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EVOLUTION OF NK-MEDIATED TARGET RECOGNITION UNDER THE PRESSURE OF PHYSIOLOGIC OR PATHOLOGIC STIMULI

Topic Editors
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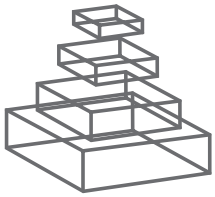

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EVOLUTION OF NK-MEDIATED TARGET RECOGNITION UNDER THE PRESSURE OF PHYSIOLOGIC OR PATHOLOGIC STIMULI

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Since their discovery NK cells have come out as potential tools to fight cancer and viruses. This finding early urged different groups to study the mechanisms governing NK cell function. The identification of the MHC-I-specific inhibitory receptors (i.e. KIRs, NKG2A and certain Ly49 molecules) allowed defining rather rapidly how NK cells could avoid self-aggression and how they could be directed towards targets that were forced, by viral infection or tumor transformation, to down-regulate MHC-I expression. In a second time, also the repertoire of surface activating receptors addressing NK cytotoxicity towards tumors and pathogens was mostly defined.

In spite of the first findings, however, most recent studies may suggest that NK cells and their receptors might not have been evolved to kill tumor targets and, perhaps, they might have been only partially influenced, in their evolution, by the need of recognizing viruses. Indeed certain NK receptors known to activate NK cell cytotoxicity (NKp30, DNAM-1, NKp80) can also participate at regulatory interactions occurring between NK and myeloid cells. In addition, a peculiar NK cell subset which intensively populate decidua during the first trimester of pregnancy, through the engagement of specific receptors and the interaction with decidual DC, produce chemokines and pro-angiogenic cytokines, and induce Tregs. Thus, in this context, NK cells favor decidua vascularization and development of the (semiallogeneic) foetus in a tolerant environment.

Viruses have nevertheless played an important role in shaping the NK cell receptor repertoire. Several studies have unveiled clues of the evolutionary struggle between these pathogens and NK cells. Different NK receptors, including NKp46, NKp30, NKp44, NKG2D, NKG2C, Ly49, and certain KIRs have been demonstrated to recognize virus-encoded or virus-induced ligands. The expression of TLR specifically recognizing microbial products, together with the unexpected role of KIR3DL2 in shuttling these products to TLR-containing endosomes have also been documented in NK cells. On the other side, different viral immune evasion molecules have been shown to interfere with the expression of ligands for T or NK cell activating receptors. In addition, viral infections can occur in the reproductive stage of life cycle, and may represent a serious threat for the species propagation.

Thus the control of viruses, together with the maintenance of foetus during pregnancy, should represent major evolutionary forces in shaping NK-receptors.

Along this line, the NK-mediated control of tumors should not be under the same evolutionary pressure, as tumors mostly appear later in the life cycle, and the recognition of tumor-encoded ligands may be less efficient (as the NK cell receptors might have not been selected for such aim). This may be the reason why, although displaying strong anti-tumor activity in vitro, NK cells could hardly contain tumor burden in vivo. In addition the pathogen-driven evolution of NK cell function may also favor the role of NK cells in the insurgence of immune-mediated diseases.

This research topic will collect contributions that may clarify the relationships between the evolution of the NK receptors and their role in an efficient recognition of viruses and tumor cells or in immune-mediated diseases.

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NK receptors: tools for a polyvalent cell family

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In these last years, along with the description of new NK cell types playing key roles within different body compartments, it is becoming conceivable that NK cell receptors might have been shaped to fulfill a complex array of functions beyond the simple recognition of pathological cells (1, 2). Such a process of adaptation to multiple needs offers new unexpected viewpoints on the evolution of NK receptors and their possible exploitation in a growing range of diseases. The first article of the present Research Topic will introduce this concept by providing a general discussion on how the major groups of receptors can be used by NK cells inside or outside the classical natural killing function (3). The following articles will go into detail of the various NK cell functions and the involved NK cell subsets, primarily considering the pressure exerted by physiological or pathological stimuli on the related receptors and their specific ligands.

Li and Mariuzza (4) describe the crystal structures of several surface receptors, belonging to the most known NK receptor families, and explain how such information could be critical for the prediction of possible molecular interactions with either extracellular ligands or intracellular signaling molecules. Cassidy et al. (5) focus on the KIR ligands, the MHC-I molecules, and analyze the role of the MHC-I peptide content in such receptor/ligand interaction suggesting that also NK cells can perceive in some ways intracellular modifications in tumor or virally infected cells.

Whether the peptide could also have a role in the HLA-I recognition by activating KIRs (aKIRs) is still unclear. Nevertheless, aKIRs are likely to play a role in infectious diseases, cancer, and autoimmunity. For example, aKIRs-mediated recognition of viruses and consequent NK cell priming has been proposed to occur and to be directly involved in the pathogenesis of the chronic lymphoproliferative disorder of NK cells (CLPD-NK) (6). Remarkably, several CLPD-NK cases present an expansion of $aKIR^+$ NK cells, which are, nevertheless, characterized by reduced NCR-mediated function. In this context, aKIRs have been recently shown to participate in the education of NK cells, rendering them hyporesponsive. Interestingly, education by aKIRs shares features with the hyporesponsiveness induced by chronic stimulation of other activating receptors expressed by NK cells (7).

All the above mentioned articles can provide hints on the process of co-evolution that may have affected KIRs, their autologous ligands and exogenous molecules or peptides, as well as the mechanisms controlling the strength of NK cell response to stimuli. A possible receptor/ligand co-evolution may have also concerned several non-MHC-specific activating receptors. A living recapitulation of how this co-evolution may have occurred is represented by the struggle between NK cells and tumors. Cerboni et al. (8) and Huergo-Zapico et al. (9) describe different molecular pathways that can convey stress or damage signals to enhance the expression of various ligands of NKG2D or DNAM-1 activating receptors. These signals are often related to the process of tumor transformation. Bottino et al. (10), Baier et al. (11), Chouaib et al. (12), and Chretien et al. (13) show that a large part of the non-MHC-specific activating NK receptors can recognize ligands overexpressed on tumors. On the other hand, they also show that really many strategies are executed by the tumor to avoid the NK cell attack. In the end, the authors indicate how a deeper understanding of the forces involved in the NK/tumor struggle is essential to develop more effective NK cell-based immunotherapy against cancer.

Remarkably, the quantitative deficiency of NK cells or the altered expression/function of their receptors witness the negative influence exerted not only by tumor microenvironment but also by viruses. Lugli et al. (14) and Marras et al. (15) show that viruses (i.e., HIV, HCV, and hCMV) are able to affect the functional status and the homeostasis of NK cell population through the modulation/engagement of several surface receptors and the expansion of unconventional poorly functional NK cell subsets.

As above mentioned, because of their polyvalent role, certain receptors may have been exposed to multiple shaping pressures. For example, NK cells that populate decidua (dNK) in the first trimester of pregnancy can use NKp44, NKp30, and KIR2DL4 both to control infections and to promote regulatory interactions [with trophoblasts or dendritic cell (DC)] important for placental development (16). The abundance of dNK cells in the decidua in a specific phase of pregnancy suggests that these cells could be determinant for a safe and successful childbirth, indicating that reproduction may have significantly intervened on the evolution of certain NK receptors. The dual role of certain NK receptors and the promiscuity of their ligands have also been recently highlighted by the characterization of the large family of innate lymphoid cells (ILCs). Cella et al. (17) and Killig et al. (18) discuss this issue. ILCs play a relevant role in the defense against invading microbes, but also in tissue remodeling including the induction of lymphoid tissues as well as the homeostasis of epithelial barrier in the mucose.

They include two different NK-R$^+$ cell types: the conventional NK cells (belonging to group 1 ILCs) and the IL22-producing NCR$^+$ILC3 cells (belonging to group 3 ILCs). Depending on the environmental mediators, engagement of NKp44 and PRRs can mediate release of the ILC3 signature cytokine IL-22 or pro-inflammatory soluble factors thus modulating both epithelial and immune cell functions at mucosal interfaces.

Also, NKp30 can fulfill different tasks, as it can serve as a tool for eliminating altered cells and promoting regulatory interaction with autologous cells. Reiners et al. (19) shows that exosomes derived from both tumor cells and activated DCs can play a relevant role in the regulation of NK cell function. Interestingly, in both cases, interaction of NKp30 with its ligand BAG6 on exosomes can activate NK cells.

Different studies have highlighted the important immunomodulatory role of the crosstalk between NK cells and DCs. Ferlazzo and Morandi (20) describe the new DC subsets recently identified in the human system and suggest that the NK/DC interactions should be considered as a complex network of cell subset cooperation acting in discrete regions of the body to fulfill complementary tasks.

The functional interaction with normal cells (such as DCs or trophoblasts) implies that several NK receptors can recognize autologous ligands. These considerations indicate that a potential role for NK cells also in the development of autoimmune diseases. Poggi and Zocchi (21) and Enk and Mandelboim (22), respectively, describe how the inappropriate interaction with APC or MSC and the ectopic/abnormal expression of certain NCR-ligands could be implied on several autoimmune diseases thus contributing to widen the range of pathologies potentially involving NK cells.

In conclusion, the analysis of the NK cell functions and receptors as discussed in this Research Topic indicates that, in spite of the continuous flow of discoveries that have significantly changed key concepts on the biology of NK cells, "the recognition of self," originally postulated by Kärre more than 20 years ago as strategy to drive NK cell cytotoxicity (23), still represents the basis of NK cell function. Exactly following this core strategy, NK cells have evolved their wide range of receptors required to fulfill their ever increasing number of functions. NK receptors mostly recognize autologous ligands whose expression could be induced/regulated by stress, viral infection, cell damage, or activating stimuli. Through these interactions, NK receptors regulate the response to pathogens or dangerous altered cells, but also take part to functional interactions with different elements of peripheral tissues.

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TLR/NCR/KIR: which one to use and when?

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By means of a complex receptor array, Natural killer (NK) cells can recognize variable patterns of ligands and regulate or amplify accordingly their effector functions. Such NK receptors include old, rather conserved, molecules, such as toll-like receptors (TLRs), which enable NK cells to respond both to viral and bacterial products, and newer and evolving molecules, such as killer Ig-like receptors and natural cytotoxicity receptors, which control NK cytotoxicity and are responsible for the elimination of virus-infected or tumor cells without damaging self-unaltered cells. In addition, to rapidly gain new functions NK cells also can acquire new receptors by trogocytosis. Thus, NK cells may have adapted their receptors to different functional needs making them able to play a key role in the modulation of critical events occurring in several compartments of human body (primarily in SLCs but also in decidua during pregnancy). In this review, we will discuss on how the various types of receptors can be used to address specific functions in different immunological contexts.

Keywords: NK cell, TLR, KIR, NCR, CCR7, anti-tumor response, anti-viral response, innate immunity

INTRODUCTION

Natural killer (NK) cells are innate lymphocytes present in all mammalian species capable of mediating multiple effector functions. NK cells express a number of receptors through which they can directly recognize microbial products, can sense aberrant/transformed cells [which lack constitutive self-human leukocyte antigen (HLA)-I molecules and express ligands for activating NK receptors], or even mediate regulatory functions (being an early source of cytokines) (1, 2).

Despite the original definition of NK cells based on their "natural" cytotoxicity against transformed cells in the absence of prior immunization, several studies indicate that NK cells require education/maturation process before killing and carrying out their biological functions (3).

Specialized NK subsets, which display distinct functions according to their typical cell-surface phenotype, exist. In particular, the $CD56^{bright}$ NK cell subset presents little cytolytic activity but releases high amounts of cytokines, whereas $CD56^{dim}$ NK cell subset displays potent cytotoxicity but also high cytokine production in response to specific stimuli (4–6).

The former subset is characterized by the $CD16^{-}KIR^{-}NKG2A^{+}$ phenotype and largely predominates in lymph nodes, according to the expression of CCR7 (the homing receptor for secondary lymphoid compartments, SLCs) (7, 8), whereas the latter subset is $CD16^{+}KIR^{+}$ and/or $NKG2A^{+}$ and prevails in peripheral blood and inflamed tissues where they can be recruited, thanks to the expression of the CXCR1, CX3CR1, and ChemR23 chemokine receptors (4, 9, 10).

Thus, during the early phases of an inflammatory response, $CD56^{dim}$ NK cells may be recruited into inflamed tissues in response to various chemokines. Notably, the extravasation of NK cells do not imply their activation, thus these cells, once reached inflammatory sites, need to be activated to carry out their full effector functions (9). Candidates for NK cell activation are cytokines, released by innate cells, which are known to interact with NK cells (e.g., IL12), and/or engagement of certain activating receptors, including toll-like receptors (TLRs) and/or natural cytotoxicity receptors (NCRs) (11–14).

The stimulatory activity mediated by these receptors can induce NK cell-mediated lysis of tumor/virus-infected cells, the release of pro-inflammatory cytokines and, importantly, can promote the interactions of NK cells with other innate cells (15–17). This latter event can help to boost the innate immune system and promote the development of efficient adaptive immune responses (18).

In this review, the contribution of the different types of NK cell receptors in NK cell activation and their cooperation has been analyzed. In addition, new mechanisms of cell communication that allow the acquisition of unexpected receptor functions and/or novel functional properties have been described (7, 19, 20).

These novel aspects, probably occurring in response to environmental stress such as viral or bacterial infections, disclose new potential implications of NK cells in physiologic and pathologic conditions.

TOLL-LIKE RECEPTORS

Toll-like receptors belong to pattern-recognition receptors (PRRs), which have evolved to recognize conserved features of microbes, the so-called pathogen-associated molecular patterns (PAMPs) (21, 22). TLRs are expressed on NK cells independently of their state of activation and can synergize with chemokine- or cytokine-mediated signals to activate NK cell function (**Table 1A**). Both NK cells and other innate cells express certain TLRs, thus allowing a coordinated response to the same pathogen-derived product. For example, viral dsRNA can act on TLR3 expressed by both myeloid dendritic cells (DCs) and NK cells, recruited by chemokine gradients to inflammatory sites. In the presence of IL12 (released by DCs

Table 1 | (A) TLRs expressed by human NK cells and relative ligands: effect of their interaction; (B) NCRs expressed by human NK cells and relative ligands: effect of their interaction; (C) KIRs expressed by human NK cells and relative ligands: effect of their interaction.

	Ligand(s) type	Ligand(s) expression	Effect of receptor/ligand interaction
(A) TLR			
TLR2	Bacterial lipoprotein (BCG)	Bacteria (27, 28)	Induction of cytotoxicity and cytokine release
TLR3	Double-stranded RNA	Viruses (13)	Induction of cytotoxicity and cytokine release
TLR5	Flagellin	Bacteria (33)	Induction of cytotoxicity and cytokine release
TLR7/8	Single-stranded RNA	Viruses (34, 35)	Induction of cytotoxicity and cytokine release
TLR9	CpG DNA motifs	Bacteria/viruses (13)	Induction of cytotoxicity and cytokine release
(B) NCR			
NKp30	B7-H6	Tumor cells (40)	Activation (cytokine/cytotoxicity)
		Monocytes/neutrophils (48) (induced by TLR engagement)	Activation/regulation?
	BAT3/BAG6	Tumor cells (39)	Activation (cytokine/cytotoxicity)
		DC (47) (induced by TLR engagement)	Activation of NK/DC cross-talk
	ECTV HA	Infected cells (44)	N.D.
	VV HA	Infected cells (44)	Inhibition
NKp44	21spe-MLL5	Tumor cells (41)	Activation (cytokine/cytotoxicity)
	Influenza virus-HA; Sendai virus-HN	Infected cells (42)	Activation (cytokine/cytotoxicity)
	PCNA	Induced on tumor cells (114)	Inhibition
	N.D.	N.D.	IL-22 and TNFα release by ILC3
	N.D.	BCG (29)	Supporting of NK cell activation
	N.D.	Trophoblasts (73, 74)	Activation (cytotoxicity)
NKp46	N.D.	Tumor cells (14, 37)	Activation (cytokine/cytotoxicity)
	Influenza virus-HA; Sendai virus-HN	Infected cells (38, 44)	Activation (cytokine/cytotoxicity)
	N.D.	CMV-infected DC (51)	Activation (cytokine/cytotoxicity)
	NDV-HN	Infected tumor cells (115)	Activation (cytokine/cytotoxicity)
	N.D.	Trophoblasts (73, 74)	Chemokine production by dNK
(C) KIR			
2DL1	C2 epitope	Normal cells	Inhibition
2DL2/DL3	C1/C2 epitope; few HLA-B (C1 epitope)	Normal cells	Inhibition
2DL4	HLA-G	Normal cells	Inhibition/IFNγ induction
	CpG ODN (+ +)	Infected cells?	N.D.
2DL5	N.D.	N.D.	Inhibition
2DS1	C2 epitope	Normal cells; Tumor cells	Activation (cytokine/cytotoxicity)
2DS2	N.D.	N.D.	N.D.
2DS3	N.D.	N.D.	N.D.
2DS4	N.D.	Melanoma cell lines; primary melanoma	Activation (cytokine/cytotoxicity)
	HLA-A11; some C1 and C2 HLA-C	N.D.	Activation (cytokine/cytotoxicity)
2DS5	N.D.	N.D.	N.D.
3DL1	HLA-B and HLA-A (Bw4+)	Normal cells	Inhibition
	CpG ODN (+ + +)	Infected cells?	N.D.
3DL2	HLA-A3, −A11	Normal cells	Inhibition
	CpG ODN (+ + +)	Infected cells?	Activation (cytokine/cytotoxicity)
3DL3	N.D.	N.D.	N.D.
3DS1	CpG ODN (+ + +)	Infected cells?	N.D.
	HIV-peptides?; HCV-peptides?	Infected cells?	N.D.

ECTV, ecteromelia virus; VV, vaccinia virus; NDV, new castle disease virus; dNK, decidual NK cells; N.D., not determined.

after TLR3 stimulation), NK cells respond to dsRNA by improving their killing capabilities: not only against abnormal target cells, but also against immature DCs (iDCs) (13). This latter effect has been proposed as a mechanism by which NK cells can "edit" the process of DC maturation by selecting those DCs that are undergoing appropriate maturation and therefore would best prime T cells after migration to SLCs (23, 24). Moreover, upon the simultaneous stimulation by TLR3 engagement and IL12, NK cells also increase their capability of secreting pro-inflammatory cytokines, which mediate several important functions, including promotion of further DC maturation, anti-viral/anti-tumor effects, and induction of Th1 responses (13, 15, 18).

Similar to DCs, NK cells also express TLR2, specific for products of bacterial origin (25). TLR2 is directly involved in the NK-mediated recognition of *Mycobacterium tuberculosis* (BCG) by NK cells (26–28). In turn, DCs, when exposed to BCG, release large amounts of IL12 that induce an amplification of the effector functions of NK cells. These include the enhancement of the NK cell cytotoxicity against both tumor cells and iDCs, and the cytokine release that can promote maturation of DCs, capable of inducing adaptive Th1 immune responses (27).

Moreover, the TLR2-mediated interaction of NK cells with BCG may induce the NK cell expression of NKp44, which, in turn, can directly bind to BCG (29). However, while TLR2 binding to *Mycobacterium* cell wall is sufficient to induce activation of NK cell effector functions (including IFN-γ production), the engagement of NKp44 by BCG cell wall components may play a role in maintaining NK cell activation (28).

In addition, it has been recently demonstrated that TLR2 may be also involved in the NK-mediated response to human CMV (30).

Microbial unmethylated CpG DNA motifs are able to stimulate both NK cells and plasmacytoid DCs (pDCs) via TLR9, which is, indeed, expressed by both cell types. IFN-α, released by pDCs upon TLR9 engagement, supports the triggering of TLR9-responsive NK cells (31, 32). This activation may be further amplified by IL12, released by DCs (31).

It has been reported that NK cells may also express TLR5. Flagellin, a typical TLR5 ligand, may directly act on NK cells, and induces the release of IFN-γ, contributing to activate surrounding cells, and α-defensins, mediating pathogen destruction (33).

Human NK cells may also express functional TLR7 and TLR8 (34). In this context, it has been shown that NK cell stimulation by the TLR7/8 ligand ssRNA derived from HIV-1 depends on a direct contact between NK cells and pDCs or monocytes (35).

Thus, although NK cells can be directly activated by some TLR agonists, the microenvironment in which they lie, during TLR-mediated activation, may play an important role not only in the activation of their cytotoxic activity but also in their regulatory functions, able to modulate subsequent adaptive immune responses (11, 22, 36).

NATURAL CYTOTOXICITY RECEPTORS

NKp46, NKp44, and NKp30 were among the first identified activating receptors on human NK cells. These structurally unrelated surface molecules were collectively defined as NCRs for their common ability to strongly activate NK cell cytolytic activity (37). The generation of NCR-specific blocking monoclonal antibodies (mAbs) and the identification of an NCR^{dim}-phenotype (with impaired NK-mediated tumor killing capabilities) in some individuals (14), rapidly allowed the demonstration that these receptors were recognizing ligands on a large array of NK-susceptible targets, primarily tumor cells. The first NCR ligands to be discovered, however, were of viral origin (38), while only recently some of the tumor-expressed cellular ligands (39–41) have been identified (**Table 1B**). Different viral hemagglutinins (HAs) bind one or more NCRs and trigger NK cell functions (42). The pressure exerted by NCRs on viruses is witnessed by the onset of specific escape mechanisms (43, 44). Thus, for example, the CMV-encoded pp65 molecule gives rise to intracellular inhibitory interaction with NKp30 (43) and the vaccinia virus HA has been recently shown to bind NKp30 and block NKp30-mediated activation (44). In addition, NK cells in HIV-infected patients may show various alterations, including a reduced expression and function of NCRs (45, 46).

