64 (2011).
599. Stojanovic, A., Correia, M. P. & Cerwenka, A. Shaping of NK Cell Responses by the Tumor Microenvironment. *Cancer Microenviron.* **6,** 135–146 (2013).
600. Halama, N. *et al.* Natural killer cells are scarce in colorectal carcinoma tissue despite high levels of chemokines and cytokines. *Clin. Cancer Res.* **17,** 678–89 (2011).
601. Platonova, S. *et al.* Profound coordinated alterations of intratumoral NK cell phenotype and function in lung carcinoma. *Cancer Res.* **71,** 5412–22 (2011).
602. Kelly, J. M. *et al.* Induction of tumor-specific T cell memory by NK cell–mediated tumor rejection. *Nat. Immunol.* **3,** 83–90 (2002).
603. Smyth, M. J., Hayakawa, Y., Takeda, K. & Yagita, H. New aspects of natural-killer-cell surveillance and therapy of cancer. *Nat. Rev. Cancer* **2,** 850–861 (2002).
604. Gorelik, E., Wiltrout, R. H., Okumura, K., Habu, S. & Herberman, R. B. Role of NK cells in the control of metastatic spread and growth of tumor cells in mice. *Int. J. Cancer* **30,** 107–112 (1982).
605. López-Soto, A., Gonzalez, S., Smyth, M. J. & Galluzzi, L. Control of Metastasis by NK Cells. *Cancer Cell* **32,** 135–154 (2017).
606. Ruggeri, L. *et al.* Effectiveness of Donor Natural Killer Cell Alloreactivity in Mismatched Hematopoietic Transplants. *Science (80-.).* **295,** 2097–2100 (2002).
607. Ruggeri, L., Aversa, F., Martelli, M. F. & Velardi, A. Allogeneic hematopoietic transplantation and natural killer cell recognition of missing self. *Immunol. Rev.* **214,** 202–218 (2006).
608. Ljunggren, H.-G. G. & Malmberg, K.-J. J. Prospects for the use of NK cells in immunotherapy of human cancer. *Nat. Rev. Immunol.* **7,** 329–339 (2007).
609. Pahl, J. & Cerwenka, A. Tricking the balance: NK cells in anti-cancer immunity. *Immunobiology* **222,** 11–20 (2017).
610. Vivier, E., Ugolini, S., Blaise, D., Chabannon, C. & Brossay, L. Targeting natural killer cells and natural killer T cells in cancer. *Nat. Rev. Immunol.* **12,** 239–252 (2012).
611. Böttcher, J. P. *et al.* NK Cells Stimulate Recruitment of cDC1 into the Tumor Microenvironment Promoting Cancer Immune Control. *Cell* **172,** 1022–1037.e14 (2018).
612. Crouse, J., Xu, H. C., Lang, P. A. & Oxenius, A. NK cells regulating T cell responses: mechanisms and outcome. *Trends Immunol.* **36,** 49–58 (2015).
613. Domogala, A., Madrigal, J. A. & Saudemont, A. Natural Killer Cell Immunotherapy: From Bench to Bedside. *Front. Immunol.* **6,** 264 (2015).
614. Austin, R., Smyth, M. J. & Lane, S. W. Harnessing the immune system in acute myeloid leukaemia. *Crit. Rev. Oncol. Hematol.* **103,** 62–77 (2016).
615. Pittari, G., Filippini, P., Gentilcore, G., Grivel, J.-C. & Rutella, S. Revving up Natural Killer Cells and Cytokine-Induced Killer Cells Against Hematological Malignancies. *Front. Immunol.* **6,** 230 (2015).
616. Albertsson, P. A. *et al.* NK cells and the tumour microenvironment: implications for NK-cell function and anti-tumour activity. *Trends Immunol.* **24,** 603–609 (2003).
617. Hölsken, O., Miller, M. & Cerwenka, A. Exploiting natural killer cells for therapy of melanoma. *J. der Dtsch. Dermatologischen Gesellschaft* **13,** 23–28 (2015).
618. Handgretinger, R., Lang, P. & André, M. C. Exploitation of Natural Killer (NK) cells for the treatment of acute leukemia. *Blood* **127,** 3341–3349 (2016).
619. Cichocki, F. *et al.* in *Current Topics in Microbiology and Immunology* **395,** 225–243 (2015).
620. Chabannon, C. *et al.* Manufacturing Natural Killer Cells as Medicinal Products. *Front. Immunol.* **7,** 504 (2016).
621. Rezvani, K. & Rouce, R. H. The Application of Natural Killer Cell Immunotherapy for the Treatment of Cancer. *Front. Immunol.* **6,** 578 (2015).

622. Knorr, D. A. & Kaufman, D. S. Pluripotent stem cell-derived natural killer cells for cancer therapy. *Transl. Res.* **156,** 147–54 (2010).