The so far identified tumor-expressed NCR-ligands are represented by self-antigens whose expression/exposure at the outer cell surface can be induced by cell stress or activation, or by still unknown mechanisms related to tumor transformation (**Table 1B**). Thus, the NKp30-ligand HLA-B-associated transcript-3/BCL-2-associated athanogene 6 (BAT3/BAG6) is a nuclear factor that can be released via exosomes and exposed at the cell surface by many tumor cells or, in response to stress, by DCs (39, 47). Another known ligand of NKp30, B7-H6, is expressed on transformed cells, and its expression can also be induced on normal cells (including monocytes and neutrophils) following stimulation with TLR ligands or pro-inflammatory cytokines (40, 48). Finally, an NKp44-ligand has been identified as an exon 21spe-containing isoform (21spe) of mixed lineage leukemia-5 (MLL5) protein. While the MLL5 is expressed in the nucleus of normal cells and is involved in the regulation of cell cycle and hematopoietic differentiation, the 21spe-MLL5 is located in the cytoplasm and at the cell surface, where it cannot conceivably exert its physiologic function (41). Remarkably, this isoform appears to be exclusively transcribed in tumor cells.

Although it is presently unknown whether the tumor-associated NCR-ligands could also be induced by viral infections, there are evidences that certain viruses, such as filovirus or HIV, can promote the expression of still undefined NKp46- or NKp30-ligands on DCs or T cells (49, 50) Moreover, CMV-infected DCs can activate NK cells via NKp46 and DNAM-1 (51). Intriguingly, the mAb recognizing 21spe-MLL5 had been previously shown to react with $CD4^+$ T cells treated with a specific peptide of HIV gp41 protein or derived from HIV-infected patients (50). Whatever could be the pathogenic process that mainly shapes the specificity of the NCRs, it is quite evident that these receptors are nevertheless involved in tumor surveillance *in vivo*. Experiments on NKp46KO mice support this evidence (52). In addition, various human studies have shown that both solid and hematologic malignancies can be frequently associated to the presence of NCR^{dim} NK cells (either in PB or at the tumor site) (53–56). In this context, different tumor-orchestrated mechanisms capable of suppressing NCR functionality have been described. Hypoxia, which frequently characterizes tumor tissues (57), or various factors produced by tumor cells or

induced on by-stander cells, can down-regulate NCR expression or function. These factors include: IDO, TGFβ, PGE_2 (58–60), or inhibitory NCR-ligands, such as the soluble form of BAT3 (61), and the proliferating cell nuclear antigene (PCNA) (which has been recently shown to induce NKp44-mediated inhibitory signaling) (see also **Table 1B**).

Besides inducing natural cytotoxicity, the NCRs can also orchestrate regulatory functions. Indeed, since their first discovery, these receptors were known to induce cytokine release. In addition, in the early 2000s NK cells were shown to participate to regulatory interactions with DCs, pDCs, neutrophils, macrophages, and T cells, involving the engagement of various receptors including NKp30 and/or NKp46 (12, 58, 62–67). Finally, over the past 10–15 years, different NCR-expressing NK cell types, poorly cytolytic, and prominently devoted to regulatory functions, have been described. According to recent findings, these various NK cell types and the classical NK cells appear to be part of the larger family of innate lymphoid cells (ILCs), which show, as unifying trait, the expression of the transcriptional repressor Id2 during their development from a putative common hematopoietic precursor (68, 69). The different ILCs can be induced in selected tissues and are characterized by unique cytokine patterns and surface phenotypic profiles (including or not NK cell markers). Roughly, in both mice and humans, three groups of ILCs, ILC-group 1, 2, or 3, can be defined according to their ability to release Th1-, Th2-, or Th17/22-type cytokines respectively. NK or NK-like cells are comprised within group 1 and 3. The ILC-group 1 includes classical circulating $CD56^{dim}CD16^{+}$ NK cells, and $CD56^{bright}CD16^{dim}$ cells (which prominently populate lymph nodes). Both these cell types can produce large amounts of IFN-γ and TNF-α upon NCR engagement (6). The ILC-group 3 includes the $CD56^{+/-}NKp44^{+}NKp46^{+}$ IL-22-producing NK-like cells ($NCR^{+}ILC3$), which populate mucosal tissues. $NCR^{+}ILC3$ are not cytotoxic but, by producing cytokines including IL-22, maintain epithelial-cell barrier function and contrast bacterial dissemination. The role of NCR on these cells is still poorly defined, however, a recent report by Glazer et al. (70) indicated that NKp44 engagement could both induce TNF-α release and synergize with other cytokines to induce IL-22 production.

Another poorly cytotoxic NK cell population endowed with regulatory functions is represented by $CD56^{bright}CD16^{dim}NCR^{+}$ NK cells that populate the decidua in the first trimester of pregnancy. These cells produce defined pattern of cytokines, chemokines, and pro-angiogenic factors [favoring the appropriate placenta and fetus development (71)], and participate at the induction of tolerance at the maternal/fetal interface (72). The interaction with trophoblasts by the engagement of NCRs would activate their regulatory functions (73, 74). Thus, the definition of the above-described NCR^{+} NK cell types, along with their new functions, would suggest that NCRs could recognize ligands expressed on different cells, to fulfill multiple tasks. In this context, for each receptor, different portions of the molecule and/or different polymorphism may have been shaped or selected to ensure the putative pleiotropy of NCRs (75–77).

HLA-SPECIFIC RECEPTORS: KIRs

Natural killer cells are equipped with inhibitory receptors able to interact specifically with human leukocyte antigen (HLA) class-I molecules on potential target cells. These receptors prevent NK cell-mediated attack against normal autologous cells and allow the killing of cells that upon tumor transformation or viral infection present compromised HLA class-I expression ("missing self hypothesis") (78) (**Figure 1**). In humans, two different types of HLA class-I-specific inhibitory receptors exist: (i) killer Ig-like receptors (KIRs), also referred to as CD158, that belong to the Ig-superfamily and, in most instances, recognize the polymorphic HLA-A, -B, and -C molecules (79–81), and (ii) CD94/NKG2A (CD94/CD159a), a heterodimer related to C-type lectins that recognizes HLA-E (82), a non-classical MHC molecule characterized by a limited polymorphism.

In addition, activating forms of KIRs have been also identified, but their specificity is still largely elusive (see relating paragraph). Related to the function performed, the intracytoplasmatic domains of KIRs can feature either a short (activating KIRs) or a long (inhibitory KIRs) cytoplasmic tail, "S" or "L" in the nomenclature, respectively (79, 83) (**Table 1C**).

Each individual may differ strongly in the activating KIR content. In particular, two different types of KIR haplotypes (A and B) can be distinguished in humans. Both haplotypes share inhibitory KIRs, but haplotype A (in 50% of individuals) includes a single activating KIR (KIR2DS4) whereas haplotype B has up to five activating KIRs (83, 84). In general, haplotypes A are beneficial in NK responses to pathogens, whereas haplotypes B are associated with low frequencies of pregnancy disorders (85, 86).

In humans, 13 KIR genes and 2 KIR pseudogenes (KIR2DP1 and 3DP1) are expressed. Three conserved genes (KIR3DL3, 2DL4, and 3DL2) form the common framework.

Only some of the 13 human KIRs have been demonstrated to recognize HLA class I. In contrast, no ligand has yet been identified for KIR2DS2, 2DS3, 2DS5, 2DL5, 3DS1, and 3DL3 (87)(**Table 1C**).

Regarding the Ig-domains content, each KIR displays two (KIR2D) or three (KIR3D) extracellular Ig-domains. Two types of KIR2D can be determined. KIR2D of the first type are composed by D1 and D2 domains and include the majority of KIRs (KIR2DL1/L2/L3 and KIR2DS1/S2/S3/S4/S5), whereas KIR2D of the second type are composed by D0 and D2 domains and include KIR2DL4/L5.

The high level of polymorphism in the KIR gene complex and the low conservation of KIR genes between species (only the three KIR genes KIR2DL4, KIR2DS4, KIR2DL5 have been preserved through hominid evolution) have suggested that KIRs have been undergone to a rapid evolution (87). Following this process, new KIR alleles, generated from existing ones under evolutionary pressure, are likely maintained when providing advantages (88).

A clear diversity in the KIR gene complex between modern populations indicates that geographically distinct diseases have recently exerted a selection on KIR repertoires (89).

In addition, the KIR diversification is thought to be more rapid than HLA diversification. Indeed, HLA genes are more similar in humans and chimpanzees than their KIR counterparts. In humans, HLA-C seems to have evolved as a superior and more specialized ligand for KIRs as compared to HLA-A and -B. This fact is supported by two considerations: (i) all HLA-C allotypes are KIR ligands, whereas only 45% of HLA-A and 36% of HLA-B allotypes are recognized by KIRs and (ii) HLA-C is of more recent origin as compared to HLA-A and -B (90, 91).

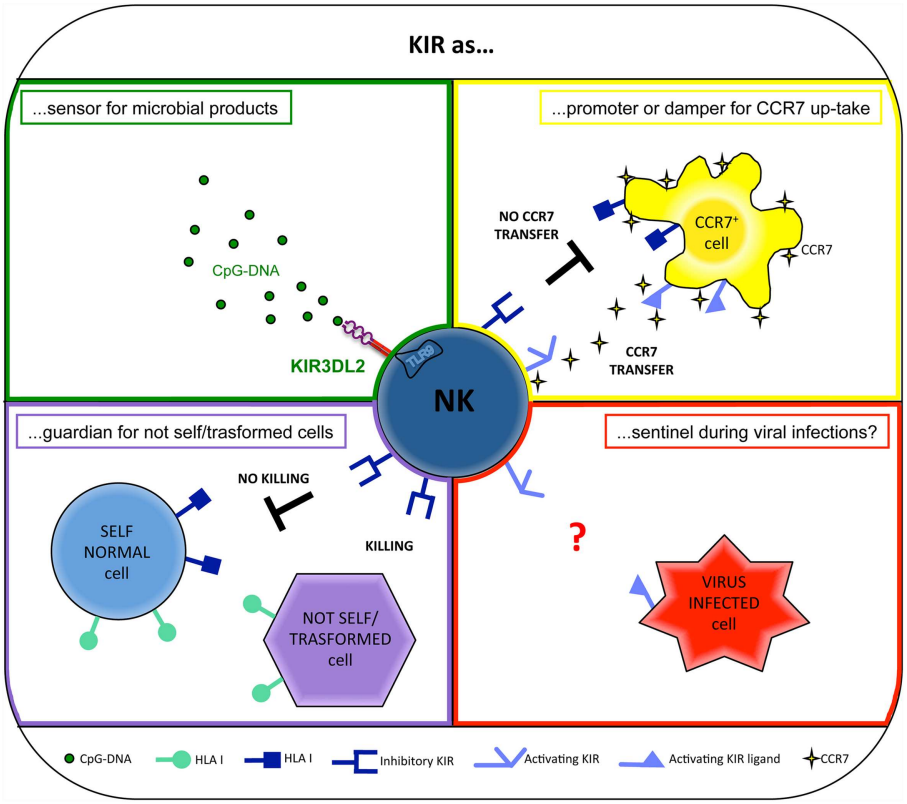

FIGURE 1 | Different roles for KIRs.

KIRs AS SENSOR FOR MICROBIAL PRODUCTS

Recently, KIRs have been shown to exert a novel and surprising function. Indeed, primarily KIR3DL2 (but also other KIRs, including KIR3DL1, KIR3DS1, and KIR2DL4) has been shown to work as sensors for microbial products and as chaperons for TLR9 ligands. In particular, KIR3DL2 can bind CpG ODNs at the NK cell surface and shuttle them to early endosomes where TLR9 translocates upon CpG ODN cell stimulation. In this intracellular compartment, KIR3DL2 gives CpG ODN to TLR9, thus allowing NK cell activation both in terms of increment of cytotoxicity and cytokine production (11, 19) (**Figure 1**).

The KIR Ig-domain involved in the direct recognition of microbial CpG ODN is represented by the D0, which is expressed by all the CpG ODN-binding KIRs. The interactions between negative charges of DNA sequences and positive charges of D0 domain probably are responsible for this binding. Remarkably, IFN-γ release induced by CpG ODN stimulation was mostly confined to KIR3DL2$^+$ NK cell subset, suggesting that this receptor may be more efficient in CpG ODN-shuttling and NK cell triggering than the other CpG ODN-binding KIRs (19).

In this context, it is worth noting that KIR3DL2 represents a framework gene (92) and, as a consequence, NK cells of all individuals can bind CpG ODN. This novel functional capability of KIR3DL2 may provide an important clue to understanding the driving forces that led to conservation of KIR3DL2-encoding gene in all haplotypes, in spite of the low frequency of its HLA ligands (HLA-A3 or -A11 alleles) (93) in the human population. Indeed, the need of rapid NK-mediated anti-microbial responses may represent an important factor of selective pressure. Moreover, KIR3DL2 is characterized by poor inhibitory capability because of its low affinity for HLA-A ligands. This weak inhibitory function may explain why KIR3DL2, in most NK cells, is coexpressed with other inhibitory KIRs or NKG2A (19).

Remarkably, some studies have indicated a three-domain surface molecule carrying a D0 domain as the putative ancestral mammalian KIR and have suggested that all KIR2D encoding genes have evolved from a KIR3D-encoding gene. Considering these evidences, it is possible to hypothesize that, in origin, certain KIRs could have a role different from that to recognize MHC-class-I molecules (94).

ACTIVATING KIRs AS SENTINELS DURING VIRAL INFECTIONS?

The main differences between activating and inhibitory HLA-specific receptors are located in their cytoplasmic tails. Indeed, the activating KIRs are characterized by a short cytoplasmic tail devoid of ITIMs and by a transmembrane domain with a charged amino-acid residue that allows association with an ITAM-bearing molecule (79, 95). The role of activating KIRs in immune response is still enigmatic and so far little information about it is available. Only for two of them (KIR2DS1 and KIR2DS4) the specificity for HLA class-I molecules has been demonstrated (96–100), despite

their extracellular domains are characterized by a high degree of sequence homology with their inhibitory counterparts.

However, a number of experimental evidences suggest that activating KIRs may play a role in NK-mediated response against viral infections (**Figure 1**). In particular, protection against hepatitis C virus infection (101) and delayed progression to AIDS (102) have been described in individuals coexpressing the activating receptor KIR3DS1 and its putative ligand, HLA-Bw4 with an isoleucine at position 80 (HLA-Bw4-80I). In addition, high levels of degranulation by KIR3DS1$^+$ NK cells in response to HIV-infected HLA-Bw4-80I$^+$ CD4$^+$ T cells (103) and a preferential expansion of KIR3DS1$^+$ NK cells in HLA-Bw4-80I$^+$ subjects during acute HIV-1 infection (104, 105) have been observed. However, the direct physical interaction between KIR3DS1 and HLA-Bw4-80I has not been demonstrated (106).

Moreover, different studies have suggested that the activating KIRs may interact with HLA class I at a lower affinity than their inhibitory counterparts. However, during viral infections, their HLA affinity may be increased by the presentation of viral peptides, thus allowing NK-mediated killing of infected cells (105). Thus, activating KIRs function could be modulated by the nature of the HLA class I presented peptide. In this context, KIR2DS1 has been described to display a certain degree of peptide selectivity in its binding to HLA-Cw4 (97).

It has been also speculated that additional molecules, up-regulated on target cells upon cellular stress/transformation, may also favor the triggering signals delivered by activating KIRs, acting as costimuli, or as non-HLA class-I ligands for these receptors. Notably, KIR2DS4 is able to interact with a non-HLA class-I protein expressed on melanoma cell lines and on a primary melanoma (107).

In some individuals, the increment in the signals generated by the low-affinity activating KIRs may overcome normal NK cell self-tolerance, thus inducing autoimmune diseases (108).

KIR AS A PROMOTER OR DAMPER FOR A PHENOTYPE CHANGE

Since cellular function is often linked to phenotype, it is of particular interest that the recent discovery of a mechanism by which NK cells would be able to capture target cell membrane components, and incorporate them in its own membrane, thus enriching their phenotypic/functional features (109). This process, known as trogocytosis, involves several cell-surface molecules in different cell types including lymphocytes as well as DCs and tumor cells and may represent a vector for rapid intercellular communication (110). The capture of target cell membrane fragments by NK cells is likely to reflect specific ligand recognition by NK cell receptors and, in particular, activating and inhibitory receptors control this process. Importantly, surface molecules acquired from other cell types could modify not only phenotypic but also functional characteristics of the recipient cells (111).

Through this mechanism, some peculiar characteristics of the two major NK cell subsets (CD56^{dim}/CD56^{bright}) (10) can be subverted. In this context, recently, it has been shown that the highly cytotoxic CD56^{dim} KIR$^+$ NK cell subpopulation that is characterized by the CCR7$^-$ phenotype, can acquire surface CCR7 upon interaction with CCR7$^+$ cells, becoming able to migrate in response to the SLC chemokines CCL19/CCL21 (7).

This novel NK cell ability occurs via the immunological synapse, precedes the NK-mediated cytolysis, and is finely controlled by the specific interaction between KIRs on NK cells and HLA class-I molecules on CCR7$^+$ cells. In particular, inhibitory KIRs block this transfer (7), whereas activating KIRs (e.g., KIR2DS1) are able to strongly promote the CCR7 acquisition by NK cells (8, 20). In addition, other surface NK receptors, including the NCR NKp46, may play a crucial role in promoting this phenomenon (7). Therefore, in some individuals, collaboration between activating KIRs and other non-HLA-specific triggering receptors may occur to further potentiate the CCR7 acquisition.

Remarkably, unlike the non-HLA-specific receptors (whose function could be limited by inhibitory signals delivered by both KIRs/NKG2A receptors), KIR2DS1 could promote CCR7 uptake even in NKG2A$^+$ NK cells, since the KIR2DS1-dependent uptake of CCR7 can override the inhibition provided by this receptor. This event allows a substantial expansion of the NK cell fraction capable of migrating to lymph nodes (20).

The key role of KIRs during the CCR7 transfer has important implications during haploidentical hematopoietic stem cell transplantation, in which donor-derived alloreactive NK cells expressing KIRs may play a relevant role in preventing graft vs. host disease (by killing recipient DCs) and host vs. graft reactions (by killing recipient T cells) (112). However, because KIR$^+$ NK cells normally do not express CCR7, it was unclear how alloreactive KIR$^+$ NK cells could reach lymph nodes and kill these cells directly in this compartment. In this context, the CCR7 acquisition by trogocytosis may represent a new way by which alloreactive KIR$^+$ NK cells can migrate to the site where they can kill mature DCs and T cell blasts (20, 113) (**Figure 1**).

CONCLUSION

Several data have demonstrated that the different NK cell receptors can reciprocally coordinate and regulate their functions, contributing to the initiation of innate responses and to the subsequent priming of adaptive immune responses. NK cell activation may be mediated by the engagement of TLRs and/or NCRs, two different types of receptors that may cooperate in inducing NK cell triggering, and thus in controlling viral/bacterial infection and cancer. For example, it has been demonstrated that TLR2 and NKp44 are directly involved in the recognition of *Mycobacterium* and in the consequent promotion of NK cell effector functions (28).

Natural killer cell triggering is under the control of receptors (e.g., KIRs) specific for self-HLA class-I molecules. The missing recognition of specific HLA ligands by inhibitory KIRs enables NK cell triggering upon NCRs engagement (37). Most tumor cell-surface ligands for NCRs have remained elusive; however, a critical role for NCRs in the control of both tumors and viral infections has been clearly demonstrated.

On the other hand, collaboration between TLRs and KIRs may also exist. Indeed, certain KIRs can function as sensor for microbial products and as chaperons for TLR9 ligands. Remarkably, an inhibitory KIR, such as KIR3DL2, may unexpectedly induce NK cell activation, favoring TLR-mediated response (19).

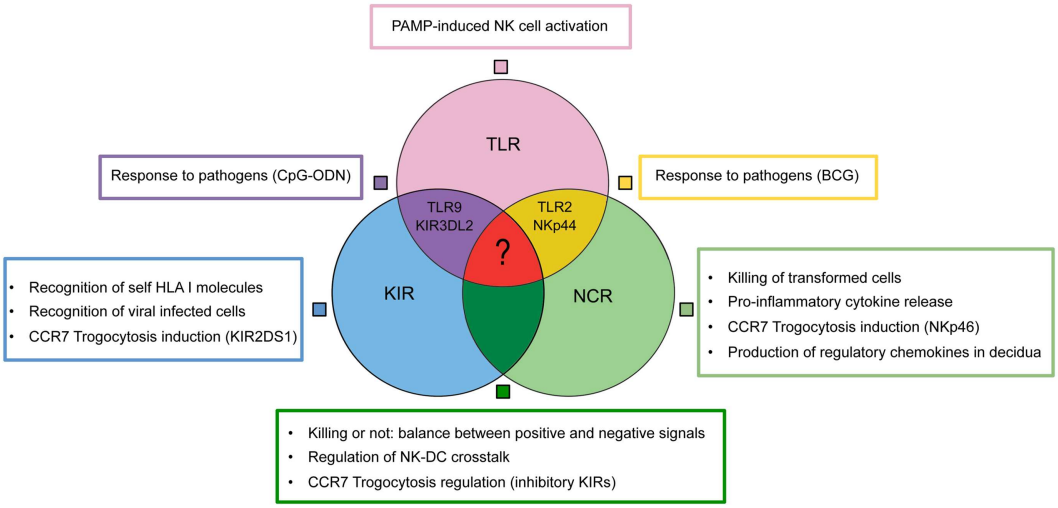

FIGURE 2 | Collaboration model between TLRs, KIRs, NCRs for NK cell activation.

Activating and inhibitory NK receptors control other important mechanisms, such as trogocytosis, by which NK cells can modify their phenotypic/functional features. For example, KIR^+ NK cells can acquire CCR7 and thus migratory properties toward SLCs, by interacting with $CCR7^+$ cells (e.g., mature DCs). This process is blocked by inhibitory KIRs and promoted by NCRs and, even further, by activating KIRs (7, 20).

The NK-mediated capability of releasing chemokines and cytokines in different compartments endowed NK cells with regulatory functions, affecting subsequent adaptive immune responses. In decidual tissues, the engagement of NCRs during NK–trophoblast interactions induces the release of regulatory chemokines, involved in tissue building and remodeling, and in the formation of new blood vessels (74).

In conclusion, a wide and heterogenous group of receptors allow NK cell to fulfill multifaceted functions (**Figure 2**). The nodes of this intricate functional network may represent new therapeutic targets in different pathological conditions including not only tumors or infections but also immune-mediated diseases or pregnancy failure.

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Structural basis for recognition of cellular and viral ligands by NK cell receptors

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Natural killer (NK) cells are key components of innate immune responses to tumors and viral infections. NK cell function is regulated by NK cell receptors that recognize both cellular and viral ligands, including major histocompatibility complex (MHC), MHC-like, and non-MHC molecules. These receptors include Ly49s, killer immunoglobulin-like receptors, leukocyte immunoglobulin-like receptors, and NKG2A/CD94, which bind MHC class I (MHC-I) molecules, and NKG2D, which binds MHC-I paralogs such as the stress-induced proteins MICA and ULBP. In addition, certain viruses have evolved MHC-like immunoevasins, such as UL18 and m157 from cytomegalovirus, that act as decoy ligands for NK receptors. A growing number of NK receptor–ligand interaction pairs involving non-MHC molecules have also been identified, including NKp30–B7-H6, killer cell lectin-like receptor G1–cadherin, and NKp80–AICL. Here, we describe crystal structures determined to date of NK cell receptors bound to MHC, MHC-related, and non-MHC ligands. Collectively, these structures reveal the diverse solutions that NK receptors have developed to recognize these molecules, thereby enabling the regulation of NK cytolytic activity by both host and viral ligands.

Keywords: NK receptor, MHC, virus, KIR, Ly49, NKG2, structure

INTRODUCTION

Natural killer (NK) cells are essential components of the innate immune response against viral infections and tumors (1–5). They not only eliminate virally infected or malignantly transformed cells by means of their cytolytic capabilities, but also produce cytokines and chemokines such as interferon-γ that modulate immune responses and help maintain tissue homeostasis. To perform these diverse functions, NK cells express a multitude of activating and inhibitory receptors that act in concert to regulate their activities (6, 7). NK receptors belong to two distinct structural families: the immunoglobulin (Ig) superfamily and the C-type lectin superfamily. In humans, NK receptors of the Ig superfamily are encoded in the leukocyte receptor complex (LRC) on chromosome 19 (7 in mouse) (8) and the NK gene complex (NKC) on chromosome 12 (6 in mouse) (9). Both superfamilies include inhibitory and activating receptors. In addition, NK receptors have been shown to recognize both cellular and viral ligands, including major histocompatibility complex (MHC), MHC-like, and non-MHC molecules.

The cytolytic activity of NK cells is regulated by positive signaling activating receptors (resulting in target cell lysis) and negative signaling inhibitory receptors (preventing lysis). It is the dynamic interplay between these signals that ultimately determines the outcome of NK cell–target cell encounters (4, 6, 7). The dominant signal received by an NK cell is inhibitory, provided by the interaction of its receptors with normal levels of MHC class I (MHC-I) molecules. If MHC-I expression is reduced by infectious or tumorigenic processes, this inhibitory signal is attenuated and the NK cell undergoes activation. As a consequence, cells with reduced MHC-I expression become subject to lysis by NK cells (1–5). The process by which NK receptors direct the cytolytic activity of NK cells against virally infected or tumor cells that have lost MHC-I expression is known as "missing-self" recognition.

Several receptor families on primate and rodent NK cells are responsible for monitoring MHC-I expression on surrounding cells (2–5, 10–13). These include the killer immunoglobulin-like receptors (KIRs) in humans, members of the Ly49 family (Ly49s) in rodents, NKG2/CD94 receptors, and leukocyte immunoglobulin-like receptors (LILRs). Although most Ly49s and KIRs inhibit NK function on binding to MHC-I ligands, some are activating (6, 7). Furthermore, the activating NK receptor NKG2D binds paralogs of MHC-I molecules, including MICA and RAE-I that are selectively upregulated in stressed tissues (14). The interaction of activating Ly49s with MHC-like proteins encoded by mouse cytomegalovirus (MCMV) has demonstrated a direct role for Ly49 receptors in anti-viral immunity (15–17).

Besides receptors specific for MHC-I or MHC-related ligands, a number of other receptors that recognize non-MHC proteins are involved in regulating NK cell cytotoxic activity (2, 7). These include CD16 (18), CD69 (19), NKR-P1 (20, 21), NTB-A (22, 23), 2B4 (22–24), DNAM-1 (25), NKp30 (26), NKp44 (27), NKp46 (28), NKp65 (21, 29), and NKp80 (21, 30, 31), which contribute to NK cell activation, and the inhibitory receptors, killer cell lectin-like receptor G1 (KLRG1) (32, 33) and LAIR-1 (34). The biological ligands for most (but not all) of these receptors have now been identified, including IgG Fc for CD16, Clr for NKR-P1, CD48 for 2B4, CD155 for DNAM-1, B7-H6 for NKp30, keratinocyte-associated C-type lectin (KACL) for NKp65, AICL for NKp80, E-cadherin for KLRG1, and collagen for LAIR-1 (2, 7).

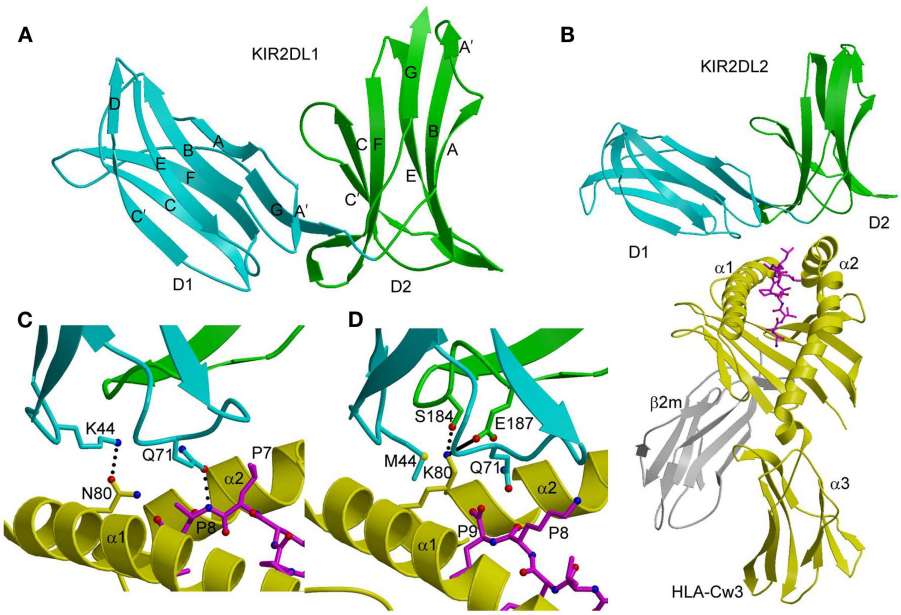

FIGURE 1 | Three-dimensional structures of KIR2DL and KIR2DL–HLA-C complexes. **(A)** Ribbon diagram of KIR2DL1 (PDB accession code 1NKR). The D1 domain is cyan; D2 is green. The secondary structural elements are labeled. **(B)** Ribbons diagram of KIR2DL2 bound to HLA-Cw3 (1EFX). The α1, α2, and α3 domains of the HLA-Cw3 heavy chain are yellow; β_2m is gray; the peptide is magenta. **(C)** Basis for allelic specificity and peptide selectivity of KIR2D receptors. The dotted lines represent hydrogen bonds formed by Asn80 of HLA-Cw3 with Lys44 of KIR2DL2, and by Gln71 of HLA-Cw3 with P8 of the peptide. **(D)** Interactions of Lys80 of HLA-Cw4 (yellow) with specificity-determining residues of KIR2DL1 (D1 domain in cyan, D2 domain in green) in the KIR2DL1–HLA-Cw4 complex (1IM9). The solid line represents a salt bridge.

Considerable progress has been made over the past few years in determining crystal structures of representative NK receptors, both in isolation and bound to MHC, MHC-related, or non-MHC ligands. These include both Ig-like (e.g., KIRs, LILRs, NKp30) and C-type lectin-like (e.g., Ly49s, NKG2D, NKG2/CD94, NKp65) receptors. These structures have revealed the multiplicity of solutions that NK receptors have evolved to recognize MHC, MHC-like, and non-MHC molecules, which collectively mediate crucial interactions for regulating the cytolytic activity of NK cells by host and viral ligands.

MHC-I RECOGNITION BY KIR RECEPTORS

The highly polymorphic KIR receptor family encodes the main MHC-monitoring molecules on primate NK cells and includes both inhibitory and activating members. KIRs are transmembrane glycoproteins containing two (D1 and D2) or three (D0, D1, and D2) extracellular C2-type Ig-like domains (10, 12). KIRs with two Ig-like domains are designated KIR2D; KIRs with three Ig-like domains are designated KIR3D. Whereas KIR2D receptors bind HLA-C alleles, KIR3D receptors bind HLA-A and HLA-B alleles. Crystallographic studies of KIR2D molecules, both in free form (35–39) and bound to HLA-C ligands (40, 41), have provided a framework for understanding the specificity of KIR2D receptors for HLA-C at the atomic level. In addition, the structure of KIR3DL1 in complex with HLA-B*5701 has revealed the basis for HLA recognition by three-domain KIRs (42).

The two N-terminal domains (D1 and D2) of KIR2D receptors are linked by a short hinge segment of three to five amino acids (**Figure 1A**). These Ig-like domains are each formed by two anti-parallel β-sheets, such that a β-sheet of four (in D1) or three (in D2) anti-parallel strands (ABED and ABE, respectively) juxtaposes a β-sheet of four anti-parallel strands (CC′ FG). The relative disposition of D1 and D2 is similar to that found in hematopoietic receptors (43, 44), with the angle between D1 and D2 ranging from 60° to 80° in different KIRD2 receptors.

In both the KIR2DL2–HLA-Cw3 (40) and KIR2DL1–HLA-Cw4 (41) complexes, the KIRs engage HLA-C through the α1 and α2 helices of the α1/α2 platform domain and the C-terminal portion of the MHC-bound peptide, with the D1–D2 axis orthogonal to the axis of the peptide (**Figure 1B**). This docking mode roughly resembles the way T-cell receptors (TCRs) bind MHC, but is completely distinct from the docking mode of LILR and Ly49 NK receptors (see below). Each KIR2D binds HLA-C using six loops from D1 and D2, which contact the α1 and α2 helices of the MHC-I molecule, respectively.

The structures of the KIR2DL2–HLA-Cw3 and KIR2DL1–HLA-Cw4 complexes explain the allelic specificity of KIR2DLs (40, 41). Of 12 HLA-Cw3 residues in contact with KIR2DL2, 11 are invariant in HLA-Cw4 and in all other HLA-C alleles. The only exception is Asn80, which defines the allelic specificity of KIR2DLs. Similarly, on the receptor side of the interface, 14 of 16 KIR2DL2 residues that contact HLA-Cw3 are conserved in KIR2DL1. The two exceptions are at positions 44 and 70. In the KIR2DL2–HLA-Cw3 structure, KIR2DL2 Lys44 makes a hydrogen bond with HLA-Cw3 Asn80; this hydrogen bond cannot be formed with KIR2DL1 Met44 (**Figure 1C**). In the KIR2DL1–HLA-Cw4 structure, the side chain of HLA-Cw4 Lys80 is situated in a negatively charge pocket of KIR2DL1 that includes Met44, which contacts HLA-Cw4 Lys80

(**Figure 1D**). Replacement of Met44 by lysine, as in KIR2DL2, would cause charge repulsion with HLA-Cw4 Lys80, resulting in loss of binding.

KIR2DS4 is an activating receptor that specifically recognizes HLA-A*11, as well as HLA-C allotypes bearing the C1 and C2 epitopes (39). A comparison of the unbound KIR2DS4 structure with the KIR2DL2–HLA-Cw3 and KIR2DL1–HLA-Cw4 complexes revealed two features that likely explain the binding specificity of KIR2DS4. First, a backbone displacement of one of the predicted HLA-contacting loops (L2) of KIR2DS4, relative to its position in KIR2DL2, may disrupt the interaction between Lys44 of KIR2DS4 and Asn80 of the HLA-C C1 epitope, resulting in weaker binding of KIR2DS4 to $C1^+$ allotypes than KIR2DL2 (39). Conversely, this displacement could increase avidity for $C2^+$ allotypes by accommodating Lys80 of the C2 epitope. Second, the Pro71–Val72 motif of KIR2DS4 that confers reactivity with HLA-A*11 is part of a loop (L3), which in KIR2DL2 contacts HLA-C using the Gln71–Asp72 motif (40). Replacement of Gln71–Asp72 by Pro71–Val72 in KIR2DS4, which is the result of gene conversion with KIR3DL2, reduces avidity for $C1^+$ allotypes but increases avidity for HLA-A*11 and $C2^+$ allotypes (39).

The binding of KIR2D receptors to HLA-C molecules displays preferences for certain peptides (45, 46); however, whether peptide selectivity has a role in NK receptor function is not clear. Intriguingly, KIR-associated HIV-1 sequence polymorphisms in chronically infected individuals have been found to increase the binding of inhibitory KIRs to $CD4^+$ T cells infected with HIV-1, and to decrease the anti-viral activity of KIR-positive NK cells (47). Consistent with the observation that the KIR binding site is centered near C-terminal residues P7 and P8 of the MHC-bound peptide (40, 41), the KIR–HLA interaction is most sensitive to substitutions at these two peptide positions. By contrast, TCRs, which exhibit much greater peptide specificity than KIRs, typically focus on the central P5 position of the peptide (48). Since the peptide positions recognized by KIRs are not usually directly involved in TCR binding, MHC molecules may be able to evolve their polymorphic regions to present diverse microbial (i.e., foreign) peptides for T-cell-mediated immunity, while at the same time maintaining non-variant regions to bind self-peptides for KIR recognition and NK-cell-mediated immune defense.

In the KIR3DL1–HLA-B*5701 complex (42), KIR3DL1 engages HLA-B*5701 with its D1 and D2 domains situated over the C-terminal half of the peptide-binding groove in an overall orientation highly similar to that of the KIR2D–HLA-C complexes (**Figure 2A**). KIR3DL1 adopts an elongated conformation, which allows D0 to extend down toward β_2-microglobulin (β_2m) and engage a region of the MHC-I molecule that is nearly invariant

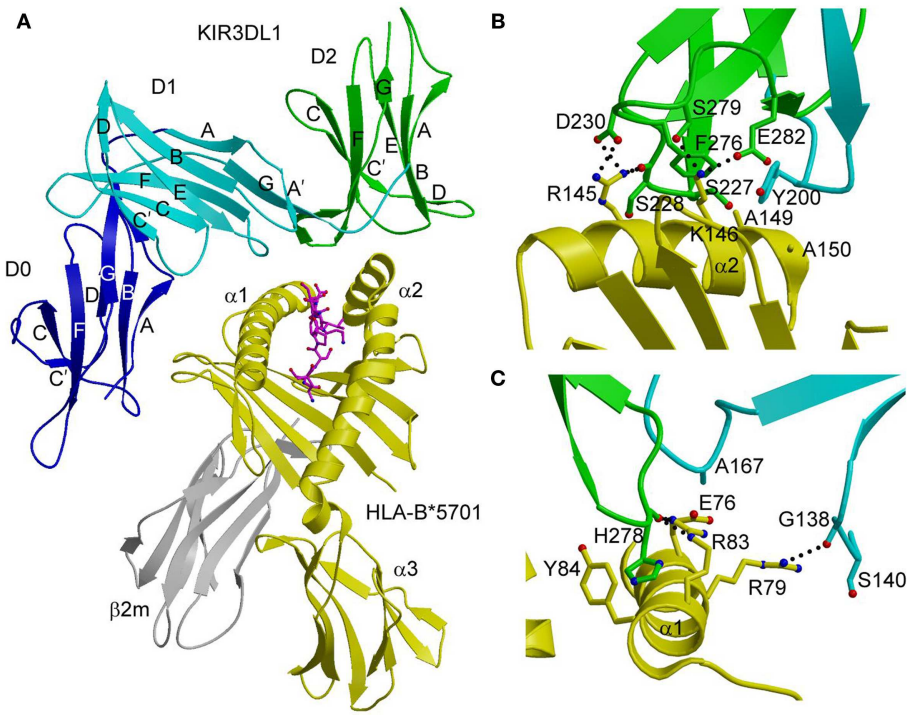

FIGURE 2 | Structure of the KIR3DL1–HLA-B*5701 complex. **(A)** Ribbon diagram of KIR3DL1 bound to HLA-B*5701 (3VH8). The orientation of the MHC-I ligand is similar to that of HLA-Cw3 in the KIR2DL2–HLA-Cw3 complex (**Figure 1B**). The HLA-B*5701 heavy chain is yellow; β_2m is gray; the peptide is magenta. The KIR3DL1 D0 domain is dark blue; D1 is cyan; D2 is green. The secondary structural elements of KIR3DL1 are labeled. **(B)** Contacts between KIR3DL1 and the HLA-B*5701 α2 helix. The D2 domain mainly interacts with HLA-B*5701 residues 142–151, which display limited polymorphism among HLA-B alleles. At the center of the D2–HLA-B*5701 interface, KIR3DL1 residues Tyr200 and Phe276 form an aromatic cluster that converges on the α2 helix. **(C)** Contacts between KIR3DL1 and the HLA-B*5701 α1 helix. KIR3DL1 recognizes HLA allotypes that contain the Bw4 epitope-defining residues 77–83 on the α1 helix, which likely accounts for the allelic specificity of KIR3DLs.

across HLA-A and HLA-B allotypes. The D1 domain contacts the α1 helix and the self-peptide, while the D2 domain contacts the α2 helix. The D2 domain mainly interacts with HLA-B*5701 residues 142–151, which display restricted polymorphism across HLA-B alleles. At the center of the D2–HLA-B*5701 interface, KIR3DL1 residues Tyr200 and Phe276 form an aromatic cluster that converges on the α2 helix (**Figure 2B**). Alanine substitution of these residues abrogated binding to HLA-B*5701, demonstrating the importance of this central core to HLA recognition.

KIR3DL1 recognizes HLA allotypes that contain the Bw4 epitope, which is defined by residues 77–83 of the α1 helix. In the structure (42), KIR3DL1 contacts residues 79, 80, and 83 within the Bw4 epitope through its D1 domain (**Figure 2C**), which likely accounts for the allelic specificity of KIR3DLs. The D1 domain also makes limited contacts with the self-peptide at position P8, analogous to the interaction of KIR2D receptors with peptides bound to HLA-C (see above).

Unexpectedly, the extensive polymorphisms found within individual KIR3D families are located predominantly at positions not implicated in HLA binding. This implies that most KIR3D polymorphisms, a number of which are subject to positive selection (49), are unlikely to impact affinity directly, but could potentially affect HLA binding indirectly by altering the clustering or expression levels of KIR3D receptors on the NK cell surface. In this way, evolutionary pressures may drive the diversification of KIR3D sequences at sites remote from the HLA-binding site.

MHC-I RECOGNITION BY LILRs

The human LILR family of immunoreceptors (also referred to as Ig-like transcripts, or ILTs) is broadly expressed on NK cells, T cells, monocytes, B cells, and dendritic cells (50). The mouse orthologs of LILRs are known as paired immunoglobulin receptors (PIRs). Like KIRs, LILR receptors contain either two or four tandem extracellular Ig-like domains. LILRA1, LILRA2, LILRA3, LILRB1, and LILRB2 bind classical MHC-I proteins (HLA-A, -B, and -C), whereas LILRA4, LILRA5, LILRA6, LILRB3, LILRB4, and LILRB4 do not appear to recognize MHC-I. The inhibitory LILRB1 and LILRB2 receptors bind multiple MHC-I molecules, both classical and non-classical (HLA-E, -F, and -G), with comparable kinetics and affinities (51, 52). By contrast, individual KIR receptors display allelic specificity, as discussed above. In addition to their role as MHC-I sensors, LILRs may be involved in immune responses to viral infections, as suggested by the finding that LIRLB1 is a receptor for UL18 (53). This immunoevasin is an MHC-I homolog encoded by human cytomegalovirus (HCMV). The crystal structure of LILRB1 (domains D1 and D2 only) has been solved in free form (54) and bound to HLA-A2 (55) and UL18 (56). Structures have also been reported for LILRB2 (D1 and D2) in unbound form (57) and in complex with HLA-G (58).

Similar to KIR2D (**Figure 1A**), the two tandem Ig-like domains of both LILRB1 and LILRB2 form a bent structure characterized by an acute interdomain angle (**Figure 3A**). Each domain comprises two anti-parallel β-sheets arranged in a topology like that of KIRs. In the LILRB1–HLA-A2 complex (55), LILRB1 D1D2 binds the side of HLA-A2, forming two contact surfaces that include residues from β_2m, which is invariant, and the HLA-A2 α3 domain, which is relatively non-polymorphic. The D1–D2 interdomain hinge region contacts β_2m, while the tip of LILRB1 D1 contacts the HLA-A2 α3 domain, (**Figure 3A**). Similar to LILRB1, LILRB2 recognizes β_2m and the HLA-G α3 domain using the interdomain hinge and D1, respectively (58). The docking mode utilized by LILRB1 and LILRB2, which differs completely from that of KIRs (**Figure 1B**), is consistent with MHC-I recognition in a peptide-independent manner. The focus by LILRs on conserved elements of MHC-I molecules, both classical and non-classical, accounts for the broad specificity of these NK receptors for numerous HLA alleles.