623. Zeng, J., Tang, S. Y., Toh, L. L. & Wang, S. Generation of "Off-the-Shelf" Natural Killer Cells from Peripheral Blood Cell-Derived Induced Pluripotent Stem Cells. *Stem Cell Reports* **9,** 1796–1812 (2017).
624. Glienke, W. *et al.* Advantages and applications of CAR-expressing natural killer cells. *Front. Pharmacol.* **6,** 21 (2015).
625. Cheng, M., Zhang, J., Jiang, W., Chen, Y. & Tian, Z. Natural killer cell lines in tumor immunotherapy. *Front. Med.* **6,** 56–66 (2012).
626. Tarn, Y. K., Martinson, J. A., Doligosa, K. & Klingernann, H.-G. Ex vivo expansion of the highly cytotoxic human natural killer cell line NK-92 under current good manufacturing practice conditions for clinical adoptive cellular immunotherapy. *Cytotherapy* **5,** 259–272 (2003).
627. Klingemann, H.-G. Natural killer cell-based immunotherapeutic strategies. *Cytotherapy* **7,** 16–22 (2005).
628. Koehl, U. *et al.* Clinical grade purification and expansion of NK cell products for an optimized manufacturing protocol. *Front Oncol* **3,** 118 (2013).
629. Klingemann, H. Challenges of cancer therapy with natural killer cells. *Cytotherapy* **17,** 245–249 (2015).
630. Becker, P. S. A. *et al.* Selection and expansion of natural killer cells for NK cell-based immunotherapy. *Cancer Immunol. Immunother.* **65,** 477–484 (2016).
631. Koehl, U. *et al.* Advances in clinical NK cell studies: Donor selection, manufacturing and quality control. *Oncoimmunology* **5,** e1115178 (2016).
632. Childs, R. W. & Berg, M. Bringing natural killer cells to the clinic: ex vivo manipulation. *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program* **2013,** 234–246 (2013).
633. Knorr, D. A., Bachanova, V., Verneris, M. R. & Miller, J. S. Clinical utility of natural killer cells in cancer therapy and transplantation. *Semin. Immunol.* **26,** 161–172 (2014).
634. Granzin, M. *et al.* Fully automated expansion and activation of clinical-grade natural killer cells for adoptive immunotherapy. *Cytotherapy* **17,** 621–632 (2015).
635. Klöß, S. *et al.* Optimization of Human NK Cell Manufacturing: Fully Automated Separation, Improved Ex Vivo Expansion Using IL-21 with Autologous Feeder Cells, and Generation of Anti-CD123-CAR-Expressing Effector Cells. *Hum. Gene Ther.* **28,** 897–913 (2017).
636. Granzin, M. *et al.* Highly efficient IL-21 and feeder cell-driven ex vivo expansion of human NK cells with therapeutic activity in a xenograft mouse model of melanoma. *Oncoimmunology* **5,** e1219007 (2016).
637. Law, T. M. *et al.* Phase iii randomized trial of interleukin-2 with or without lymphokine-activated killer cells in the treatment of patients with advanced renal cell carcinoma. *Cancer* **76,** 824–832 (1995).
638. Rosenberg, S. A. *et al.* Observations on the Systemic Administration of Autologous Lymphokine-Activated Killer Cells and Recombinant Interleukin-2 to Patients with Metastatic Cancer. *N. Engl. J. Med.* **313,** 1485–1492 (1985).
639. Rayner, A. A., Grimm, E. A., Lotze, M. T., Chu, E. W. & Rosenberg, S. A. Lymphokine-activated killer (LAK) cells. Analysis of factors relevant to the immunotherapy of human cancer. *Cancer* **55,** 1327–1333 (1985).
640. Parkhurst, M. R., Riley, J. P., Dudley, M. E. & Rosenberg, S. A. Adoptive Transfer of Autologous Natural Killer Cells Leads to High Levels of Circulating Natural Killer Cells but Does Not Mediate Tumor Regression. *Clin. Cancer Res.* **17,** 6287–6297 (2011).
641. Burns, L. J. *et al.* IL-2-based immunotherapy after autologous transplantation for lymphoma and breast cancer induces immune activation and cytokine release: a phase I/II trial. *Bone Marrow Transplant.* **32,** 177–86 (2003).
642. Bachanova, V. *et al.* Clearance of acute myeloid leukemia by haploidentical natural killer cells is improved using IL-2 diphtheria toxin fusion protein. *Blood* **123,** 3855–3863 (2014).
643. Bachanova, V. *et al.* Allogeneic natural killer cells for refractory lymphoma. *Cancer Immunol. Immunother.* **59,** 1739–1744 (2010).
644. Sim, G. C. *et al.* IL-2 therapy promotes suppressive ICOS+ Treg expansion in melanoma patients. *J. Clin. Invest.* **124,** 99–110 (2014).