Whereas LILRB1 undergoes an interdomain angle change of ~15° after binding MHC-I, LILRB2 maintains the same interdomain angle (55, 58). Overall, however, LILRB2 exhibits greater

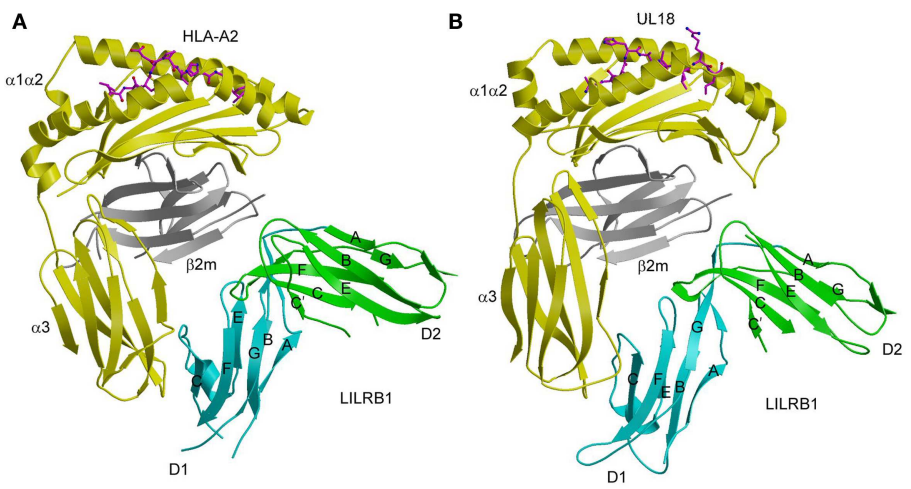

FIGURE 3 | Interaction of LILRB1 with MHC-I and a viral MHC-I mimic. **(A)** Structure of LILRB1 bound to HLA-A2 (1P7Q). The α1, α2, and α3 domains of the HLA-A2 heavy chain are yellow; β_2m is gray; the peptide is magenta. The D1 and D2 domains of LILRB1 are colored in cyan and green, respectively. The secondary structural elements of LILRB1 are labeled. **(B)** Structure of LILRB1 bound to the HCMV MHC-I mimic UL18 (3D2U).

conformational changes than LILRB1 upon complex formation. In particular, free LILRB2 contains only one 3_{10} helix (residues 52–55) involving binding site residues, whereas bound LILRB2 contains two such helices in the interface with HLA-G (residues 46–50 and 53–57). By contrast, LILRB1 contains two 3_{10} helices in both free and bound states. Although affinity measurements indicate that the membrane-distal D1 and D2 domains are mainly responsible for HLA binding (54, 55), a role for the membrane-proximal D3 and D4 domains cannot be formally excluded in the absence of a structure of a four-domain LILR in complex with ligand. A complete D1–D4 LILR structure is also required to understand the apparent ability of LILR/PIR receptors to bind not only MHC-I molecules on opposing cells (*trans* interaction), but also ones on the same cell (*cis* interaction) (59, 60), as discussed below.

LILR RECOGNITION OF UL18, A VIRAL MHC-I MIMIC

Among the microorganisms that have achieved great success in inventing strategies for immune evasion are the cytomegaloviruses, whose genomes encode proteins that interfere with both NK cell and T-cell recognition, as well as antigen processing and presentation (61–63). These include proteins that are known, or predicted to be, structural homologs of host MHC-I molecules. HCMV encodes an MHC-I homolog, UL18, that binds the inhibitory receptor LILRB1 (64). This interaction is believed to allow HCMV-infected cells to avoid NK-cell-mediated lysis (65). UL18 is a heavily glycosylated transmembrane protein that associates with β_2m, and with endogenous peptides derived from host cytoplasmic proteins that resemble those bound to HLA alleles (66). Remarkably, UL18 binds LILRB1 >1000-fold more tightly than MHC-I proteins, enabling this decoy ligand to compete effectively with MHC-I for binding to LILRB1 (67).

Despite sharing only ~25% sequence with its MHC-I counterparts, the structure of UL18 bound to LILRB1 shows striking similarity to the LILRB1–HLA-A2 and LILRB2–HLA-G complexes, with the tip LILRB1 D1 domain contacting the UL18 α3 domain and the D1–D2 interdomain hinge contacting β_2m (**Figure 3B**) (56). Variable residues in the UL18 α1 domain, which were identified by sequence analysis of laboratory and clinical HCMV strains, do not contact LILRB1, although domains D3 and D4, which are not present in the structure, could potentially engage this region of UL18. Most contacts between LILRB1 and U18 involve the UL18-specific portion of the UL18/β_2m heterodimer (i.e., the heavy chain), whereas the majority of LILRB1 interactions with HLA-A2 involve the invariant β_2m light chain. Additional salt bridges and better surface complementarity in the LILRB1–UL18 interface compared with the LILRB1–HLA-A2 interface likely explain the >1000-fold higher affinity of UL18.

A major difference between UL18 and MHC-I molecules is the exceptionally high carbohydrate content of UL18, which is attributable to its 13 potential N-glycosylation sites, compared to only one *N*-glycan attached to human MHC-I molecules. In fully glycosylated UL18 (the protein used for crystallization was minimally glycosylated), most of the surface of UL18 was predicted to be covered by carbohydrate, with the notable exceptions of the binding site for LILRB1 and the docking interface with β_2m (56). This suggests that UL18 evolved a glycan shield to prevent neutralization by antibodies, while preserving the binding site for LILRs. Such a strategy for reducing immunogenicity is analogous to that employed by other viruses with heavily glycosylated envelope proteins, notably HIV and influenza (68).

NATURAL CYTOTOXICITY RECEPTORS

Natural cytotoxicity receptors (NCRs) were discovered in a search for receptors that activated NK cells independently of MHC (69). To date, the NCR family includes NKp30 (NCR3, CD337), NKp44 (NCR2, CD336), and NKp46 (NCR1, CD335). In humans, NKp44 and NKp30 are encoded in the class III region of the MHC locus, while NKp46 is encoded in the LRC (69). Mice only possess a functional gene for NKp46. These very potent activating receptors comprise one (NKp30 and NKp44) or two (NKp46) Ig-like extracellular domains (69, 70). NCRs contain charged residues in their transmembrane regions for association with immunoreceptor tyrosine-based activation motif (ITAM)-bearing signaling polypeptides: ζ–γ for NKp30 and NKp46; and DAP12 for NKp44 (71). In humans, NCRs play a major role in NK-cell-mediated lysis of diverse tumor cells, including carcinomas, neuroblastomas, and leukemias (69, 70). In addition, NCRs have been implicated in protective responses against various viruses, including influenza (72), hepatitis C (73), West Nile (74), and Ebola (75).

Despite intensive efforts over many years, ligands for the NRC family have proven very elusive and, in some cases, controversial. NKp44 and NKp46 bind influenza and other viral hemagglutinins (HAs) mainly through recognition by the HA of terminal sialic acid moieties (the cellular receptor for HAs) on N-linked glycans of these NCRs (72, 76, 77). Although this mechanism would allow NKp44 and NKp46 to bind a wide variety of viruses, due to the ability of HAs to bind sialic acid-containing glycoproteins in general, this is probably not the full story, since recognition would not depend on the NCR ectodomain itself, but only on the fact that NKp44 and NKp46 are glycoproteins with terminal sialic acids (13). Binding of NKp46 to heparan sulfate proteoglycans has also been described (78), but the biological relevance of this interaction is unclear. Recently, a novel isoform of the mixed-lineage leukemia-5 protein (MLL5) was identified as a cellular ligand for NKp44 (79). This MLL5 isoform was not expressed on cells from healthy individuals, but was detected on a large panel of tumor and transformed cell lines. Moreover, MLL5 expression on target cells triggered NKp44-mediated NK cell cytotoxicity.

NKp30 binds the nuclear factor BAT3 (80) and the tumor cell surface protein B7-H6 (81). BAT3 (also known as BAG-6) has been implicated in the induction of apoptosis after endoplasmic reticulum stress or DNA damage (82). B7-H6 is a member of the B7-family (81), which includes ligands (B7-1 and B7-2) for the T-cell co-inhibitory receptor CTLA-4 and the co-stimulatory receptor CD28 (83). The B7-family also encodes PD-L1 and PD-L2, which are ligands for the T-cell co-inhibitory receptor PD-1. B7-H6 is not expressed in normal human tissues, but can be detected on a variety of human tumor cell lines that includes T and B lymphomas, melanomas, and carcinomas (81). Importantly, B7-H6 expression on tumor cells triggered NK cell cytotoxicity that was mediated specifically by NKp30. These results implicate B7-H6 as tumor-induced self-protein, analogous to MICA (2),

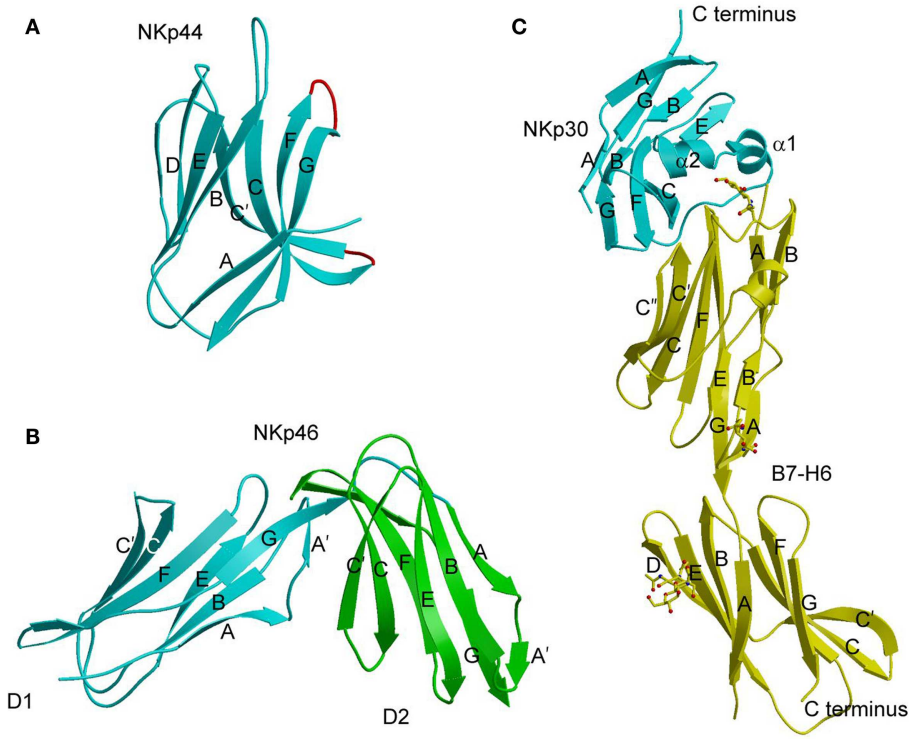

FIGURE 4 | Natural cytotoxicity receptors. **(A)** Structure of NKp44 (1HKF). The β-strands are labeled. The CC′ and FG loops, drawn in red, define a positively charged surface groove that may serve as a binding site for anionic ligands. **(B)** Structure of NKp46 (1P6F). D1 is cyan; D2 is green. **(C)** Structure of NKp30 bound to its tumor cell ligand B7-H6 (3PV6). N-linked glycans at B7-H6 residues Asn43 and Asn57 in the V-like domain and Asn208 in the C-like domain are shown in ball-and-stick representation.

which alerts NK cells to cellular transformation (81). NKp30 also recognizes the tegument pp65 protein of HCMV, indicating a role for this NCR in anti-viral immunity (84). Recently, NKp30 was shown to be responsible for the recognition and killing of the opportunistic fungi *Cryptococcus* and *Candida* (85). Although the fungal ligand recognized by NKp30 remains to be identified, possible candidates include β-1,3 glucans, which are major components of fungal cell wells and are highly conserved across fungal species. Thus, NKp30 interacts with multiple ligands, as do the activating NK receptors NKG2D and DNAM-1 (86, 87).

At present, crystal structures have been determined for NKp30, NKp44, and NKp46 in unbound form (88–90), and for NKp30 bound to B7-H6 (91). NKp44 comprises a single V-type Ig-like domain that features a prominent groove formed by two facing β-hairpin loops (CC′ and FG) projecting from the Ig fold core (**Figure 4A**) (88). The solvent accessibility of the groove, and its electropositive nature, suggest a possible binding site for anionic ligands, such as sialic acid, although no structure of a complex has been reported. NKp46 consists of two C2-set Ig-like domains whose overall fold and disposition are similar to those of the D1D2 domains of KIRs and LILRs (**Figure 4B**) (89). This structural resemblance suggests that similar receptor surfaces may be involved in ligand binding. The region of NKp46 analogous to the KIR or LILR ligand-recognition site is located at the interdomain hinge and comprises residues from both Ig-like domains. However, confirmation of this hypothesis awaits structural studies of NKp46–ligand complexes.

The Ig-like domain of NKp30 exhibits the chain topology found in C1-set domains (**Figure 4C**) (90, 91). The closest structural homolog of NKp30 is PD-L1, a ligand for PD-1. Like PD-1, NKp30 is a member of the CD28 family, which also includes CTLA-4, ICOS, and B and T lymphocyte attenuator (BTLA) (81). Similar to other B7-family members, the extracellular portion of B7-H6 consists of a V-like and a C-like domain, with the V-like domain distal from the membrane (91).

The structure of the NKp30–B7-H6 complex revealed a binding interface formed by the front β-sheet of the B7-H6 V-like domain and the front and back β-sheets of the NKp30 C-like domain (**Figure 4C**) (91). The overall architecture of the NKp30–B7-H6 complex differs considerably from those of the PD-1–PD-L1 (or PD-1–PD-L2) (92, 93) and CTLA-4–B7-1 (or CTLA-4–B7-2) complexes (94, 95), as is evident from superposing these complexes (**Figures 5A,B**). Relative to NKp30, PD-1 and CTLA-4 bind their ligands at angles of ~90° and ~60°, respectively. Whereas the PD-1–PD-L2 and CTLA-4–B7-1 interfaces are dominated by strand-to-strand interactions (**Figures 5D,E**), B7-H6 engages NKp30 in an antibody-like manner, with greater involvement by the loops of the B7-H6 V-like domain (**Figure 5C**). Thus, the protruding FG loop of B7-H6, which corresponds to complementarity-determining region (CDR) 3 of antibodies, fits

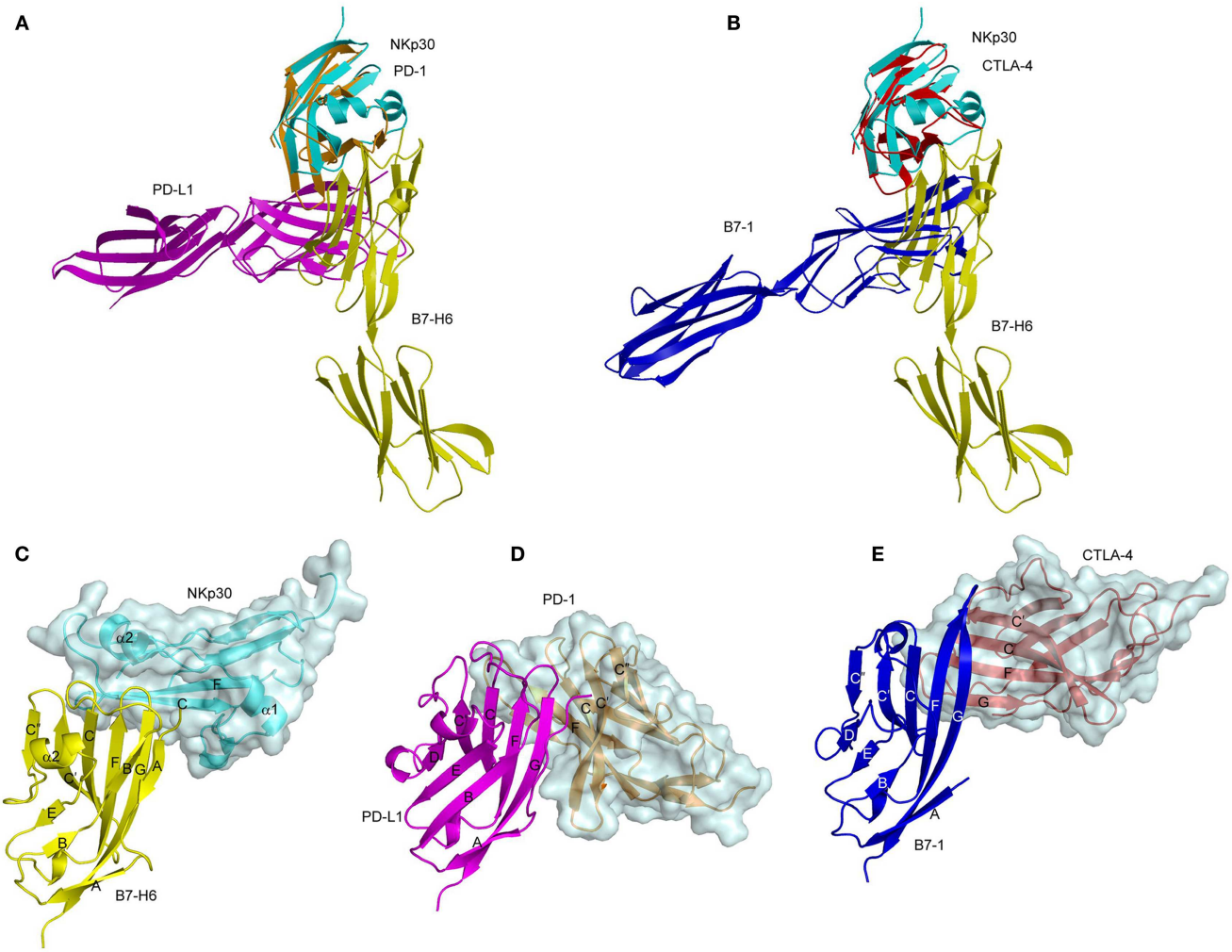

FIGURE 5 | Comparison of the NKp30–B7-H6, PD-1–PD-L1, and CTLA-4–B7-1 complexes. **(A)** Overlay of the NKp30–B7-H6 (3PV6) and PD-1–PD-L1 (3BIK) complexes by superposing NKp30 (cyan) onto PD-1 (brown). B7-H6 is yellow; PD-L1 is violet. **(B)** Overlay of the NKp30–B7-H6 and CTLA-4–B7-1 (1I8L) complexes by superposing NKp30 (cyan) onto CTLA-4 (red). B7-H6 is yellow; B7-1 is dark blue. **(C–E)** Docking modes in the NKp30–B7-H6, PD-1–PD-L1, and CTLA-4–B7-1 complexes. The three complexes were overlaid by superposing the IgV domains of B7-H6 (yellow), PD-1-L1 (violet), and B7-1 (dark blue), then translated horizontally for viewing.

into a deep groove on NKp30, with additional contacts provided by the BC (CDR1-like) and C′C″ (CDR2-like) loops of B7-H6.

Besides B7-H6, NKp30 recognizes the tegument pp65 protein of HCMV (84) and the nuclear factor BAT3 (80). Remarkably, B7-H6, pp65, and BAT3 appear completely unrelated, both in terms of three-dimensional structure and origin (B7-H6 is a host surface protein; pp65 is a viral structural protein; BAT3 is a host nuclear protein). How NKp30, a relatively small receptor comprising a single Ig-like ectodomain, can bind such disparate ligands is at present a mystery.

MHC-I RECOGNITION BY LY49 RECEPTORS

The highly polymorphic Ly49 receptors are the principle MHC-monitoring molecules on rodent NK cells. In mice, the Ly49 family includes at least 23 members (Ly49A–W), along with multiple allelic variants (96, 97). Most Ly49s inhibit NK-cell-mediated cytolysis upon recognizing one or more H-2D or H-2K alleles (96, 98, 99). However, some Ly49s are activating. In general, Ly49s recognize MHC-I independently of the bound peptide, although Ly49C and Ly49I display considerable peptide specificity (100, 101).

Like NKG2D and NKG2/CD94, Ly49 receptors are members of the C-type lectin-like family of proteins (10, 11). However, none of these NK receptors have a functional calcium-binding site. Ly49s are homodimeric type II transmembrane proteins (N-terminus inside the NK cell), with each chain containing a C-type lectin-like domain (CTLD), known as the natural killer receptor domain (NKD). Each NKD of the Ly49 homodimer is linked by a stalk of ~70 residues to the transmembrane and cytoplasmic domains. Activating and inhibitory receptors differ with regards to their cytoplasmic domains: whereas inhibitory Ly49s transduce signals via immunoreceptor tyrosine-based inhibitory motifs (ITIM),

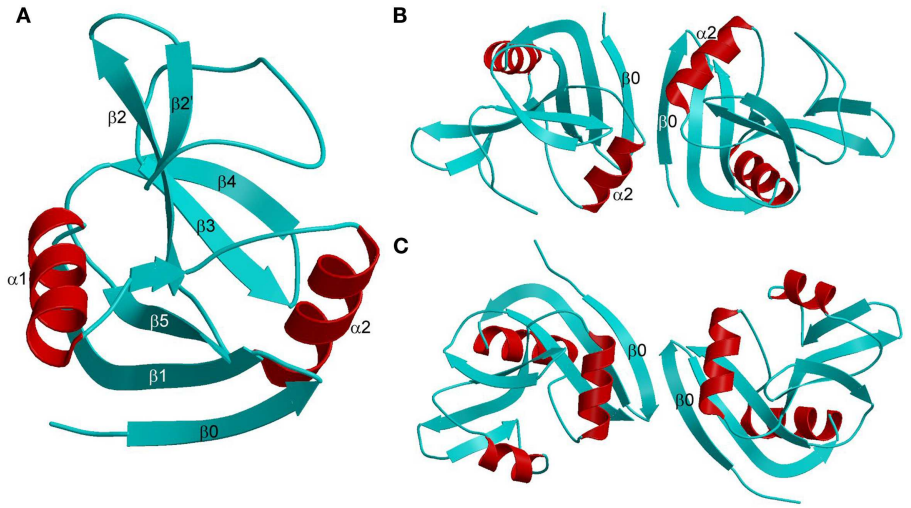

FIGURE 6 | Structures of Ly49 NK receptors. **(A)** Ribbon drawing of the Ly49A C-type lectin-like domain (1QO3). Secondary structure elements are labeled. β-strands and loops are cyan; α-helices are red. **(B)** Structure of the "closed" Ly49A homodimer. Secondary structure elements that participate in formation of the dimer interface are labeled. The α2 helices are juxtaposed. **(C)** Structure of the "open" Ly49C homodimer (3C8J). The α2 helices do not make contact across the dimer interface.

activating Ly49s instead use the associated signaling homodimer DAP12, which possesses an ITAM (96, 98).

Extensive structural information is available for Ly49 receptors in MHC-bound and unbound forms. Crystal structures have been reported for Ly49A NKD in complex with H-2D^d (102), Ly49C NKD bound to H-2K^b (103, 104), Ly49C NKD (104), Ly49I NKD (105), Ly49G2 NKD (104), Ly49L NKD (106), Ly49L NKD with the stalk region (106), and Ly49H bound to the MCMV immunoevasin m157 (107). Together, these structures have revealed the molecular architecture of the MHC-binding site of Ly49 receptors, the basis for MHC-I engagement in *trans* and *cis*, and the means by which viral immunoevasins target Ly49s.

The Ly49 NKD is composed of two α-helices, designated α1 and α2, and two anti-parallel β-sheets formed by seven β-strands (**Figure 6A**). Ly49 receptors exist exclusively as dimers on the NK cell surface. In the dimers, two NKDs associate through strand β0 to create an extended anti-parallel β-sheet. Ly49 dimers may adopt two distinct conformations: "closed" and "open," as exemplified by Ly49A (**Figure 6B**) and Ly49C (**Figure 6C**), respectively. In the closed dimer, the C-terminal ends of the α2 helices are juxtaposed, whereas in the open dimer the α2 helices do not contact each other. As explained below, this variability in Ly49 dimerization geometry serves to modulate the way NK receptors bind MHC (102–104).

Although Ly49s and other C-type lectin-like NK receptors (e.g., NKG2D, NKG2/CD94, NKp65) retain a lectin-like fold, specific structural features required for lectin activity are absent, enabling these receptors to recognize proteins as opposed to carbohydrates. Most notably, C-type lectin-like NK receptors do not contain bound calcium ions due to missing calcium-coordinating residues.

In the Ly49A–H-2D^d complex (**Figure 7A**), the Ly49A homodimer engages a single H-2D^d molecule using only one of its subunits, at a site beneath the peptide-binding platform of the MHC-I ligand (102). This site partially overlaps the binding sites for CD8 and LILRB1 (**Figure 3A**). In the Ly49C–H-2K^b complex (103, 104), by contrast, the Ly49C dimer binds H-2K^b bivalently, with each subunit making identical contacts with MHC-I at a site equivalent to Ly49A binding site on H-2D^d (**Figure 7B**).

The different dimerization geometries of Ly49A and Ly49C account for the different modes of MHC engagement in the Ly49A–H-2D^d and Ly49C–H-2K^b complexes. The closed Ly49A dimer cannot simultaneously bind two MHC ligands, like the open Ly49C dimer, because this would result in major steric clashes between MHC molecules, at least in the crystal. However, an NMR study revealed that Ly49A exists predominantly as an open dimer in solution that can bind two MHC-I molecules (108). The most likely interpretation of the combined results from X-ray crystallography and NMR is that Ly49 receptors are present on the NK cell surface in dynamic equilibrium between an open form, which permits bivalent MHC binding, and a closed form, which only allows engagement of one MHC ligand.

As in the case of KIRs (see above), Ly49s display specificity for different MHC alleles. Thus, whereas the promiscuous Ly49C receptor recognizes H-2K^b, H-2K^d, H-2D^b, H-2D^d, and H-2D^k, the more specific Ly49A and Ly49C receptors only bind H-2D^d and H-2D^k (97, 101). Ly49s have developed a two-tiered strategy for recognizing MHC, as deduced from the Ly49A–H-2D^d and Ly49C–H-2K^b structures (104). Primary recognition is carried out by a central region consisting of strand β3, loop L5, and strand β4 (residues 232–243). This central region has a conserved structure across Ly49s and contributes most to the binding energetics. Supplementing these primary interactions are secondary ones mediated by a region flanking the central region that comprises residues 218–231. This region, which exhibits high sequence variability across the Ly49 family, confers different MHC specificities. It adopts markedly different conformations in Ly49A (**Figure 7C**) and Ly49C (**Figure 7D**), and separates Ly49s into

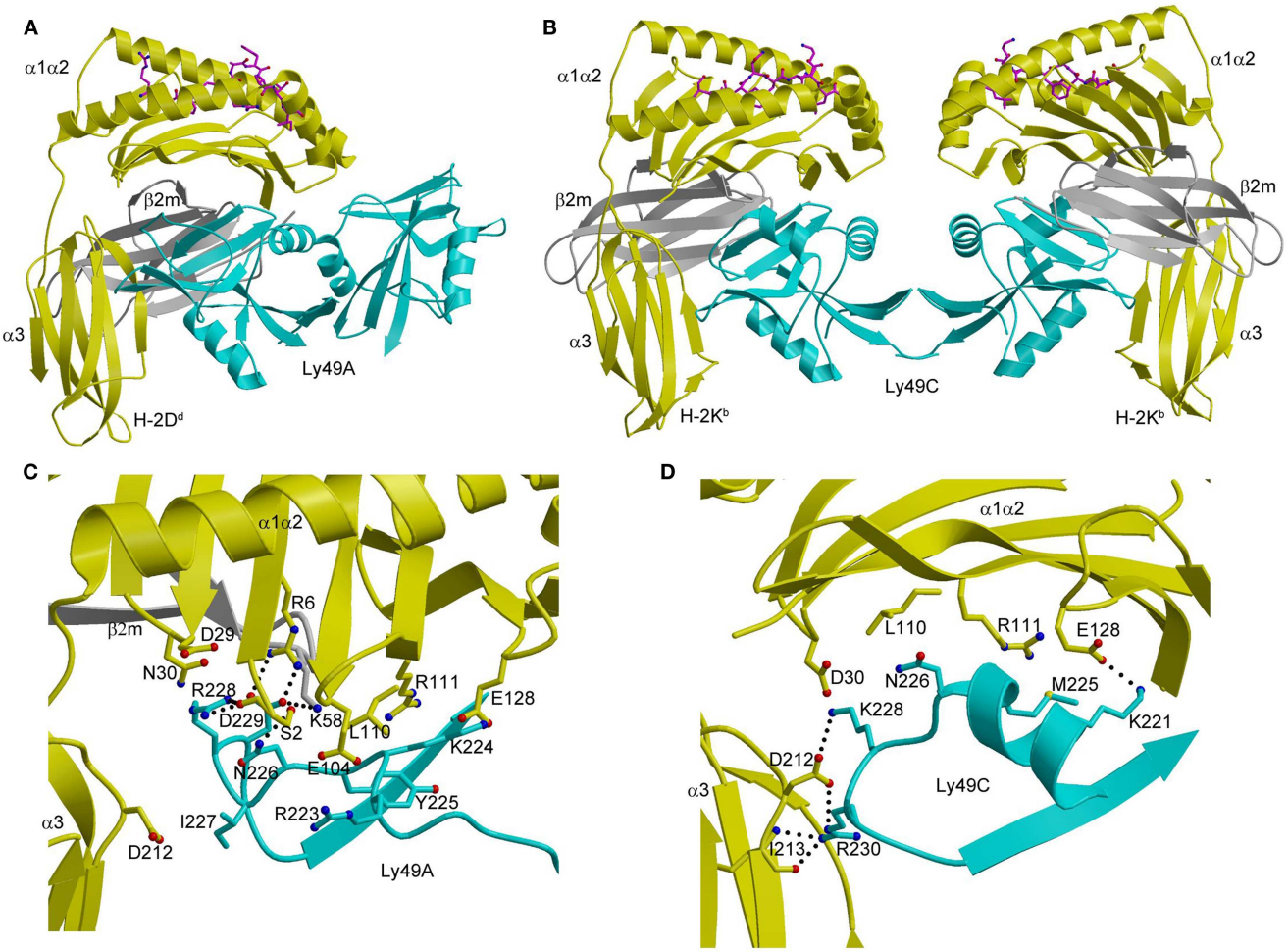

FIGURE 7 | Structures of Ly49–MHC-I complexes. **(A)** Ribbon diagram of Ly49A bound to H-2D^d (1QO3). The α1, α2, and α3 domains of the MHC-I heavy chain are yellow; β_2m is gray; the peptide is magenta; the Ly49A dimer is cyan. In this view, the complex is oriented with the H-2D^d molecule on the target cell at the bottom; the Ly49A homodimer reaches H-2D^d from an opposing NK cell at the top, to which it is connected via stalk regions projecting down to the N-termini of the subunits. **(B)** Structure of Ly49C in complex with H-2K^b (3C8K). **(C)** The Ly49A–H-2D^d interface, highlighting interactions made by residues 211–231 of Ly49A. **(D)** The Ly49C–H-2K^b interface, showing interactions made by the corresponding region of Ly49C. The side chains of interacting residues are drawn in ball-and-stick representation, with carbon atoms in cyan (Ly49A or Ly49C), yellow (H-2D^d or H-2K^b), or gray (β_2m), oxygen atoms in red, and nitrogen atoms in blue. Hydrogen bonds are represented by dotted lines.

ones that recognize both H-2D and H-2K ligands (e.g., Ly49C) versus ones that only recognize H-2D (e.g., Ly49A) (104).

The finding that MHC recognition by Ly49A is independent of the sequence of the MHC-bound peptide in both cellular and binding assays is readily explained by the total absence of direct contacts between Ly49A and the peptide in the Ly49A–H-2D^d structure (**Figure 7A**) (102). By contrast, the remarkable peptide selectivity of Ly49C is difficult to understand in terms of the Ly49C–H-2K^b complex, in which there is also a complete lack of contacts between Ly49C and the peptide (**Figure 7B**) (103, 104). Although the potential biological role of the peptide selectivity of certain Ly49s (and KIRs) remains obscure, the description of a functional interaction between Ly49P and H-2D^k on MCMV-infected cells that confers resistance to the virus suggests an intriguing possibility (17). This interaction requires the participation of the MCMV gene product m04/gp34 (109). This virulence factor associates with MHC-I in the endoplasmic reticulum and travels to the cell surface (110). Although the molecular nature of the Ly49P–H-2D^k interaction on MCMV-infected cells remains to be defined, one possibility is that m04/gp34 provides a specific peptide recognized by Ly49P in an H-2D^k-dependent manner, which would confer on NK cells a degree of specificity for viral pathogens reminiscent of that of cytotoxic T cells (11, 17, 109).

TRANS AND *CIS* INTERACTIONS OF LY49 RECEPTORS WITH MHC-I

Cell surface receptors mediate cell-to-cell communication by interacting with ligands expressed on other cells (*trans* interactions). In addition, some cell surface receptors have been shown

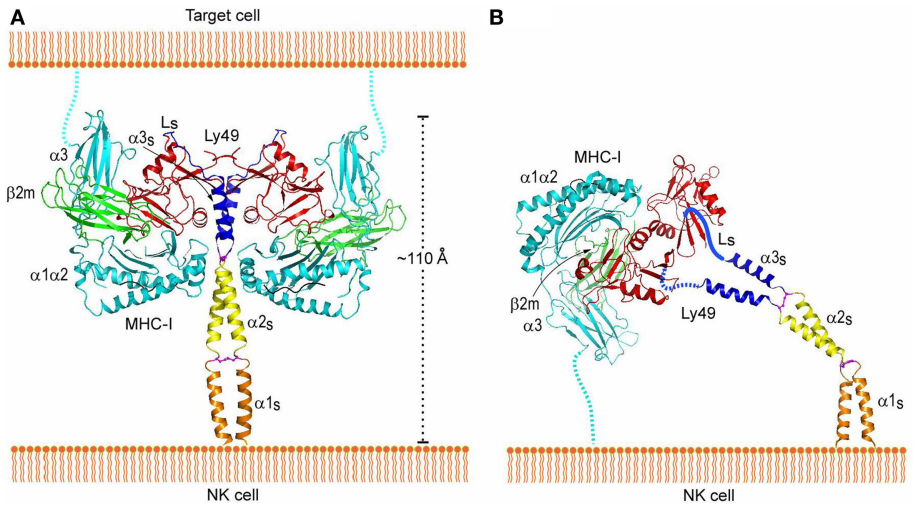

FIGURE 8 | Interaction of Ly49 receptors with MHC-I in *trans* and *cis*. **(A)** *Trans* interaction of an Ly49 receptor with two MHC-I molecules, based on the structures of Ly49L (3G8L) and the Ly49C–H-2K^b complex (3C8K). The α1, α2, and α3 domains of the MHC-I heavy chain are cyan; β_2m is green; Ly49 NKD is red; helix $\alpha3_S$ of the Ly49 stalk and loop L_S connecting $\alpha3_S$ to the NKD are blue; the disulfide bond linking the $\alpha3_S$ helices is magenta. The predicted $\alpha1_S$ and $\alpha2_S$ helices of the stalk are orange and yellow, respectively, with the disulfide bond in magenta. The Ly49 homodimer on the NK cell binds two MHC-I molecules on the target cell. To bind in *trans*, the stalks must adopt a backfolded conformation, as the N-termini of the Ly49 monomers point away from the NK cell membrane (Ly49s are type II transmembrane proteins). **(B)** *Cis* interaction of Ly49 with MHC-I, based on the structure of Ly49L and the Ly49A–H-2D^d complex (1QO3). The L_S loops connecting the $\alpha3_S$ helices to the NKDs are drawn arbitrarily. The Ly49 homodimer binds one MHC-I molecule on the NK cell itself. In this case, the stalks must assume an extended conformation, as the N-termini of the Ly49 monomers point toward the NK cell. Reproduced with permission from *Immunity* (106).

to bind ligands expressed on the same cell via *cis* interactions (60, 111). These include the structurally unrelated NK receptors Ly49 and LILRs, which interact not only with MHC-I molecules on other cells in *trans*, but also with MHC-I molecules on the same cell in *cis* (59, 112, 113). Other examples of cell surface receptors that bind ligands in both *trans* and *cis* are: siglec-2 (CD22), a negative regulator of B cell receptor signaling that recognizes sialic acid modifications of glycoproteins (114–117); herpes virus entry mediator (HVEM), which is regulated by its ligand BTLA (118); plexin receptors that bind semaphorins (119); and notch receptors that bind Delta (120, 121). The emerging theme from these studies is that *cis* interactions regulate effector cell function by modulating (decreasing or increasing) the threshold at which cellular activation signals produce a biological response (60, 111).

Cis interactions between Ly49 receptors and MHC-I ligands facilitate NK cell activation (112). This effect is not the result of inhibitory signaling through constitutive ITIM phosphorylation of Ly49s. Rather, *cis* interactions with MHC-I sequester, or mask, Ly49s to render them physically unavailable for functional *trans* interactions (113). In this way, *cis* interactions lower the threshold at which NK cell activation exceeds inhibition, considerably increasing the sensitivity of NK cells to diseased cells (60). Remarkably, in addition to modulating inhibitory function, *cis* interactions of Ly49A are necessary for NK cell education (122). As in the case of Ly49, the interaction of LILRB/PIR-B receptors with MHC-I in *cis* is constitutive (59). However, unlike Ly49, the ITIMs of LILRB/PIR-B receptors are phosphorylated constitutively, such that *cis* binding generates tonic inhibitory signals that dampen NK cell activation.

Trans and *cis* interactions by Ly49 receptors utilize the same binding site beneath the peptide-binding platform of MHC-I (112). Therefore, Ly49s must drastically reorient their NKDs relative to the NK cell membrane in order to bind MHC-I in *trans* versus *cis*. Most likely, it is the exceptionally long stalk regions of Ly49s (~70 residues) that provide the requisite flexibility. In the crystal structure of Ly49L, which includes the C-terminal 40 residues of the stalk region (designated the α3s segment), the stalk is composed of an α-helix and a 12-residue loop linking the helix to the NKD (**Figure 8A**) (106). However, instead of projecting from the NKD, as is typical for a stalk region, the Ly49L stalk backfolds onto the NKD. In a modeled Ly49–MHC-I complex (**Figure 8A**), the N-termini of the stalk regions point in a completely opposite direction from the C-termini of the MHC-I molecules. Therefore, Ly49s likely adopt the backfolded conformation to bind MHC-I in *trans*. On the other hand, *cis* binding requires the stalks to assume an extended conformation that orients the N-termini of the NKDs toward the NK cell (**Figure 8B**). Unlike the *trans* interaction, where one Ly49 dimer engages two MHC-I ligands (**Figure 3A**), this model precludes *cis* engagement of both NKDs by MHC-I, because of the orientation that binding of one MHC-I ligand would impose on the Ly49 dimer (**Figure 3B**). In agreement with the model, biochemical analyses confirmed that *trans* binding of MHC-I by Ly49 dimers occurs in a bivalent fashion, whereas *cis* binding is monovalent (106). Moreover, Ly49 receptors appear able to switch between backfolded and extended conformations (108, 123).

Cis–trans binding may typically require major structural changes analogous to those of Ly49. However, the stalk regions

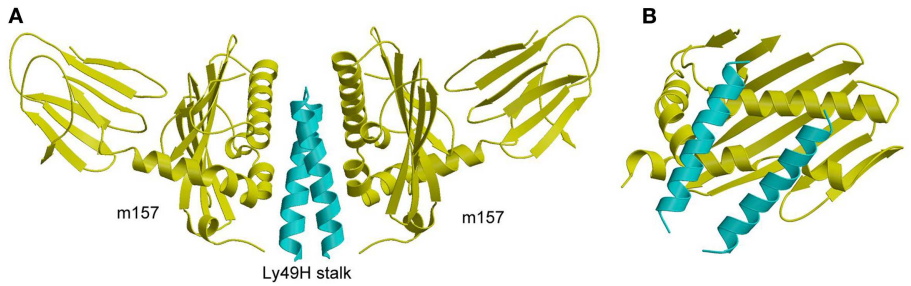

FIGURE 9 | Structure of m157 bound to the stalk region of Ly49H. **(A)** Side view of the Ly49H–m157 complex (4JO8), in which two m157 monomers (yellow) engage the Ly49H stalk (cyan). Only part of the helical stalk region of Ly49H (the α3s segment) was visible in electron density. The rest of the Ly49H stalk (segments α1s and α2s) and the NKD were not resolved in the structure. **(B)** Top view of the Ly49H–m157 complex, in which the helical stalks of Ly49H lie across the α1/α2 platform of m157.

of LILRB/PIR-B receptors are much shorter than those of Ly49. Accordingly, the ligand-binding D1 and D2 domains of LILRB would need to reverse direction relative to the surface of the NK cell in order to engage MHC-I in *cis*. To do so, LILRB would need to bend back on itself and assume a horseshoe-shaped arrangement of the four Ig domains (D1–D4), as observed for the four N-terminal Ig-like domains of *Drosophila* Dscam (124). Such a large reversal implies considerable flexibility in the segment connecting D2 with D3. In the case of PIB-B, which has two additional membrane-proximal Ig-like domains compared to LILRB, the D4–D5 or D5–D6 connecting segments might provide additional flexibility.

LY49 RECOGNITION OF A VIRAL IMMUNOEVASIN

Studies of the susceptibility of different mouse strains to infection by MCMV were the first to demonstrate direct recognition of a viral pathogen by NK cells that confers host protection (125). Resistance to infection in C57BL/6 mice is mediated by a single genetic locus in the NKC, which contains both inhibitory and activating Ly49 receptors (126). This locus encodes the activating receptor Ly49H, which impairs MCMV replication (127–130). By contrast, BALB/c mice do not restrict MCMV replication because they lack a gene for Ly49H. Subsequent studies revealed that Ly49H binds directly to a viral glycoprotein, the MHC-I homolog m157, which is expressed on MCMV-infected cells (15, 16). It was also discovered that m157 binds not only to the activating receptor Ly49H in resistant mouse strains, but also to the inhibitory receptor Ly49I in susceptible ones, which explains why MCMV would possess a gene that confers a selective disadvantage to its survival (15, 16, 131).

The crystal structure of m157 showed that this immunoevasin has an MHC-like fold, although it does not bind peptides or associate with β_2m (132). Surprisingly, m157 binds to the stalk region of Ly49H, rather than the NKDs, which recognize MHC-I (107). Although m157 was well resolved in the Ly49H–m157 structure, only the α3s segment of Ly49H could be seen in the electron density (**Figure 9A**). The lack of density for the Ly49H NKD implies considerable flexibility of the NKD in the crystal lattice. In agreement with the structure, solution binding measurements using Ly49H constructs lacking the NKD or stalk region showed that binding to m157 was mediated entirely by the α3s stalk segment, and that the NKD made no appreciable contribution to the interaction (107).

In the complex, two m157 monomers engage the Ly49H dimer, such that the helical stalks lie diagonally across the α1/α2 platform of m157 (**Figure 9B**). This binding mode is completely distinct from that used by Ly49A and Ly49C to engage MHC-I, whereby the NKDs contact MHC-I at a site beneath the α1/α2 peptide-binding platform (**Figures 7A,B**). Consequently, m157 does not compete with MHC-I for binding to the NKD. Central to the Ly49H–m157 interaction are two exposed aromatic residues in the Ly49H α3s stalk segment (Tyr115 and Trp123) that make extensive contacts with the immunoevasin. The ability of m157 to target some, but not all, members of the Ly49 receptor family can be correlated with sequence differences in the stalk region (107).