645. Geller, M. A. *et al.* A phase II study of allogeneic natural killer cell therapy to treat patients with recurrent ovarian and breast cancer. *Cytotherapy* **13,** 98–107 (2011).
646. Geller, M. A. & Miller, J. S. Use of allogeneic NK cells for cancer immunotherapy. *Immunotherapy*

3, 1445–59 (2011).
647. Waldmann, T. A. Interleukin-15 in the treatment of cancer. *Expert Rev. Clin. Immunol.* **10,** 1689–1701 (2014).
648. Conlon, K. C. *et al.* Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. *J. Clin. Oncol.* **33,** 74–82 (2015).
649. Porrata, L. F. *et al.* Interleukin-15 affects patient survival through natural killer cell recovery after autologous hematopoietic stem cell transplantation for non-Hodgkin lymphomas. *Clin. Dev. Immunol.* **2010,** 914945 (2010).
650. Kim, P. S. *et al.* IL-15 superagonist/IL-15RαSushi-Fc fusion complex (IL-15SA/IL-15RαSu-Fc; ALT-803) markedly enhances specific subpopulations of NK and memory CD8+ T cells, and mediates potent anti-tumor activity against murine breast and colon carcinom. *Oncotarget* **7,** 16130–16145 (2016).
651. Liu, B. *et al.* A Novel Fusion of ALT-803 (Interleukin (IL)-15 Superagonist) with an Antibody Demonstrates Antigen-specific Antitumor Responses. *J. Biol. Chem.* **291,** 23869–23881 (2016).
652. Dubois, S., Patel, H. J., Zhang, M., Waldmann, T. A. & Müller, J. R. Preassociation of IL-15 with IL-15R alpha-IgG1-Fc enhances its activity on proliferation of NK and CD8+/CD44high T cells and its antitumor action. *J. Immunol.* **180,** 2099–106 (2008).
653. Foley, B. *et al.* The biology of NK cells and their receptors affects clinical outcomes after hematopoietic cell transplantation (HCT). *Immunol. Rev.* **258,** 45–63 (2014).
654. Zhang, L., Chu, J., Yu, J. & Wei, W. Cellular and molecular mechanisms in graft-versus-host disease. *J. Leukoc. Biol.* **99,** 279–287 (2016).
655. Ruggeri, L. *et al.* Role of Natural Killer Cell Alloreactivity in HLA-Mismatched Hematopoietic Stem Cell Transplantation. *Blood* **94,** (1999).
656. Ruggeri, L. *et al.* Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: Challenging its predictive value. *Blood* **110,** 433–440 (2007).
657. Mohty, M. *et al.* Recovery of Lymphocyte and Dendritic Cell Subsets Following Reduced Intensity Allogeneic Bone Marrow Transplantation. *Hematology* **7,** 157–164 (2002).
658. Velardi, A., Ruggeri, L., Alessandro, Moretta & Moretta, L. NK cells: a lesson from mismatched hematopoietic transplantation. *Trends Immunol.* **23,** 438–444 (2002).
659. Ruggeri, L., Parisi, S., Urbani, E. & Curti, A. Alloreactive Natural Killer Cells for the Treatment of Acute Myeloid Leukemia: From Stem Cell Transplantation to Adoptive Immunotherapy. *Front. Immunol.* **6,** 479 (2015).
660. Cooley, S. *et al.* Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. *Blood* **116,** 2411–2419 (2010).
661. Pende, D. *et al.* Anti-leukemia activity of alloreactive NK cells in KIR ligand-mismatched haploidentical HSCT for pediatric patients: evaluation of the functional role of activating KIR and redefinition of inhibitory KIR specificity. *Blood* **113,** 3119–3129 (2009).
662. Verheyden, S., Schots, R., Duquet, W. & Demanet, C. A defined donor activating natural killer cell receptor genotype protects against leukemic relapse after related HLA-identical hematopoietic stem cell transplantation. *Leukemia* **19,** 1446–1451 (2005).
663. Bachanova, V. *et al.* Donor KIR B Genotype Improves Progression-Free Survival of Non-Hodgkin Lymphoma Patients Receiving Unrelated Donor Transplantation. *Biol. Blood Marrow Transplant.* (2016). doi:10.1016/j.bbmt.2016.05.016
664. Soiffer, R. J. Donor lymphocyte infusions for acute myeloid leukaemia. *Best Pract. Res. Clin. Haematol.* **21,** 455–466 (2008).
665. Kolb, H.-J., Simoes, B. & Schmid, C. Cellular immunotherapy after allogeneic stem cell transplantation in hematologic malignancies. *Curr. Opin. Oncol.* **16,** (2004).
666. Lee, D. A. *et al.* Haploidentical Natural Killer Cells Infused before Allogeneic Stem Cell Transplantation for Myeloid Malignancies: A Phase I Trial. *Biol. Blood Marrow Transplant.* **22,** 1290–1298 (2016).
667. Rubnitz, J. E. *et al.* NKAML: A pilot study to determine the safety and feasibility of haploidentical natural killer cell transplantation in childhood acute myeloid leukemia. *J. Clin. Oncol.* **28,** 955–959 (2010).
668. Curti, A. *et al.* Successful transfer of alloreactive haploidentical KIR ligand-mismatched natural killer cells after infusion in elderly high risk acute myeloid leukemia patients. *Blood* **118,** 3273–3279 (2011).
669. Miller, J. S. *et al.* Successful adoptive transfer and in vivo expansion of human haploidentical NK

cells in patients with cancer. *Blood* **105,** 3051–3057 (2005).
670. Passweg, J. R. *et al.* Purified donor NK-lymphocyte infusion to consolidate engraftment after haploidentical stem cell transplantation. *Leukemia* **18,** 1835–1838 (2004).
671. Shi, J. *et al.* Infusion of haplo-identical killer immunoglobulin-like receptor ligand mismatched NK cells for relapsed myeloma in the setting of autologous stem cell transplantation. *Br. J. Haematol.* **143,** 641–653 (2008).
672. Smyth, M. J., Cretney, E., Kershaw, M. H. & Hayakawa, Y. Cytokines in cancer immunity and immunotherapy. *Immunol. Rev.* **202,** 275–293 (2004).
673. Fehniger, T. A. & Cooper, M. A. Harnessing NK Cell Memory for Cancer Immunotherapy. *Trends Immunol.* **37,** 877–888 (2016).
674. Stern, M. *et al.* Pre-emptive immunotherapy with purified natural killer cells after haploidentical SCT: a prospective phase II study in two centers. *Bone Marrow Transplant.* **48,** 433–438 (2013).
675. Shah, N. N. *et al.* Acute GVHD in patients receiving IL-15/4-1BBL activated NK cells following T-cell-depleted stem cell transplantation. *Blood* **125,** 784–792 (2015).
676. Choi, I. *et al.* Donor-Derived Natural Killer Cells Infused after Human Leukocyte Antigen–Haploidentical Hematopoietic Cell Transplantation: A Dose-Escalation Study. *Biol. Blood Marrow Transplant.* **20,** 696–704 (2014).
677. Bishara, A. *et al.* The beneficial role of inhibitory KIR genes of HLA class I NK epitopes in haploidentically mismatched stem cell allografts may be masked by residual donor-alloreactive T cells causing GVHD. *Tissue Antigens* **63,** 204–211 (2004).
678. Miller, J. S. *et al.* Missing KIR ligands are associated with less relapse and increased graft-versus-host disease (GVHD) following unrelated donor allogeneic HCT. *Blood* **109,** 5058–5061 (2007).
679. Stojanovic, A., Correia, M. P. & Cerwenka, A. Shaping of NK Cell Responses by the Tumor Microenvironment. *Cancer Microenviron.* **6,** 135–146 (2013).
680. Childs, R. W. & Carlsten, M. Therapeutic approaches to enhance natural killer cell cytotoxicity against cancer: the force awakens. *Nat. Rev. Drug Discov.* **14,** 487–98 (2015).
681. Guillerey, C., Huntington, N. D. & Smyth, M. J. Targeting natural killer cells in cancer immunotherapy. *Nat. Immunol.* **17,** 1025–1036 (2016).
682. Morvan, M. G. & Lanier, L. L. NK cells and cancer: you can teach innate cells new tricks. *Nat. Rev. Cancer* **16,** 7–19 (2016).
683. Davis, Z. B., Vallera, D. A., Miller, J. S. & Felices, M. Natural killer cells unleashed: Checkpoint receptor blockade and BiKE/TriKE utilization in NK-mediated anti-tumor immunotherapy. *Semin. Immunol.* **31,** 64–75 (2017).
684. Vallera, D. A. *et al.* IL15 Trispecific Killer Engagers (TriKE) Make Natural Killer Cells Specific to CD33+Targets while Also Inducing Persistence, in Vivo Expansion, and Enhanced Function. *Clin. Cancer Res.* **22,** 3440–3450 (2016).
685. Srivastava, S. & Riddell, S. R. Engineering CAR-T cells: Design concepts. *Trends Immunol.* **36,** 494–502 (2015).
686. Kalaitsidou, M., Kueberuwa, G., Schütt, A. & Gilham, D. E. CAR T-cell therapy: toxicity and the relevance of preclinical models. *Immunotherapy* **7,** 487–497 (2015).
687. Mehta, R. S. & Rezvani, K. Chimeric Antigen Receptor Expressing Natural Killer Cells for the Immunotherapy of Cancer. *Front. Immunol.* **9,** 283 (2018).
688. Hermanson, D. L. & Kaufman, D. S. Utilizing Chimeric Antigen Receptors to Direct Natural Killer Cell Activity. *Front. Immunol.* **6,** 195 (2015).