As discussed above, Ly49 receptors can adopt two distinct conformations, backfolded or extended (106). However, the recognition mode observed in the Ly49H–m157 complex only appears possible with Ly49 in the extended state, the conformation that recognizes MHC-I in *cis* (**Figure 8B**). In the backfolded conformation, by contrast, the Ly49 α3s stalk segment would not be accessible to m157, due to its intimate association with the NKD (**Figure 8A**). For both the Ly49H–m157 and Ly49I–m157 interactions, kinetic and thermodynamic measurements showed that binding involves a conformational selection mechanism where only the extended conformation of Ly49 is able to bind a first m157 ligand, followed by binding of a second m157 (123). The interaction is characterized by strong positive cooperativity, such that the second m157 binds the Ly49 homodimer 1,000-fold more tightly than the first. The rate-limiting step in the overall mechanism is a conformational transition in Ly49 from its backfolded to extended form.

LIGAND RECOGNITION BY NKG2D

NKG2D is a homodimeric C-type lectin-like NK receptor that is expressed on NK cells and cytotoxic T cells. It recognizes multiple structural homologs of MHC-I, including MICA, MICB, ULBP13, and RAE-1β, which all lack a peptide-binding groove and β_2m (14, 133, 134). ULBP3 and RAE-1β also lack an α3 domain, and are present on the cell surface as glycophosphatidylinositol-linked α1/α2 domains. In humans, expression of MICA and MICB is upregulated in epithelial tumors and stressed cells, compared to

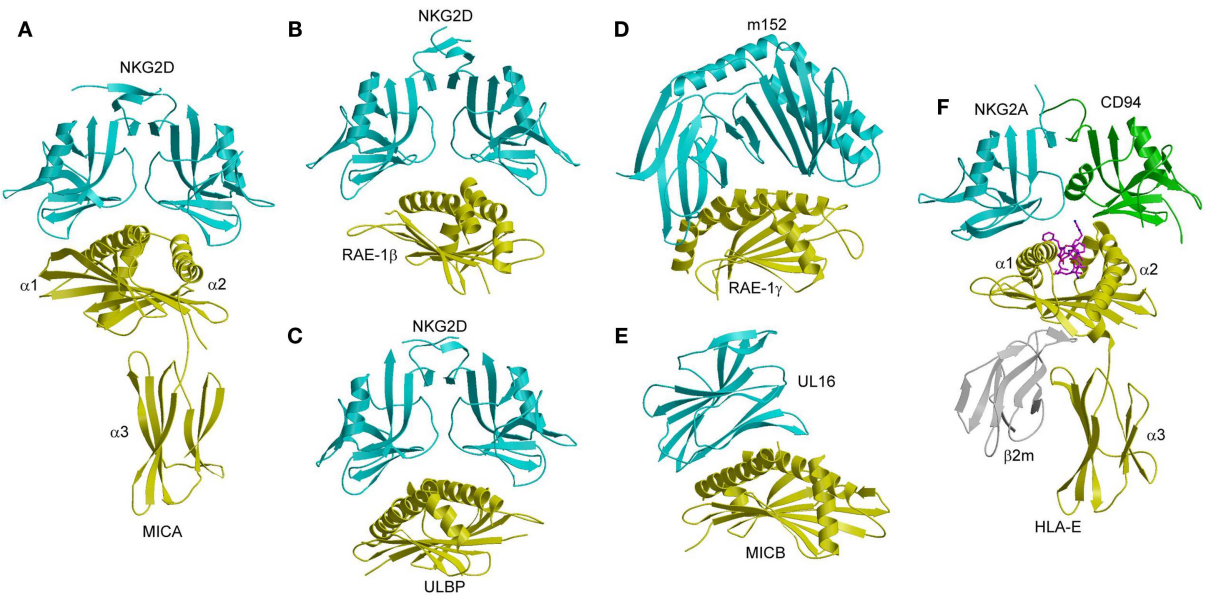

FIGURE 10 | Structures of NKG2D and NKG2A/CD94 complexes. **(A)** The human NKG2D–MICA complex (1HYR). The two subunits of the NKG2D homodimer are cyan; MICA is yellow. **(B)** The mouse NKG2D–RAE-1β complex (1JSK). **(C)** The human NKG2D–ULBP3 complex (1KCG). **(D)** Structure of the MCMV immunoevasin m152 bound to the NKG2D ligand RAE-1γ (4G59). **(E)** Structure of the Ig-like HCMV immunoevasin UL16 bound to MICA (2WY3). **(F)** The human NKG2A/CD94–HLA-E complex (3CDG). The NKG2A and CD94 subunits of the NKG2A/CD94 heterodimer are cyan and green, respectively.

little or no expression in normal tissues (135, 136). In rodents, RAE-1, MULT-1, and H-60 are upregulated in tumor cells but not normal cells, similar to MICA and MICB in humans (137, 138). The differential expression pattern of these MHC-related ligands indicates that NKG2D is a key receptor for tumor surveillance by NK cells. In mice, the MCMV-encoded immunoevasins m145, m152, and m155 are involved in downregulating surface expression of the NKG2D ligands MULT-1, RAE-1, and H-60, respectively, thereby thwarting an NKG2D-mediated anti-viral response (63, 139). In humans, the HCMV-encoded immunoevasin UL16 acts as a decoy receptor by binding the NKG2D ligands MICB, ULBP1, and ULBP2 (140). Crystal structures have been reported for human and mouse NKG2D in free form (141, 142), for human NKG2D bound to MICA and ULBP3 (134, 143), and for mouse NKG2D in complex with RAE-1β (144). In addition, structures have been determined for m152 in complex with RAE-1γ (139), and for UL16 bound to MICB (140).

MICA consists of an α1/α2 platform domain, which contains the α1 and α2 helices that define the peptide-binding groove in bona fide MHC-I molecules, and a C-type Ig-like α3 domain (**Figure 10A**) (145). The NKG2D homodimer binds MICA orthogonally to the α1 and α2 helices of the α1/α2 platform (143). This docking mode roughly resembles that of TCR onto MHC-I, but is completely distinct from the way Ly49C binds MHC-I (**Figure 7B**). Recognition of the asymmetric MICA ligand by the symmetric NKG2D receptor is mediated by similar sites on the NKG2D subunits that contact distinct sites on MICA. In particular, most (7 of 11) contact residues from each NKG2D monomer are shared by both MICA binding sites.

NKG2D binds ULBP3 and RAE-1β orthogonally to the α1/α2 domain of these MHC-like ligands, in a manner resembling the NKG2D–MICA complex (**Figures 10B,C**) (134, 144). Most notably, a shared subset of NKG2D residues mediates hydrophobic interactions with all three ligands. However, the binding interfaces also display significant differences, such that only one salt bridge and two hydrogen bonds are common to the NKG2D–ULBP3, NKG2D–RAE-1β, and NKG2D–MICA complexes (134, 143, 144).

These structural studies have demonstrated that NKG2D has a remarkable ability to recognize MICA, ULBP3, and RAE-1β using a single binding site, even though these ligands share only ~25% sequence identity. This ability is explained by a rigid adaptation recognition mechanism, rather than induced fit (146). Detailed mutational analysis of NKG2D has shown that the most energetically important residues of the receptor ("hot spots") interact with relatively conserved residues of MICA, ULBP3, and RAE-1β, without significant conformational changes in NKG2D upon ligand binding (141, 146).

In the MCMV m152–RAE-1γ complex (139), the MHC-I-like immunoevasin binds the α1 and α2 helices of RAE-1γ in a pincer-like manner that resembles the interaction of NKG2D with RAE-1β (**Figure 10D**). In the HCMV UL16–MICB complex (140), by contrast, the Ig-like UL16 protein uses a three-stranded β-sheet to engage the α1 and α2 helices of MICB, such that residues at the center of the β-sheet mimic a binding motif employed by the structurally unrelated C-type lectin-like NKG2D to bind its diverse ligands (**Figure 10E**). By competing with NKG2D for ligand binding, m152 and UL16 prevent NKG2D-mediated NK cell activation and thus promote viral survival (147–149).

RECOGNITION OF HLA-E BY NKG2/CD94 RECEPTORS

In addition to NKG2D, which exists as a homodimer on the NK cell surface, the NKG2D family includes NKG2A, NKG2B, NKG2C, and NKG2E, all of which form obligate heterodimers with CD94 (150–152). NKG2A and NKG2B contain ITIM motifs in their cytoplasmic tails and function as inhibitory receptors; NKG2C and NKG2E associate with the ITAM-containing DAP12 molecule and are activating receptors. The ligand for NKG2/CD94 receptors is the non-classical MHC-I molecule HLA-E, which binds a restricted set of peptides derived from the leader peptides of classical and non-classical MHC-I proteins (150–152). Because HLA-E does not express on the cell surface without a bound peptide, HLA-E expression depends on the production of other MHC-I molecules. Therefore, recognition of HLA-E by NKG2/CD94 receptors enables NK cells to monitor the expression of other HLA class I proteins on cells. This double-check mechanism ensures that cells are producing MHC-I molecules in a normal manner.

The crystal structure of NKG2A/CD94 has been determined in unbound form (153), and in complex with HLA-E bound to a peptide derived from the leader sequence of HLA-G (154, 155). In the complex, NKG2A/CD94 straddles the peptide-binding cleft of HLA-E, with the NKG2A and CD94 subunits mainly interacting with the α2 and α1 helices of HLA-E, respectively (**Figure 10F**). No significant conformational changes in NKG2A/CD94 or HLA-E occur upon complex formation, indicating a lock-and-key binding mechanism, as in the case of NKG2D (141, 146).

Most (~70%) of the buried surface area in the NKG2A/CD94–HLA-E complex is contributed by the invariant CD94 subunit (154, 155). Thus, CD94 dominates the interaction with HLA-E, whereas NKG2A is more peripheral to the interface. The peptide accounts for ~20% of the buried surface area on the HLA-E side of interface, in which CD94 again dominates the interactions with peptide, albeit with poor shape and chemical complementarity (154). CD94 is positioned over the P8 residue of the peptide, with additional contacts to residue P5. The focus of NKG2A/CD94 on the C-terminal half of the peptide is notable, since nearly all of the limited sequence variation among HLA-E-restricted peptides is concentrated in the C-terminal residues, which are read out primarily by the invariant CD94 subunit.

In sharp contrast to the dominant role of hydrophobic interactions in ligand recognition by NKG2D (134, 144), the NKG2A/CD94–HLA-E interface is mostly electrostatic in nature (154). The interface is characterized by a large number of polar interactions, including 19 hydrogen bonds and 8 salt bridges. This helps explain the fidelity of NKG2A/CD94 for HLA-E compared to the promiscuity of NKG2D for multiple ligands, as discussed above.

CADHERIN RECOGNITION BY KLRG1

Killer cell lectin-like receptor G1 is a C-type lectin-like inhibitory receptor that contains an ITIM motif in its cytoplasmic region (156, 157). KLRG1 is found on 50–80% of human NK cells, and its expression is highly upregulated following infection with viruses or parasites (158–161). The biological ligand for KLRG1 is E-cadherin (32, 33, 162). E-cadherin, whose extracellular region comprises five Ig-like domains (EC1–EC5), is localized at the basolateral membrane of epithelial cells where it establishes tight binding between neighboring cells in adherens junctions (163). Besides E-cadherin, KLRG1 recognizes N- and R-cadherins (32), which are present in analogous structures in other cell types. The binding of E-cadherin to KLRG1 prevents lysis of E-cadherin-expressing epithelial cells by KLRG1$^+$ NK cells, thereby preventing tissue damage (32, 71, 164). In addition, KLRG1 may play a role in tumor immunosurveillance analogous to missing self-recognition by inhibitory NK receptors that bind MHC-I (Ly49s and KIRs) (164, 165). Because the malignancy of epithelial tumors is frequently associated with down-regulation of E-cadherin, the KLRG1–E-cadherin system may serve to detect potentially metastatic tumors with abnormal cadherin expression (71, 164, 166).

In the crystal structure of the complex between KLRG1 and the EC1 domain of E-cadherin, one KLRG1 CTLD binds one EC1 molecule (**Figure 11A**) (167). In this respect, KLRG1 recognition of its non-MHC ligand is reminiscent of Ly49 recognition of MHC-I, in which each CTLD monomer contains an entire ligand-binding site (**Figure 7B**). By contrast, the binding site of NKG2D for the MHC-related ligand MICA (143) (**Figure 10A**), as well as the binding site of NKG2A/CD94 for HLA-E (154, 155) (**Figure 10F**), is formed by two CTLD subunits. E-cadherin docks onto a surface of KLRG1 that roughly corresponds to the ligand-binding site of Ly49s and other C-type lectin-like NK receptors (167). This site is formed by three loops (L3, L4, and L6) and β-strand 4 (**Figure 11A**), which interact primarily with residues Val3–Ile7 of E-cadherin (**Figure 11B**). These five residues are absolutely conserved in E-, N-, and R-cadherins, which enables NK cells bearing a single KLRG1 receptor to monitor expression of multiple cadherins on target cells, resulting in MHC-independent missing self-recognition.

The KLRG1–E-cadherin complex buries a total solvent-accessible surface of only 1140 ^{2}. This exceptionally small interface is at the lower limit of the average value of 1600 (±400) Å^2 for stable protein–protein complexes (168), and likely explains the relatively low affinity of the KLRG1–E-cadherin interaction ($K_D = 150\ \mu M$), which it is considerably weaker than for any other NK receptor–ligand pair characterized to date (167). KLRG1 may compensate for its exceptionally low monomeric affinity for cadherins through multipoint attachment to cadherin molecules on the target cell surface. Additionally, the ability of KLGR1 to form disulfide-linked dimers (169), or even multimers (170), may further increase the avidity of KLRG1–cadherin interactions. In this way, KLRG1–cadherin recognition could be achieved through the cooperativity of multiple associations, rather than by relying on the stability of individual complexes, while still allowing for dissociation of the complexes during transient NK cell–target cell encounters.

GENETICALLY LINKED C-TYPE LECTIN-LIKE RECEPTOR–LIGAND PAIRS

The NKC encodes approximately 30 type II transmembrane glycoproteins that are members of the C-type lectin-like superfamily (171). NKC genes are divided into killer cell lectin-like receptor (KLR) genes and C-type lectin receptor (CLEC) genes. KLR genes code for molecules expressed on NK cells. CLEC genes code for molecules expressed on other cell types, such as dendritic (CLEC9A) and myeloid (CLEC2B) cells.

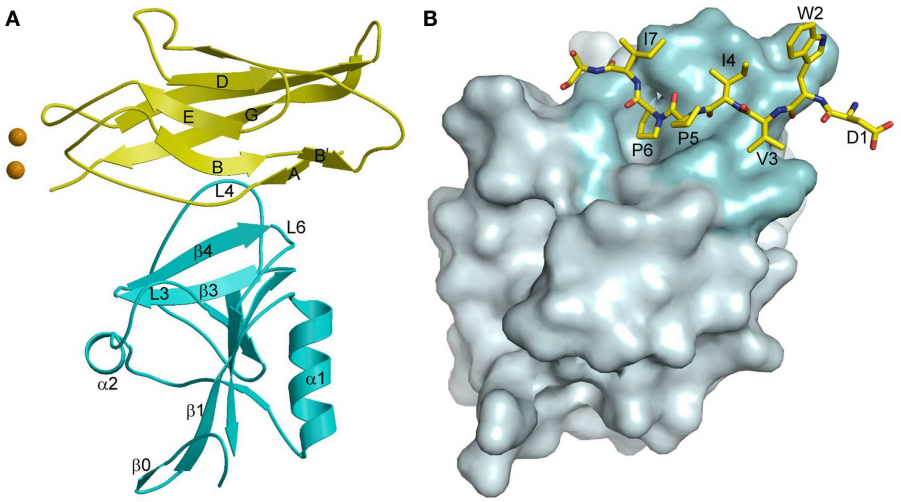

FIGURE 11 | Cadherin recognition by KLRG1. **(A)** Structure of KLRG1 in complex with the membrane-distal D1 domain of E-cadherin (3FF8). KLRG1 is cyan and E-cadherin is yellow. Secondary structure elements are labeled. Bound Ca^{2+} ions are drawn as brown spheres. **(B)** The KLRG1–E-cadherin binding interface. The KLRG1 molecular surface is shown in gray with the region contacting E-cadherin colored cyan. Residues 1–8 of E-cadherin are drawn in stick format and labeled.

The KLR family includes Ly49, NKG2D, and CD94/NKG2A receptors that bind MHC-I or MHC-I-like molecules, as discussed above. The KLR family also includes receptors that recognize non-MHC ligands. This category includes KLRG1, which binds E-cadherin (167) (**Figure 11A**), in addition to receptors that bind CLEC2 proteins which themselves belong to the C-type lectin-like superfamily (21). The genes encoding these KLR–CLEC2 receptor–ligand pairs are genetically linked in the NKC. For example, in mice, the inhibitory KLR family member receptor Nkrp1d binds Clrb (172, 173). Down-regulation of Clrb expression by genotoxic stress or tumorigenesis triggers NK-cell-mediated lysis, supporting the concept of MHC-independent control of NK cell function by Nkrp1 receptors (173, 174). In humans, the inhibitory NK receptor NKR-P1A binds the CLEC2 family member LLT1, reducing NK-cell-mediated cytotoxicity and interferon-γ secretion (175–177). Viral induction of LLT1 expression in B cells points to a role for the NKR-P1A–LLT1 interaction in modulating immune responses to pathogens (178). The human activating NK receptor NKp80 recognizes the CLEC2 family member AICL, which is genetically coupled to NKp80 in the NKC (21, 31). The NKp80–AICL pair promotes cross-talk between NK cells and monocytes (31). In addition to monocytes, AICL is expressed on monokine-activated human NK cells that also express NKp80, which may enable autonomous control of NK cell responses (179).

Keratinocyte-associated C-type lectin (KACL) is a newly identified member of the human CLEC2 family (180). Notably, KACL is expressed almost exclusively in the skin. KACL is a ligand for the activating receptor NKp65, which is genetically linked to KACL in the NKC (29). Upon binding KACL on keratinocytes, NKp65 triggers NK-mediated cytotoxicity and proinflammatory cytokine release. Thus, the NKp65–KACL interaction may contribute to the immunosurveillance of human skin (21, 29, 181).

The structure of NKp65 bound to KACL has revealed the basis for genetically coupled recognition in the NKC (182). KACL forms a homodimer similar to the NKG2D and Ly49 homodimers; NKp65, contrast, is monomeric (**Figure 12A**). KACL binds NKp65 bivalently, in a manner resembling the Ly49C–H-$2K^b$ complex (**Figure 12B**) (103, 104), except that, in the NKp65–KACL complex, it is the ligand (KACL), instead of the receptor (NKp65) that is dimeric. This bivalent binding mode is completely different from those employed by the dimeric NKG2D and Ly49A receptors. Thus, the NKG2D dimer engages one MICA molecule using a single binding site formed by the association of its two subunits (**Figure 12C**), whereas the Ly49A dimer binds a single H-$2D^d$ ligand using only one subunit (**Figure 12D**).

In the NKp65–KACL complex (182), two C-type lectin-like proteins engage each other in a head-to-head orientation utilizing similar structural elements: NKp65 uses loops L0, L3, L5, and L6 and strands β3 and β4 to contact the analogous loops and strands of KACL (**Figure 12A**). A mutational analysis of KACL residues in contact with NKp65 showed that all hotspot residues of KACL are conserved or conservatively substituted in AICL and LLT1, and that these hotspot residues contact residues on NKp65, NKp80, and NKR-P1A that are themselves conserved (182). Therefore, the docking mode observed in the NKp65–KACL complex also applies to other NKC-encoded receptor–ligand pairs, including NKp80–AICL, NKR-P1A–LLT1, and Nkrp1–Clr.