689. Oberschmidt, O., Kloess, S. & Koehl, U. Redirected Primary Human Chimeric Antigen Receptor Natural Killer Cells As an "Off-the-Shelf Immunotherapy" for Improvement in Cancer Treatment. *Front. Immunol.* **8,** 654 (2017).
690. Zhang, C. *et al.* Chimeric Antigen Receptor-Engineered NK-92 Cells: An Off-the-Shelf Cellular Therapeutic for Targeted Elimination of Cancer Cells and Induction of Protective Antitumor Immunity. *Front. Immunol.* **8,** 533 (2017).
691. Chiossone, L., Vienne, M., Kerdiles, Y. M. & Vivier, E. Natural killer cell immunotherapies against cancer: checkpoint inhibitors and more. *Semin. Immunol.* **31,** 55–63 (2017).
692. Muntasell, A. *et al.* Targeting NK-cell checkpoints for cancer immunotherapy. *Curr. Opin. Immunol.* **45,** 73–81 (2017).
693. Thielens, A., Vivier, E. & Romagné, F. NK cell MHC class I specific receptors (KIR): from biology to clinical intervention. *Curr. Opin. Immunol.* **24,** 239–245 (2012).
694. Ruggeri, L. *et al.* Effects of anti-NKG2A antibody administration on leukemia and normal hematopoietic cells. *Haematologica* **101,** 626–33 (2016).
695. Romagne, F. *et al.* Preclinical characterization of 1-7F9, a novel human anti-KIR receptor

therapeutic antibody that augments natural killer-mediated killing of tumor cells. *Blood* **114,** 2667–2677 (2009).
696. Sola, C. *et al.* Genetic and antibody-mediated reprogramming of natural killer cell missing-self recognition in vivo. *Proc. Natl. Acad. Sci.* **106,** 12879–12884 (2009).
697. Kohrt, H. E. *et al.* Anti-KIR antibody enhancement of anti-lymphoma activity of natural killer cells as monotherapy and in combination with anti-CD20 antibodies. *Blood* **123,** 678–686 (2014).
698. Nijhof, I. S. *et al.* Daratumumab-Mediated lysis of primary multiple myeloma cells is enhanced in combination with the human Anti-KIR antibody IPH2102 and lenalidomide. *Haematologica* **100,** 263–268 (2015).
699. Marie-Cardine, A. *et al.* IPH4102, a Humanized KIR3DL2 Antibody with Potent Activity against Cutaneous T-cell Lymphoma. *Cancer Res.* **74,** 6060–70 (2014).
700. Benson, D. M. *et al.* A phase I trial of the anti-KIR antibody IPH2101 in patients with relapsed/refractory multiple myeloma. *Blood* **120,** 4324–4333 (2012).
701. Benson, D. M. *et al.* IPH2101, a novel anti-inhibitory KIR antibody, and lenalidomide combine to enhance the natural killer cell versus multiple myeloma effect. *Blood* **118,** 6387–6391 (2011).
702. Vey, N. *et al.* A phase I trial of the anti-inhibitory KIR monoclonal antibody IPH2101 for acute myeloid leukemia (AML) in complete remission. *Blood* **120,** 1–3 (2012).
703. Korde, N. *et al.* A phase II trial of pan-KIR2D blockade with IPH2101 in smoldering multiple myeloma. *Haematologica* **99,** e81–e83 (2014).
704. Benson, D. M. *et al.* A Phase I Trial of the Anti-KIR Antibody IPH2101 and Lenalidomide in Patients with Relapsed/Refractory Multiple Myeloma. *Clin. Cancer Res.* **21,** 4055–4061 (2015).
705. Yalniz, F. F. *et al.* A Pilot Trial of Lirilumab With or Without Azacitidine for Patients With Myelodysplastic Syndrome. *Clin. Lymphoma Myeloma Leuk.* (2018). doi:10.1016/j.clml.2018.06.011
706. Vey, N. *et al.* A phase 1 study of lirilumab (antibody against killer immunoglobulin-like receptor antibody KIR2D; IPH2102) in patients with solid tumors and hematologic malignancies. *Oncotarget* **9,** 17675–17688 (2018).
707. Carlsten, M. *et al.* Checkpoint inhibition of KIR2D with the monoclonal antibody IPH2101 induces contraction and hyporesponsiveness of NK cells in patients with myeloma. *Clin. Cancer Res.* **22,** 5211–5222 (2016).
708. He, Y. *et al.* The combination of anti-KIR monoclonal antibodies with anti-PD-1/PD-L1 monoclonal antibodies could be a critical breakthrough in overcoming tumor immune escape in NSCLC. *Drug Des. Devel. Ther.* **Volume 12,** 981–986 (2018).
709. Binyamin, L. *et al.* Blocking NK cell inhibitory self-recognition promotes antibody-dependent cellular cytotoxicity in a model of anti-lymphoma therapy. *J. Immunol.* **180,** 6392–401 (2008).