NKp65 binds KACL with exceptionally high affinity ($K_D = 6.7 \times 10^{-10}$ M), compared to other cell–cell recognition molecules, whose K_Ds are generally micromolar (183). Indeed, the affinity of NKp65 for KACL is 70,000-fold higher than that of NKR-P1A for LLT1 (184) and 3,000-fold higher than that of NKp80 for AICL (31). In contrast to NKR-P1A and NKp80, which exist as disulfide-linked dimers, NKp65 is not disulfide-linked on the NK cell surface. Likewise, AICL and LLT1 (21, 176,

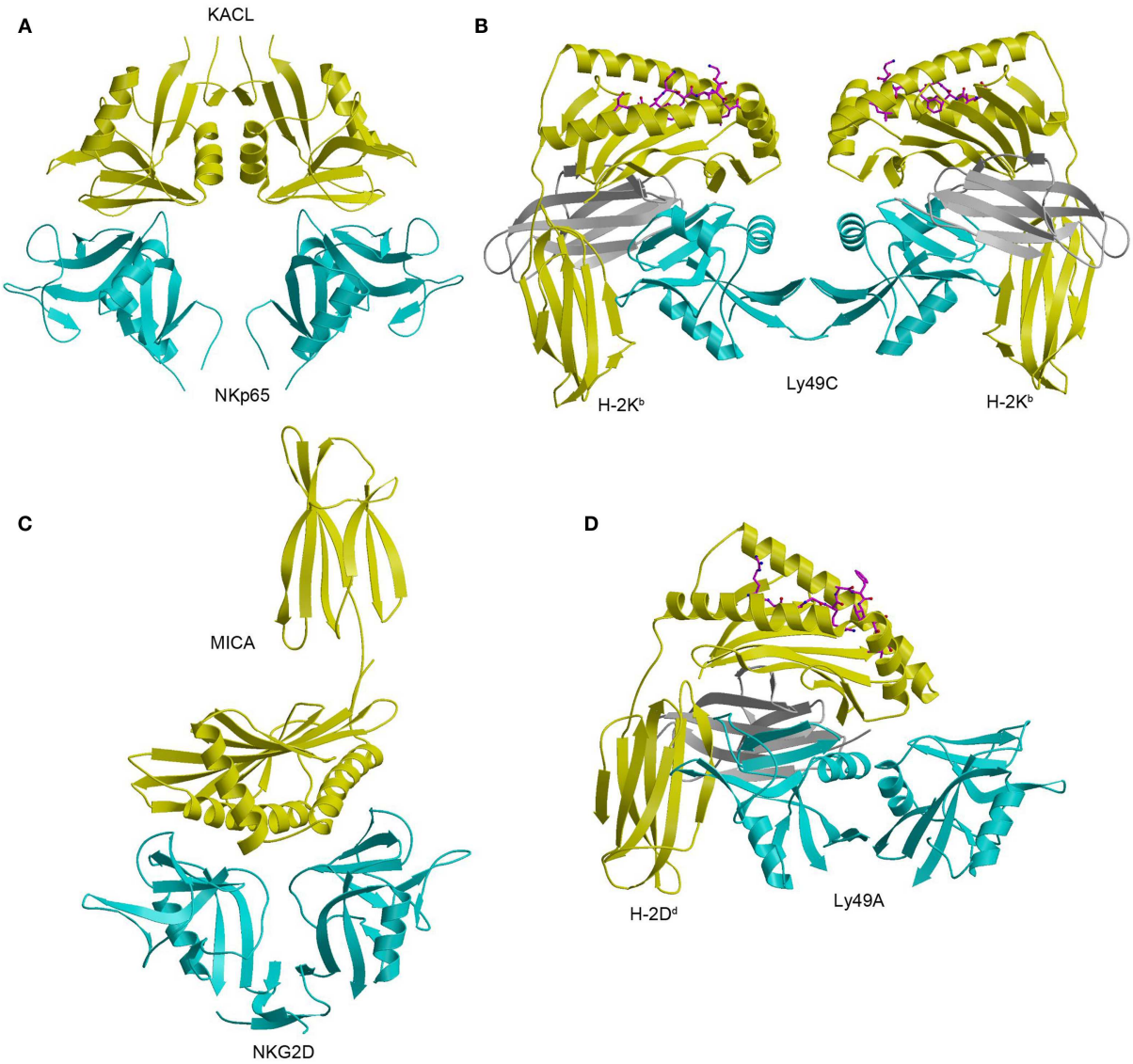

FIGURE 12 | Structure of the NKp65–KACL complex and comparison with other NKC-encoded receptor complexes. **(A)** Structure of the human NKp65–KACL complex (4IOP). NKp65 is cyan and the KACL dimer is yellow. **(B)** Structure of the Ly49C–H-2K^b complex (3C8K). The Ly49C dimer is cyan, the H-2K^b heavy chain is yellow, and β_2m is gray. **(C)** Structure of the NKG2D–MICA complex (1HYR). The NKG2D dimer is cyan and MICA is yellow. **(D)** Structure of the Ly49A–H-2D^d complex (1QO3). The Ly49A dimer is cyan, the H-2D^d is heavy chain is yellow, and β_2m is gray.

177, 181), but not KACL (29), form disulfide-linked dimers on cells. Dimerization of NKp80 and NKR-P1A may compensate for the low (micromolar) affinities of these receptors, relative to NKp65, by increasing avidity via bivalent binding of their AICL and LLT1 ligands, which are themselves dimeric. By contrast, the high (nanomolar) affinity of the NKp65–KACL interaction may overcome the need for receptor dimerization by generating complexes of half-life comparable to those of the NKp80–AICL and NKR-P1A–LLT1 complexes, resulting in efficient signaling.

FUTURE DIRECTIONS

The structural studies described in this review have enabled us to understand how representative NK receptors recognize cellular and viral ligands at the atomic level. However, the biophysical mechanisms by which inhibitory or activating signals are transmitted to the NK cell following ligand engagement remain largely a mystery. It is also unknown how inhibitory and activating signals are integrated within the NK cell to ultimately determine the outcome of NK cell–target cell encounters.

Only recently have structural studies begun to elucidate the molecular details of the signal transduction process. Crucial for NK cell triggering is the association of the transmembrane region of activating NK receptors, such as NKG2D and NKp44, with ITAM-bearing signaling molecules, such as DAP10 and DAP12. NMR has been used to determine the structure of the heterotrimeric assembly formed by the transmembrane regions

of NKG2C and DAP12 (185). The main contact site comprises an intricate electrostatic network involving five hydrophilic transmembrane residues: two aspartates and two threonines from the DAP12 dimer that together interact with a lysine from NKG2C. Such studies of membrane-embedded NK receptors, and their association with signaling proteins, promise to provide critical information for linking ligand recognition to NK cell activation or inhibition.

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Effects of peptide on NK cell-mediated MHC I recognition

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The inhibitory receptors for MHC class I have a central role in controlling natural killer (NK) cell activity. Soon after their discovery, it was found that these receptors have a degree of peptide selectivity. Such peptide selectivity has been demonstrated for all inhibitory killer cell immunoglobulin-like receptor (KIR) tested to date, certain activating KIR, and also members of the C-type lectin-like family of receptors. This selectivity is much broader than the peptide specificity of T cell receptors, with NK cell receptors recognizing peptide motifs, rather than individual peptides. Inhibitory receptors on NK cells can survey the peptide:MHC complexes expressed on the surface of target cells, therefore subsequent transduction of an inhibitory signal depends on the overall peptide content of these MHC class I complexes. Functionally, KIR-expressing NK cells have been shown to be unexpectedly sensitive to changes in the peptide content of MHC class I, as peptide:MHC class I complexes that weakly engage KIR can antagonize the inhibitory signals generated by engagement of stronger KIR-binding peptide:MHC class I complexes. This property provides KIR-expressing NK cells with the potential to recognize changes in the peptide:MHC class I repertoire, which may occur during viral infections and tumorigenesis. By contrast, in the presence of HLA class I leader peptides, virus-derived peptides can induce a synergistic inhibition of CD94:NKG2A-expressing NK cells through recruitment of CD94 in the absence of NKG2A. On the other hand, CD94:NKG2A-positive NK cells can be exquisitely sensitive to changes in the levels of MHC class I. Peptide antagonism and sensitivity to changes in MHC class I levels are properties that distinguish KIR and CD94:NKG2A. The subtle difference in the properties of NK cells expressing these receptors provides a rationale for having complementary inhibitory receptor systems for MHC class I.

Keywords: KIR, NKG2A, CD94, peptides, MHC class I, natural killer cells, antagonism, synergy

INTRODUCTION

Our knowledge of the functional role of natural killer (NK) cells has greatly increased in recent years. Originally thought to mainly recognize infected or neoplastic cells, NK cells are now known to help shape the adaptive immune response through direct interactions with dendritic cells, macrophages, and T cells (1, 2). NK cells integrate the signals derived from cellular contacts to determine whether or not effector functions are initiated. Due to a dominance of inhibitory over activating signals, healthy or quiescent cells do not activate NK cells. The ability to recognize changes in target cell state has been related to up-regulation of ligands for activating receptors ("induced self-recognition"), and down-regulation of ligands for inhibitory receptors ("missing self-recognition") (3, 4). In humans, the most important inhibitory receptors for MHC class I comprise molecules from the killer cell immunoglobulin-like receptor (KIR) or C-type lectin-like receptor (CD94:NKG2A) families.

The inhibitory KIR recognize specific HLA-A, -B, and -C alleles. In particular, KIR3DL2 binds HLA-A*03 and HLA-A*11; KIR3DL1 binds HLA-B alleles with the Bw4 serological motif; KIR2DL1 binds HLA-C alleles with lysine at position 80 (group 2 HLA-C); and both KIR2DL2 and KIR2DL3 bind HLA-C alleles with asparagine at position 80 (group 1 HLA-C alleles) (5). Thus, it was originally considered that simple structural motifs determined the engagement of KIR with MHC. However, detailed analysis of KIR binding has shown that KIR2DL2 can bind the recombinant HLA-B*4601 and B*7301 alleles, which have HLA-C-type motifs at residues 77–83 (6). Furthermore, KIR2DL2 can interact with a number of group 2 HLA-C alleles, as can KIR2DL3, albeit to a lesser extent, as the affinity of KIR2DL3 for MHC is lower than that of KIR2DL2 (7–9). Therefore, although motifs at residues 77–83 appear to dominate the specificity of the interaction between KIR and MHC, it is clear that these effects can be modified by additional contacts between KIR and the MHC class I heterotrimer.

PEPTIDE SELECTIVITY OF INHIBITORY RECEPTORS

Key experiments performed in the mid 1990s demonstrated that the KIR are sensitive to the peptide bound by MHC class I. This was originally shown for KIR3DL1 and HLA-B*2705, and then for KIR2DL1 (10–12). Subsequent work extended these findings to KIR2DL2, KIR2DL3, and KIR3DL2 (13–18). These functional experiments are supported by co-crystal structures of KIR and MHC class I. The co-crystal of KIR2DL2 and HLA-Cw*03 with the GAVDPLLAL peptide demonstrated that specific residues of KIR directly contact P7 and P8 residues of the bound peptide (13). Similarly, P8 of the LSSPVTKSF peptide in HLA-B*5701 contacts residue 166 of KIR3DL1 (19). In the crystal structure of KIR2DL1 with HLA-Cw4, direct contacts between KIR and MHC class I

peptide are not observed (20). Nevertheless, P8 is solvent accessible and changes in this residue do lead to alterations in NK cell function, implying secondary effects of MHC class I peptide on KIR2DL1:HLA-C binding.

The C-type lectin-like receptor NKG2A forms a heterodimer with a related family member CD94 to recognize the non-classical MHC class I molecule HLA-E (21–25). In general, this molecule binds leader peptides derived from HLA-A, -B, and -C molecules (26). Inhibitory signaling by CD94:NKG2A is also critically dependent on the peptide presented by HLA-E, and a hierarchy of HLA-E-binding peptides with different inhibitory properties for NKG2A-positive NK cells has been established (27–29). The peptide dependence of CD94:NKG2A was confirmed in its co-crystal structure with HLA-E and the HLA-G leader peptide VMAPRTLFL (30, 31). These studies showed that binding of CD94:NKG2A is dominated by the non-signaling CD94 moiety, and crucially P5, P6, and P8 have contacts with CD94 and P8 contacts NKG2A. The importance of these specific residues has been confirmed in surface plasmon resonance studies (32). Therefore, both inhibitory KIR and NKG2A are peptide selective. Furthermore, despite the rapid evolution of the KIR alongside that of classical MHC class I, this peptide dependence is a feature that has been retained across divergent KIR lineages (33).

PEPTIDE ANTAGONISM OF KIR-POSITIVE NK CELLS

Using a model system, we have investigated the functional consequences of KIR peptide selectivity. The T2 and 721.174 cell lines both synthesize HLA-Cw*0102 but have lost the ability to load peptide onto MHC due to a deficiency in transporter associated with antigen processing (TAP) (34). In these cell lines, MHC class I contains low affinity hydrophobic peptides derived from signal sequences, and reaches the cell surface but dissociates rapidly (35, 36). These TAP-deficient cell lines can be readily loaded with exogenous peptide as was originally demonstrated in CTL cell assays. HLA-Cw*0102 is of the HLA-C1 group specificity and hence engages with KIR2DL2 and KIR2DL3. These cell lines have allowed detailed examination of how KIR-positive NK cells respond to changes in the peptide content of MHC class I.

VAPWNSLSL is a peptide derived from TIMP1 that was eluted from an HLA-Cw*0102 transfectant of the MHC class I deficient 721.221 cell line (37). This peptide was used as a backbone to screen derivatives that differ only at the KIR-binding residues of P7 and P8 (38). Overall, although all peptides stabilized HLA-Cw*0102, in assays using KIR-fusion constructs only approximately a third of the peptides induced significant binding to KIR2DL2 and KIR2DL3. This allowed the definition of strong, weak, and null KIR binders, and this binding reactivity correlated well with their inhibitory potential in assays of NK cell function. We used the strongest inhibitory peptide VAPWNS*F*AL (VAP-FA), and the non-inhibitory peptide VAPWNS*D*AL (VAP-DA) to study how NK cells may respond to changes in peptide repertoire (38). Although it would be predicted that VAP-DA would have a null effect on the inhibition due to VAP-FA, it was found to modulate the inhibition of KIR2DL2/3-positive NK cells. We termed this phenomenon "peptide antagonism" to indicate that peptides that act alone have no effect on NK cell function, can modulate the inhibition due to inhibitory peptides. The mechanism for peptide antagonism may be related to a low affinity interaction between KIR and peptide:MHC. This phenomenon was confirmed using a tyrosine P8 substitution in VAP-DA, as tyrosine P8 substantially reduces binding of KIR2DL2 to HLA-Cw*03 in surface plasmon resonance studies (13). Further studies have shown that although VAP-DA does not bind KIR in the fusion construct assay, it can induce diffuse clustering of KIR2LD3 at the interface between NK cells and 721.174 cells (39). Additionally, it induces recruitment of SHP-1 to KIR2DL3, but it abrogates the formation of KIR2DL3 microclusters by VAP-FA, and thus it prevents downstream inhibitory signaling (**Figure 1**).

The precise molecular mechanism of antagonism is not clear at present (40). It appears to be dependent on the presence of immunoreceptor tyrosine-based inhibitory motifs (ITIMs), as in their absence there is no effect of the VAP-DA peptide on the microcluster formation by VAP-FA, although notably much lower levels of microcluster formation are observed in these experiments (39). Therefore, intracellular signaling events are likely to be important. It has been shown that rearrangement of the actin cytoskeleton is important for KIR accumulation at the NK cell synapse and it may be that this rearrangement is impaired by antagonist peptides (41). SHP-1 requires tyrosine phosphorylation for full activation (42). Therefore, although SHP-1 may be recruited by VAP-DA, it may not be phosphorylated and so not fully activated so that downstream inhibitory signaling events remain uninitiated. Alternatively, the low affinity KIR:HLA-C:VAP-DA complexes could rapidly dissociate before productive inhibitory clustering has taken place. By recruiting SHP-1 to these transient complexes, it could sequester SHP-1 away from the more stable KIR:HLA-C:VAP-FA complexes and thus prevent an inhibitory signal being generated. These events require additional investigation to determine the precise mechanisms governing the phenomenon of "peptide antagonism."

PEPTIDE SYNERGY OF NKG2A-POSITIVE NK CELLS

In contrast to the KIR, the C-type lectin-like receptors are relatively conserved in terms of evolution. The human receptor binds the non-classical HLA-E molecule which in healthy cells presents peptides derived from the leader sequences of other MHC class I molecules, including HLA-A, -B, -C, and -G (26). A homologous receptor:ligand partnership is present in the mouse.

Murine CD94:NKG2A interacts with the non-classical Q-a1 molecule, which also binds a leader sequence, Qdm, derived from MHC class I (43–45). In the absence of TAP, HLA-E has been shown to bind a wider variety of peptides (46), and can also bind viral peptides derived from CMV (VMAPRTLIL), EBV (SQAPLPCVL), HIV (AISPRTLNA), and HCV (YLLPRRGPRL) (47–50). In terms of peptide sequence, the CMV peptide is derived from the signal sequence of UL-40 and the common variant is identical to the HLA-Cw*03 leader sequence (51). The peptides from EBV, HIV, and HCV have less sequence homology to MHC class I leader peptides and were identified by functional approaches. Detailed investigation of the HCV core$_{35–44}$ peptide YLLPRRGPRL demonstrated that although it stabilized HLA-E on the surface of the TAP-deficient 721.174 cell line, these peptide-loaded targets did not inhibit NKG2A-positive NK cells (52). However, it was noted that relatively small amounts of leader

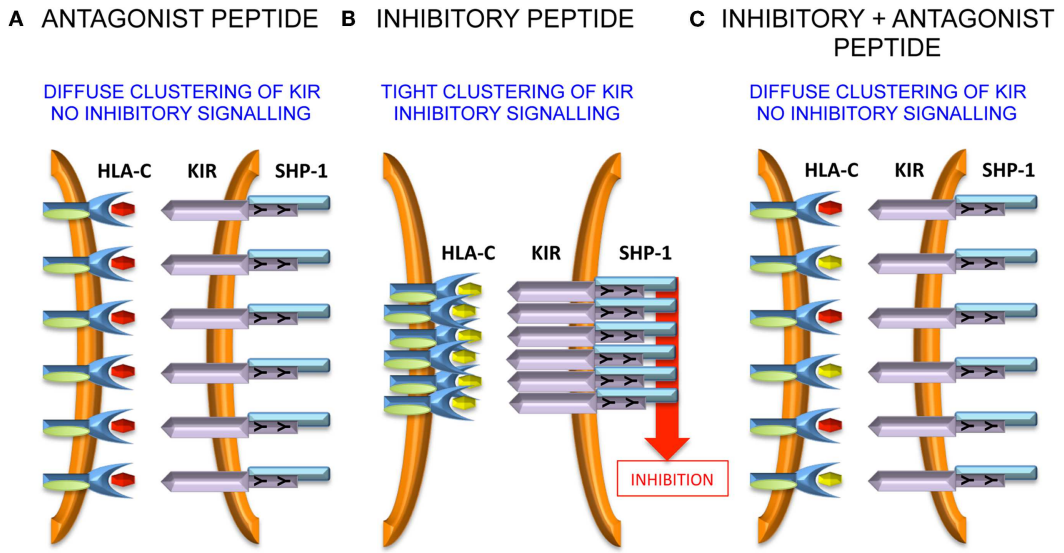

FIGURE 1 | Peptide antagonism is associated with loss of tight clustering of KIR. (A) The antagonist peptide induces clustering of KIR and SHP-1 recruitment to the immune synapse. However, the clustering is diffuse and there is no inhibitory signal generated. **(B)** An inhibitory peptide induces tight clustering of KIR, SHP-1 recruitment, and inhibitory signaling. **(C)** The antagonist peptide abrogates tight clustering and thence inhibitory signaling.

sequences derived from HLA-A, -B, and -G could inhibit a fraction of NKG2A-positive NK cells and that addition of HCV core$_{35–44}$ increased that inhibition. This was also true for the HIV and EBV-derived peptides as well as the Hsp60 leader peptide, which had previously been shown to bind to HLA-E and engage the activating receptor NKG2C. In experiments studying the clustering of CD94:NKG2A at the interface between NK cells and peptide-loaded 721.174 cells, it could be demonstrated that HCV core$_{35–44}$ induced clustering of CD94, but not of NKG2A. Furthermore, this clustering could be abrogated by mutating P5 of the peptide from arginine to lysine, a substitution that would be predicted to prevent binding to CD94. As CD94 can exist on the cell surface as a homodimer, we proposed that the HLA-E:YLLPRRGPRL complex engages the CD94 homodimer, but not the CD94:NKG2A heterodimer (53, 54). Although CD94 does not have a signaling motif in its cytoplasmic tail and is not thought to mediate signaling on its own, stabilization of CD94 homodimers could lead to higher order receptor clustering and augment inhibitory signaling in NKG2A-positive NK cells. Additionally, an HLA-E tetramer loaded with the HLA-Cw*03 peptide VMAPRTLIL binds well to NKL which express CD94:NKG2A heterodimers, but not to Jurkat cells expressing only CD94. This, combined with the functional data, indicates that CD94:NKG2A and CD94 homodimers have different peptide specificities.

Further work needs to be performed to define precisely how the "non-signaling" CD94 molecule influences inhibitory signaling. Unlike antagonism for KIR, it is less likely that intracellular effects are important because CD94 possesses only a short intra-cytoplasmic tail, and is not thought to have a signaling function in isolation or in combination with a signaling adaptor protein. Therefore, extracellular effects may be relevant and one hypothesis could be that CD94 homodimers assist in the formation of macromolecular aggregates of the CD94:NKG2A heterodimer. Such aggregates may stabilize receptor:ligand contacts at the immune synapse and augment inhibitory signaling (**Figure 2**).

Comparison of the response of NKG2A+ and KIR+ NK cells to changes in cell-surface MHC class I demonstrate an additional important difference. The stoichiometry of KIR-mediated inhibition and MHC class I cell-surface expression is linear, whereas that of MHC class I with NKG2A exhibits saturation kinetics (**Figure 3**). This can be expressed as follows:

$$\text{Degranulation}_{(\text{KIR+NK Cells})} = k_1(\text{MHC I})$$

but

$$\text{Degranulation}_{(\text{NKG2A+NK Cells})} = k_2(\text{MHC I})/[\text{x} + (\text{MHC I})]$$

Thus, an additional factor "x" is required to explain the relationship of NKG2A-mediated degranulation to MHC class I cell-surface levels. This additional factor may be a constant or a variable. However, the key feature is that it is more dominant at low rather than high cell-surface MHC class I levels, thus reflecting the greater sensitivity of NKG2A-positive NK cells to changes in MHC class I cell-surface levels as compared to KIR-positive NK cells (**Figure 3**). One potential factor could be that CD94:NKG2A forms macromolecular aggregates, which are facilitated by CD94 homodimers. Conversely, the 2Ig domain KIR may not form aggregates spontaneously. Aggregation of KIR is a contentious issue. KIR binding to MHC is dependent on the presence of zinc ions, with KIR having a zinc-binding motif (55, 56). Furthermore, both zinc and cobalt can mediate aggregate formation *in vitro* (57, 58). In crystallographic studies of KIR2DL2 with HLA-Cw*03, KIR2DL2

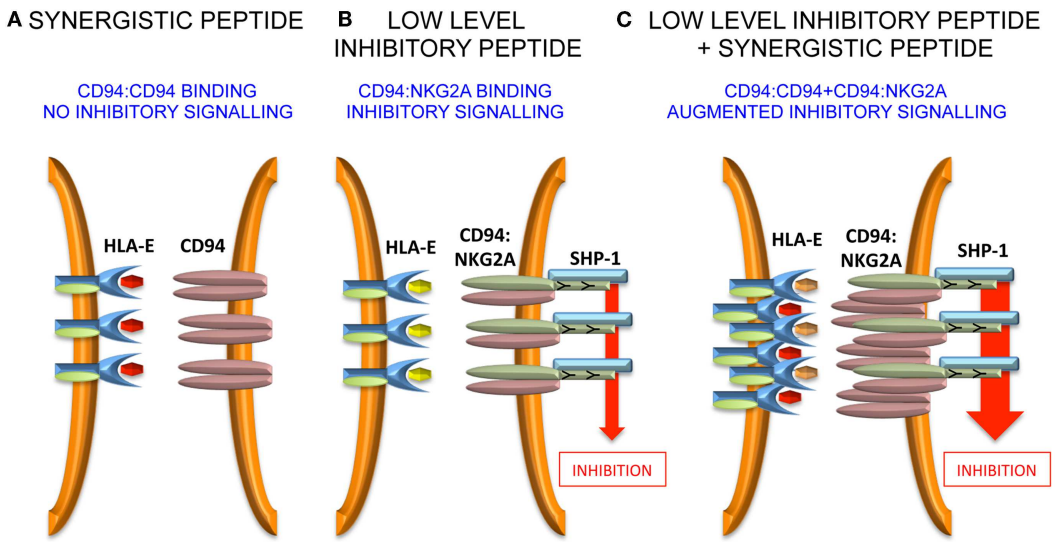

FIGURE 2 | Model for peptide synergy. **(A)** A synergistic peptide induces recruitment of CD94 homodimers, but not CD94:NKG2A to the immune synapse. Therefore, there is no inhibitory signal generated. **(B)** At low levels of inhibitory peptide CD94:NKG2A is recruited to the immune synapse and there is inhibitory signaling. **(C)** The synergistic peptide augments the inhibitory signaling due to low levels of inhibitory peptide by stabilizing the immune synapse.