710. Ewen, E.-M., Pahl, J. H. W., Miller, M., Watzl, C. & Cerwenka, A. KIR downregulation by IL-12/15/18 unleashes human NK cells from KIR/HLA-I inhibition and enhances killing of tumor cells. *Eur. J. Immunol.* **48,** (2018).
711. Romee, R. *et al.* NK cell CD16 surface expression and function is regulated by a disintegrin and metalloprotease-17 (ADAM17). *Blood* **121,** 3599–3608 (2013).
712. Smith, M. a *et al.* PRDM1/Blimp-1 Controls Effector Cytokine Production in Human NK Cells. *J. Immunol.* **185,** 6058–6067 (2010).
713. Allard, M. *et al.* HLA-E-restricted cross-recognition of allogeneic endothelial cells by CMV-associated CD8 T cells: a potential risk factor following transplantation. *PLoS One* **7,** e50951 (2012).
714. Guerra, N. *et al.* Killer inhibitory receptor (CD158b) modulates the lytic activity of tumor-specific T lymphocytes infiltrating renal cell carcinomas. *Blood* **95,** (2000).
715. Binstadt, B. A. *et al.* SLP-76 is a direct substrate of SHP-1 recruited to killer cell inhibitory receptors. *J. Biol. Chem.* **273,** 27518–23 (1998).
716. Peruzzi, G. *et al.* Membrane-Type 6 Matrix Metalloproteinase Regulates the Activation-Induced Downmodulation of CD16 in Human Primary NK Cells. *J. Immunol.* **191,** 1883–1894 (2013).
717. Shimizu, Y. & DeMars, R. Production of human cells expressing individual transferred HLA-A,-B,-C genes using an HLA-A,-B,-C null human cell line. *J. Immunol.* **142,** 3320–8 (1989).
718. Rölle, A. & Brodin, P. Immune Adaptation to Environmental Influence: The Case of NK Cells and HCMV. *Trends Immunol.* **37,** 233–243 (2016).
719. Gumá, M. *et al.* Expansion of CD94/NKG2C+ NK cells in response to human cytomegalovirus-infected fibroblasts. *Blood* **107,** 3624–31 (2006).
720. Yoon, S. R. *et al.* Generation of donor natural killer cells from CD34+ progenitor cells and subsequent infusion after HLA-mismatched allogeneic hematopoietic cell transplantation: a

feasibility study. *Bone Marrow Transplant.* **45,** 1038–1046 (2010).
721. Vendrame, E., Fukuyama, J., Strauss-Albee, D. M., Holmes, S. & Blish, C. A. Mass Cytometry Analytical Approaches Reveal Cytokine-Induced Changes in Natural Killer Cells. *Cytom. Part B Clin. Cytom.* **92,** 57–67 (2017).
722. Hromadnikova, I., Pirkova, P. & Sedlackova, L. Influence of In Vitro IL-2 or IL-15 Alone or in Combination with Hsp-70-Derived 14-mer Peptide (TKD) on the Expression of NK Cell Activatory and Inhibitory Receptors. *Mediators Inflamm.* **2013,** 405295 (2013).
723. Duggan, M. C. *et al.* Co-stimulation of the fc receptor and interleukin-12 receptor on human natural killer cells leads to increased expression of cd25. *Oncoimmunology* **7,** e1381813 (2018).
724. André, P. *et al.* Comparative analysis of human NK cell activation induced by NKG2D and natural cytotoxicity receptors. *Eur. J. Immunol.* **34,** 961–971 (2004).
725. ROSENBERG, S. A. *et al.* Experience with the Use of High-Dose Interleukin-2 in the Treatment of 652 Cancer Patients. *Ann. Surg.* **210,** (1989).
726. Kohrt, H. E. *et al.* CD137 stimulation enhances the antilymphoma activity of anti-CD20 antibodies. *Blood* **117,** 2423–32 (2011).
727. Wu, A. A., Drake, V., Huang, H.-S., Chiu, S. & Zheng, L. Reprogramming the tumor microenvironment: tumor-induced immunosuppressive factors paralyze T cells. *Oncoimmunology* **4,** e1016700 (2015).
728. Gays, F., Martin, K., Kenefeck, R., Aust, J. G. & Brooks, C. G. Multiple Cytokines Regulate the NK Gene Complex-Encoded Receptor Repertoire of Mature NK Cells and T Cells. *J. Immunol.* **175,** 2938–2947 (2005).
729. de Rham, C. *et al.* The proinflammatory cytokines IL-2, IL-15 and IL-21 modulate the repertoire of mature human natural killer cell receptors. *Arthritis Res. Ther.* **9,** R125 (2007).
730. Kogure, T. *et al.* Killer-cell inhibitory receptors, CD158a/b, are upregulated by interleukin-2, but not interferon-gamma or interleukin-4. *Mediators Inflamm.* **8,** 313–318 (1999).
731. Decot, V. *et al.* Natural-killer cell amplification for adoptive leukemia relapse immunotherapy: Comparison of three cytokines, IL-2, IL-15, or IL-7 and impact on NKG2D, KIR2DL1, and KIR2DL2 expression. *Exp. Hematol.* **38,** 351–362 (2010).
732. Shin, E. C., Choi, K. S., Kim, S. J. & Shin, J. S. Modulation of the Surface Expression of CD158 Killer Cell Ig-like Receptor by Interleukin-2 and Transforming Growth Factor-β. *Yonsei Med. J.* **45,** 510 (2004).
733. van Bergen, J., Stewart, C. A., van den Elsen, P. J. & Trowsdale, J. Structural and functional differences between the promoters of independently expressed killer cell Ig-like receptors. *Eur. J. Immunol.* **35,** 2191–2199 (2005).
734. Presnell, S. R., Zhang, L., Ramilo, C. A., Chan, H.-W. & Lutz, C. T. Functional redundancy of transcription factor-binding sites in the killer cell Ig-like receptor (KIR) gene promoter. *Int. Immunol.* **18,** 1221–32 (2006).
735. Vendelbosch, S. *et al.* Novel insights in the genomic organization and hotspots of recombination in the human KIR locus through analysis of intergenic regions. *Genes Immun.* **16,** 103–111 (2015).
736. Gómez-Lozano, N. *et al.* Epigenetic silencing of potentially functional KIR2DL5 alleles: Implications for the acquisition of KIR repertoires by NK cells. *Eur. J. Immunol.* **37,** 1954–1965 (2007).
737. Vilches, C., Gardiner, C. M. & Parham, P. Gene Structure and Promoter Variation of Expressed and Nonexpressed Variants of the KIR2DL5 Gene. *J. Immunol.* **165,** 6416–6421 (2000).
738. Xu, J., Vallejo, A. N., Jiang, Y., Weyand, C. M. & Goronzy, J. J. Distinct Transcriptional Control Mechanisms of Killer Immunoglobulin-like Receptors in Natural Killer (NK) and in T Cells. *J. Biol. Chem.* **280,** 24277–24285 (2005).
739. Otto, F., Lübbert, M. & Stock, M. Upstream and downstream targets of RUNX proteins. *J. Cell. Biochem.* **89,** 9–18 (2003).
740. Felices, M. *et al.* Notch Signaling at Later Stages of NK Cell Development Enhances KIR Expression and Functional Maturation. *J. Immunol.* **193,** 3344–3354 (2014).
741. Bouchard, C. *et al.* Regulation of cyclin D2 gene expression by the Myc/Max/Mad network: Myc-dependent TRRAP recruitment and histone acetylation at the cyclin D2 promoter. *Genes Dev.* **15,** 2042–7 (2001).
742. Cichocki, F. *et al.* Cutting edge: microRNA-181 promotes human NK cell development by regulating Notch signaling. *J. Immunol.* **187,** 6171–6175 (2011).
743. Ishitani, T. *et al.* Nemo-like kinase suppresses Notch signalling by interfering with formation of the Notch active transcriptional complex. *Nat. Cell Biol.* **12,** 278–285 (2010).
744. Hüber, C. M. *et al.* IL-12/15/18-preactivated NK cells suppress GvHD in a mouse model of

mismatched hematopoietic cell transplantation. *Eur. J. Immunol.* **45,** 1727–1735 (2015).
745. Song, Y. *et al.* IL-12/IL-18-preactivated donor NK cells enhance GVL effects and mitigate GvHD after allogeneic hematopoietic stem cell transplantation. *Eur. J. Immunol.* **48,** 670–682 (2018).
746. Liu, L. L. *et al.* Harnessing adaptive natural killer cells in cancer immunotherapy. *Mol. Oncol.* **9,** 1904–17 (2015).
747. Malmberg, K.-J., Sohlberg, E., Goodridge, J. P. & Ljunggren, H.-G. Immune selection during tumor checkpoint inhibition therapy paves way for NK-cell "missing self" recognition. *Immunogenetics* **69,** (2017).
748. Sarhan, D. *et al.* Adaptive NK Cells with Low TIGIT Expression Are Inherently Resistant to Myeloid-Derived Suppressor Cells. *Cancer Res.* **76,** 5696–5706 (2016).
749. Wiernik, A. *et al.* Targeting Natural Killer Cells to Acute Myeloid Leukemia In Vitro with a CD16 x 33 Bispecific Killer Cell Engager and ADAM17 Inhibition. *Clin. Cancer Res.* **19,** 3844–3855 (2013).
750. Zhou, Q., Gil-Krzewska, A., Peruzzi, G. & Borrego, F. Matrix metalloproteinases inhibition promotes the polyfunctionality of human natural killer cells in therapeutic antibody-based anti-tumour immunotherapy. *Clin. Exp. Immunol.* **173,** 131–9 (2013).
751. Chan, C. J., Smyth, M. J. & Martinet, L. Molecular mechanisms of natural killer cell activation in response to cellular stress. *Cell Death Differ.* **21,** 5–14 (2014).

9. ABBREVIATIONS

221	721.221 EBV transformed lymphoblastoid cell line
7-AAD	7-aminoactinomycin D
ADCC	Antibody-dependent cellular cytotoxicity or antibody-dependent cell-mediated cytotoxicity
AICL	Activation-induced C-type lectin
AML	Acute myeloid leukemia
APC	Antigen presenting cell
Bat-3	Human leukocyte antigen-b-associated transcript 3
Bcl-2	B-cell lymphoma 2
BCR	B cell receptor
BCR	B cell receptor
BiKEs	bispecific killer engagers
BM	Bone marrow
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CLP	Common lymphoid progenitor
CTLA-4	Cytotoxic t-lymphocyte-associated protein 4
DAMPs	Damage-associated molecular patterns
DC	Dendritic cell
DD	Death domain
DISC	Death-inducing signaling complex
DKFZ	Deutsches Krebsforschungszentrum / German Cancer Research Center
DLL1	Delta-like protein 1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAM-I	Dnax accessory molecule-1
dsRNA	Double-stranded RNA
E:T	Effector-to-target
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting

FcR	Fragment, crystalizable-receptor
FCS	Fetal calf serum
FcγR	Fragment, crystallizable γ-receptor
FoxP3	Forkhead box protein P3
GM-CSF	Granulocyte macrophage colony-stimulating factor
Grb-2	Growth factor receptor-bound protein 2
GvHD	Graft-vs-host disease
GvL	Graft-vs-leukemia
H-60	Histocompatibility antigen 60
HCMV	Human cytomegalovirus
hESC	Human embryonic stem cell
HLA	Human leukocyte antigen
HMGB1	Chromatin-associated protein high-mobility group box 1
HSC	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
IFN-α/γ	Interferon-alpha/gamma
Ig	Immunoglobulin
IL	Interleukin
IL-2R	Interleukin-2 receptor
ILC	Innate lymphoid cell
iNK	Immature NK cells
iPSC	Induced pluripotent stem cell
IRAK	Interleukin-1 receptor-associated kinase
ITAM	Immune tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITT	Immunoglobulin tail tyrosine-like motif
JAK	Janus tyrosine-kinase
KIR	Killer immunoglobulin-like receptor
KIR	Killer cell immunoglobulin-like
LAK	Lymphokine-activated killer
LFA1	Leukocyte functional antigen 1
lncRNA	Long non-coding RNA
LPS	Lipopolysaccharides
LRC	Leukocyte receptor complex
LTi	Lymphoid tissue inducer cell
mAb	Monoclonal antibody
MACS	Magnetic activated cell sorting
MAPK	Mitogen-activated protein kinase

MCMV	murine cytomegalovirus
MDSC	Myeloid-derived suppressor cell
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MIC-A/B	MHC class I chain-related gene A/B
mIgG	Mouse immunoglobulin G
min	Minute
miRNA	Micro RNA
MM	Multiple myeloma
MMP	Matrix-metalloproteinase
MMPi	Matrix-metalloproteinase inhibitor
mNK	Mature NK cell
MULT-1	Murine UL16-binding-like transcript-1
MyD88	Myeloid differentiation primary response gene 88
n.d.	Not detectable
NCAM	Neural cell adhesion molecule
NCR	Natural cytotoxicity receptor
NF-κB	Nuclear factor-κB
NK cell	Natural killer cell
NKG2A/C/D	Natural killer group 2 A/C/D
NKP	NK cell precursor
NLK	Nemo-like kinase
p.i.	Post infection
PAMPs	Pathogen-associated molecular patterns
PB	Peripheral blood
pbNK	Peripheral blood NK cells
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death 1 ligand 1
PI3K	Phosphatidylinositol-3 kinase
PKC	Protein kinase C
PRR	Pattern recognition receptor
Rae-1	Retinoic acid early transcript-1 molecules
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RT	Room temperature
SHIP	SH2 domain-containing inositol-5-phosphatase

SHP	Src homology region 2 domain-containing phosphatases
STAT	Signal transducer and activator of transcription
TAM	Tumor-associated macrophage
TCR	T cell receptor
TGF-β	Transforming growth factor β
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TILs	Tumor-infiltrating lymphocytes
TLR	Toll-like receptor
TNFRSF	Tumor necrosis factor receptor superfamily
TNF-α	Tumor necrosis factor alpha
TRAF	TNF receptor associated factor
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
Tregs	Regulatory T cell
TYK2	Tyrosine kinase 2
UCB	Umbilical cord blood
ULBP	UL16 binding protein
VEGF-A	Vascular endothelial growth factor-A
w/o	Without
α-a	Anti-

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