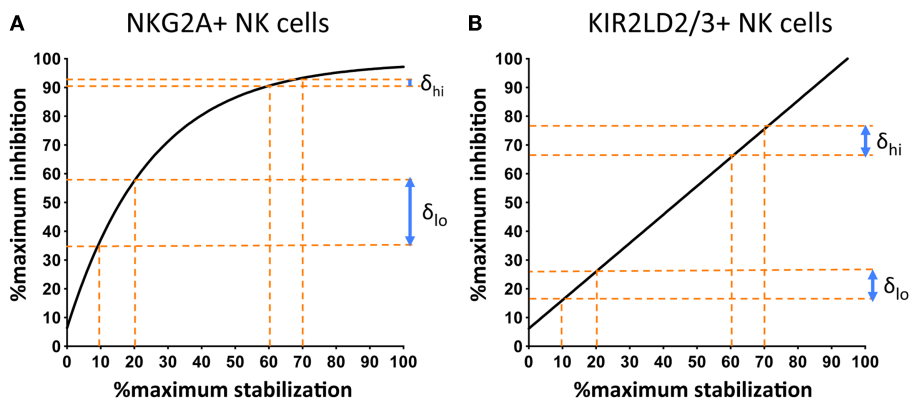

FIGURE 3 | NKG2A+ NK cells are more sensitive to changes in the cell-surface levels of MHC class I at low, as compared to high, surface levels of MHC class I. 721.174 cells were loaded with increasing concentrations of peptide and used as targets in CD107a assays. The levels of degranulation of NKG2A+ **(A)** or KIR2D:2/3+ **(B)** NK cells were plotted against levels of cognate MHC class I:peptide. In **(A)** HLA-E was loaded with increasing concentrations of the HLA-G leader peptide and in **(B)** HLA-Cw*0102 was loaded with increasing concentrations of the VAP-FA peptide. At between 10 and 20% of maximal HLA-E levels, there is an increase of ~20% in the fraction of degranulating NKG2A+ NK cells (δ_{lo}) but between 60 and 70% of maximal HLA-E levels, this change is less than 5% (δ_{hi}). For KIR+ NK cells, δ_{lo} and δ_{hi} are similar at ~10%. Data were derived from Cheent et al. (47).

have also demonstrated the formation of multimers and, based on this feature, a model for KIR aggregation was proposed (59, 60). Subsequently little additional data has been generated on this model and its *in vivo* significance is less clear. Conversely for the 3Ig domain KIR, the D0 domain appears to assist in signaling, even though it does not contact its MHC class I ligand (19, 61). Therefore, it has been proposed that the D0 domain assists in aggregation of KIR. The ability to form, or not to form, multi-molecular aggregates may be relevant to the differences we have observed in how KIR- and NKG2A-positive NK cells respond to changes in peptide. However, clearly these mechanisms require additional structural and functional investigation.

CONSIDERATIONS FOR VIRAL INFECTIONS

As both KIR and CD94:NKG2A are peptide selective receptors, this implies that NK cells may be sensitive to changes in the peptide repertoire presented by MHC class I. Therefore, the content and economics of peptide presentation is a key consideration in

determining if this could be a feasible mechanism for changing NK cell reactivity.

The MHC class I peptidome is a complex mixture of host peptides. The MHC class I repertoire on the cell surface is the result of several processes: cellular protein degradation; access of peptides to nascent MHC class I molecules; and the multi-step process of peptide loading. Viral infection can alter this at many levels, including switching off host protein synthesis, turning on viral peptide synthesis, interfering with MHC class I peptide loading, and changing the recycling of MHC class I, leading to cell-surface down-regulation (62).

Until recently, it was thought that peptides presented by MHC class I were derived from the degradation of mature proteins or "retirees." However, there can be marked changes in the efficiency by which specific peptide epitopes are generated (63–65) and recent data suggest that a substantial fraction of MHC class I peptide derives from defective protein synthesis or "DRiPs" (defective ribosomal products) (66, 67). Up to 70% of proteins synthesized may be degraded before forming functional proteins as the result of defective transcription, failed assembly, mistakes by amino-acyl t-RNA synthetases, or altered ubiquitin modifications (68).

DRiPs may additionally be derived from alternative open-reading frames, and the presentation of these "cryptic" epitopes may make understanding the peptide repertoire more difficult (69). In a system in which the MHC class I peptidome is derived from mature proteins, the turnover and abundance of cellular proteins will determine the nature of peptides presented. However, in the case of DRiPs, this becomes less predictable and the peptide repertoire becomes determined by both mRNA abundance and also error rates in protein synthesis. Errors in protein transcription that ultimately lead to proteins with aberrant sequences are more likely to be more common for viral, as opposed to host proteins, as viral RNA polymerases may lack proof reading capacity. Thus, for the HCV RNA dependent-RNA polymerase, estimated error rates may be as high as one per 1000 per nucleotide site (70). As the HCV genome is only 9.3 kb long, there is a substantial probability of mutation, which on the one hand favors viral escape mutation, but may also lead to the synthesis of DRiPs. Favoring the DRiPs model, viral epitopes for CTL have been shown to be generated from recently synthesized peptides, rather than from mature proteins, confirming the potential of this mechanism for altering the host peptide repertoire (71). Additionally, the efficiency of presentation of an epitope may depend on the source, viral or cellular, of the mRNA and there may also be compartmentalization in the subcellular localization of peptides for class I presentation (65, 72).

Thus, generating a peptide repertoire in the context of a viral infection is a complex procedure that is not readily predictable. Analysis of the MHC class I peptidome reveals that after HIV infection the majority of peptides are self-peptides (73). Conversely, in some infections, there can be substantial numbers of viral peptides presented by MHC class I. For instance, in measles virus infection, the HLA-A*0201 epitope KLWESPQEI epitope has been suggested to be as abundant as 5×10^4 copies per cell (74). Quantitation of viral epitopes is therefore a key factor, as although both KIR-positive and CD94:NKG2A-positive NK cells are sensitive to changes in peptide repertoire, the relative magnitude of these changes will likely be important. Additionally, it has been shown that KIR2DL2 can be a driving force on HIV sequence (75) and the selection of a strong inhibitory peptide may "tip the balance" in terms of evasion of the immune response by the virus. However, accurate quantitation is required to determine whether this is due solely to viral peptides or a combination of host and viral peptides. Indeed, the broad peptide specificity of KIR implies that host peptides would be as effective as viral peptides in altering NK cell reactivity. Interference with host protein synthesis by virus infection may enhance the formation of DRiPs that could then lead on to large changes in peptide repertoire (76, 77). Furthermore, a hold-up in protein degradation can feedback negatively on protein synthesis and translation, additionally modifying the peptides available for presentation by MHC class I (78). Thus, formation of a peptide repertoire, and how a virus interferes with it, is a complex procedure, which at present requires much more detailed understanding before we can learn how this can impact inhibitory receptor signaling by NK cells.

A number of key questions remain to be answered with respect to peptide antagonism. At present, this phenomenon has only been demonstrated for one receptor:ligand system, and whether this extends to other KIR, or even KIR2DL2/3 with other group 1 HLA-C ligands needs to be examined. When the breadth of peptide antagonism is understood, then it will be possible to determine the physiological relevance of it for viral infections, and in particular how commonly antagonism affects the balance between inhibition and activation of an NK cell in physiological and pathological situations. Furthermore, individual peptides will need to be examined in greater detail to understand precisely which peptides are antagonistic and how this correlates with binding. Comparing peptides eluted from group 1 HLA-C molecules as described in the SYFPEITHI database (79), with binding studies using KIR2DL2 fusion constructs (38) suggests that about half of these peptide are unlikely to bind KIR, which speculatively would correlate with the number considered to be antagonist. This estimate has the condition that at present we do not know the limits of binding-affinity to KIR for antagonist peptides; that is at which point a peptide has a high enough affinity to act as a weak binder or conversely an affinity so low that it may be null. This may be determined by the overall binding of the peptide:MHC complex for KIR, rather than just the peptide, so different HLA-C alleles may have different frequencies of peptides that fall into the inhibitory, antagonistic, and null categories. One study in HIV has shown that the majority of peptides is non-KIR binders and hence could fall into the antagonistic or null categories (80). In this work of 217 HIV-derived peptides tested, 11 were identified that bound HLA-Cw*0102, and only one of these bound KIR2DL2.

SUBTLY DIFFERENT FUNCTIONS OF KIR AND NKG2A

For CD94:NKG2A, the broadening in peptide specificity afforded by engagement of CD94 homodimers could be exploited by viruses, to augment inhibition of NKG2A-positive NK cells. This contrasts with the observations for KIR, as to date we have found that peptides engaging KIR2DL2/3 that do not inhibit directly, can perturb inhibitory the signaling generated by high affinity KIR:MHC:peptide complexes. As the majority of peptide variants that we tested are non-KIR binders, this suggests that changes in peptide repertoire that affect KIR are more likely to result in loss of

inhibition. This raises the possibility that KIR and CD94:NKG2A may have subtly different functions. As discussed above, NKG2A-positive and KIR2DL2/3-positive NK cells respond with different stoichiometries to changes in the levels of cell-surface MHC class I. These data imply that NKG2A-positive NK cells are exquisitely sensitive to changes in MHC class I cell-surface levels at low levels of MHC class I, in our peptide titration experiments at <1% of maximal cell-surface levels. We thus propose that NKG2A is a receptor well adapted to changes in the cell-surface quantity of MHC class I. Conversely, KIR are not specialized for this function but may be more sensitive to changes in peptide repertoire. It has been proposed that KIR have a specialization to recognize cells that have down-regulated specific HLA-A, -B, -C molecules. However, as most HLA molecules have leader peptides cognate for HLA-E and CD94:NKG2A, then NKG2A-positive NK cells would serve this function adequately and, at low levels of MHC class I, most likely better than KIR. We propose that the HLA-C specific KIR are specialized to detect changes in peptide repertoire and that this function complements the role of NKG2A in detecting MHC class I down-regulation. If KIR-positive and NKG2A-positive NK cells have these subtly different functions *in vivo* then this would provide a rationale for having two distinct inhibitory receptor systems for MHC class I.

CONCLUSION

In depth study of the peptide selectivity of KIR2DL2/3 and CD94:NKG2A have given novel insights into the functions of these receptors. In addition to induced self- and missing self-recognition, it may be that an "altered self-recognition" is also important for NK cells expressing these receptors. Testing of these models *in vivo* is now required to establish the significance of these observations for disease.

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Activating KIRs in chronic lymphoproliferative disorder of NK cells: protection from viruses and disease induction?

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Human natural killer (NK) cells are functionally regulated by killer cell immunoglobulin-like receptors (KIRs) and their interactions with HLA class I molecules. As KIR expression in a given NK cell is stochastically established, KIR repertoire perturbations reflect a dominance of discrete NK-cell subsets as the consequence of adaptation of the NK-cell compartment to exogenous agents, more often represented by virus infection. Although inhibitory interactions between KIR and their cognate HLA class I ligands abrogate effector responses of NK cells, they are also required for the functional education of NK cell. The biology and molecular specificities of the activating KIRs are less well defined, and most interactions with presumed HLA class I ligands are weak. Interestingly, epidemiologic studies link activating KIR genes to resistance against numerous virus infections. Chronic lymphoproliferative disorder of NK cells (CLPD-NK) is an indolent NK cell disease characterized by a persistent increase of circulating NK cells (usually exceeding 500 NK cells/mm^3). The mechanism through which NK cells are induced to proliferate during CLPD-NK pathogenesis is still a matter of debate. Accumulating data suggest that exogenous agents, in particular viruses, might play a role. The etiology of CLPD-NK, however, is largely unknown. This is likely due to the fact that not a single, specific agent is responsible for the NK cells proliferation, which perhaps represents the expression of an abnormal processing of different foreign antigens, sharing a chronic inflammatory background. Interestingly, proliferating NK cells are typically characterized by expression of a restricted pattern of KIR, which have been demonstrated to be mostly represented by the activating form. This finding indicates that these receptors may be directly involved in the priming of NK cells proliferation.

Keywords: CLPD-NK, activating KIRs, viral infections, NK cell licensing, pathogenesis in NK disorders

INTRODUCTION

The chronic lymphoproliferative disorders of NK cells (CLPD-NK) are included among the novelties of the current World Health Organization (WHO) classification (1). These rare and heterogeneous disorders are characterized by a chronic expansion of mature appearing NK cells (usually more than 500/μl) in peripheral blood for more than 6 months (2–6), without a clearly identified cause (**Figure 1**). Patients are usually adults with a mean age of 60 years without gender and racial predisposition (7). The circulating cells show typical large granular lymphocyte (LGL) morphology, with moderate amount of pale cytoplasm that contains ≥3 azurophilic granules. Bone marrow biopsy is characterized by interstitial infiltration of cells with small nuclei and pale cytoplasm, which are difficult to recognize without the help of immunohistochemical techniques. Pathological NK cells express CD16 and usually low levels of CD56 and CD57. As expected, cells express T-cell intracellular antigen 1 (TIA1), granzyme and perforins, which correlate with the cytotoxic potential of these cells displayed *in vitro* cytotoxic assays. CD94 antigen is expressed at high density on patients' NK cells; this antigen is usually associated with the inhibitory subunit NKG2A, although in a relevant number of cases the association CD94/NKG2C has been reported (8). Patients' NK cells characteristically express functional β and γ chains of IL-2/IL-15 receptor, which are strictly related to the role of these cytokines in the pathogenesis of disease (9). Cytogenetic is normal in most cases (9) and the germ line configuration of TCR is usually demonstrated, as expected for normal NK cells. Since clonality of proliferating cells is difficult to detect in these patients, the analysis of restriction fragment length polymorphism (RFLP) has been used as marker to demonstrate the clonality in some but not all patients. The evidence of a restricted pattern of clonally distributed KIR genes expression by proliferating NK cells might also provide indirect demonstration of clonality (10). In rare case in which EBV can be demonstrated in plasmid form within NK cells, the clonality of cells might be easily examined by Southern Blot analysis using probes recognizing the EBV terminal repeats (11).

Most patients are asymptomatic, and the disease has a chronic indolent clinical course, similar to that reported for patients with T-LGL leukemia (T-LGLL) (2–6); in these cases, the disease is diagnosed by blood analysis performed for other purposes. In some cases, this disorder is associated with other conditions, including pure red cell aplasia, vasculitic syndromes, solid and hematologic

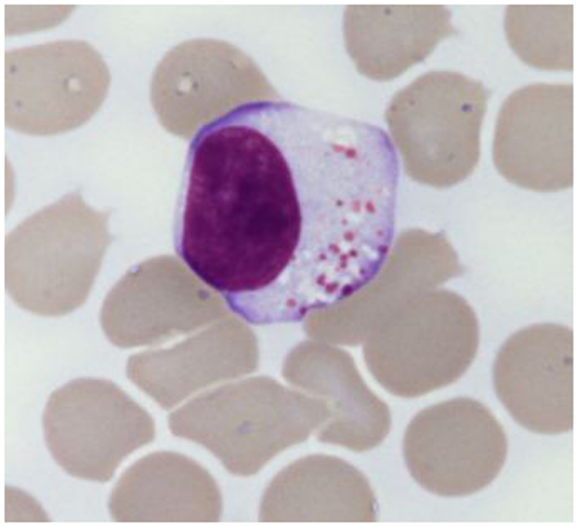

FIGURE 1 | Typical large granular lymphocyte morphology as detected in a CLPD-NK patient.

tumors, splenectomy, neuropathy, and autoimmune disorders (2–6). Recently in patients with chronic myelogenous leukemia, the association has been reported between treatment with dasatinib and the development of CLPD-NK. It has been suggested that the development of CLPD-NK might have a therapeutic effect on Ph positive leukemic cells (12). Systemic symptoms, such as cytopenia (mostly neutropenia and anemia), are uncommon. Lymphadenopathy, hepatomegaly, splenomegaly, and cutaneous lesions are uncommon. Occasionally, patients present with a slowly progressive increase of peripheral blood NK cells and with organ involvement. In rare cases, the disease transforms to aggressive NK cell leukemia (13). These EBV positive patients usually suffer from chronic active EBV infection and should carefully be monitored for the emergence of clonal cells (11). Several cases with a spontaneous complete remission have been reported (14). Patients with CLPD-NK usually have an indolent clinical course and respond to immunosuppressive therapy with low doses of methotrexate (usually 10 mg/m^2/week) or cyclophosphamide (50 or 100 mg/day) or cyclosporin (3–5 mg/kg/day) with or without inclusion of low doses of steroid (15). Because of the potential long-term side effects of immunosuppressive therapy, limiting specific therapy only to patients with symptomatic disease is recommended.

CLPD-NK EXPRESS ACTIVATING KIR

In recent years, several studies have been published focusing on the pathogenetic mechanisms of this disease (9, 16–20). A genetic susceptibility for this disease has been suggested and has been related to the detection in these patients of type B *KIR* gene repertoire, which is characterized by a high number of activating genes (21). As indicated above, a restricted pattern of KIR expression has usually been reported in these patients, which is characterized either by a dominant expression of a relevant KIR, or by a lack of KIR expression (22). A typical feature of these patients is the preferential expression of the KIR activating receptors isoforms (10, 23). Together with a bias toward activating KIR expression, a deep silencing of inhibitory KIR through increased gene methylation has been demonstrated by our group (19). More specifically, we showed the complete lack of KIR3DL1 expression in most analyzed patients, being the receptor expressed in 13% of patients as compared to 90% of controls ($p < 0.01$). Interestingly, the results of methylation patterns of *KIR3DL1* promoter showed a significantly higher methylation status (0.76 ± 0.12 SD) in the patients with respect to the healthy subjects (0.49 ± 0.10 SD, $p < 0.01$). These data suggest that together with the increased expression of activating receptors, the lack of the inhibitory signal could also play a role in the pathogenesis of disease (19). Only few studies addressed the expression of NCR, NKG2D, and other activating receptors. We investigated the expression of these receptors in a series of 18 cases of LDGL patients and showed that, among NCR antigen expression, NKp30 was strongly down regulated in all but one of the cases analyzed. Similarly the NKp46 receptor in most instances was detected only in small fractions of NK cells (10). These peculiar phenotypic results in these patients suggested the occurrence of a defect in NCR expression that was reminiscent of that reported in acute myeloid leukemia patients (24). Regarding NKp44, which is normally expressed only in activated NK cells, this receptor was not expressed at significant levels on LGL surface of the patients analyzed. A completely different pattern was observed for NKG2D, NKp80, and 2B4 molecules that were homogeneously present on NK cells in the majority of the patients. All together, these data indicate that in most cases patients' NK cells express normal levels of NKG2D while NCR molecules are generally present at low density. In addition, although in most patients NK cells were characterized by the CD94/NKG2A+ phenotype, a minor fraction of cases (nearly 20%) expressed CD94+/NKG2C+ phenotype (10).

CLPD-NK AND VIRAL INFECTIONS

Natural killer cell activation in response to an unknown stimulus, likely of viral origin, is postulated to play a role in the initial steps of CLPD-NK by selecting NK clones (**Figure 2**) (25). Although no prototypical HTLV infection was demonstrated in these patients, the evidence that in 73% of cases sera from a series of patients from Europe and USA reacted with the recombinant HTLV env protein p21E suggests that exposure to a protein containing homology to BA21 may be important in the pathogenesis of this lymphoproliferative disorder (16, 26).

In contrast with other mature NK cell neoplasms, EBV DNA is not usually detected within affected lymphocytes in USA and Europe countries (27) (6/16 NK LGL positive for EBV DNA), whereas a significative high incidence has been reported in Japanese patients, usually correlating with a more aggressive clinical behavior (11). Anyway the link between EBV infection and LGL disease is sustained by the observation that spontaneous resolution of KIR restricted LGL associated with disappearance of EBV DNA (28). Among Herpes viruses, Human CMV has been reported to be crucial in influencing NK receptor expression in NK cells (29). Remarkably, elevated numbers of CD94/NKG2C+ NK cells, previously shown to expand in association to CMV infection (30), were preferentially found in Vbeta13.1+ CD4+ T-LGLL, further supporting its role in the pathogenesis of a subset of T-LGLL (31).

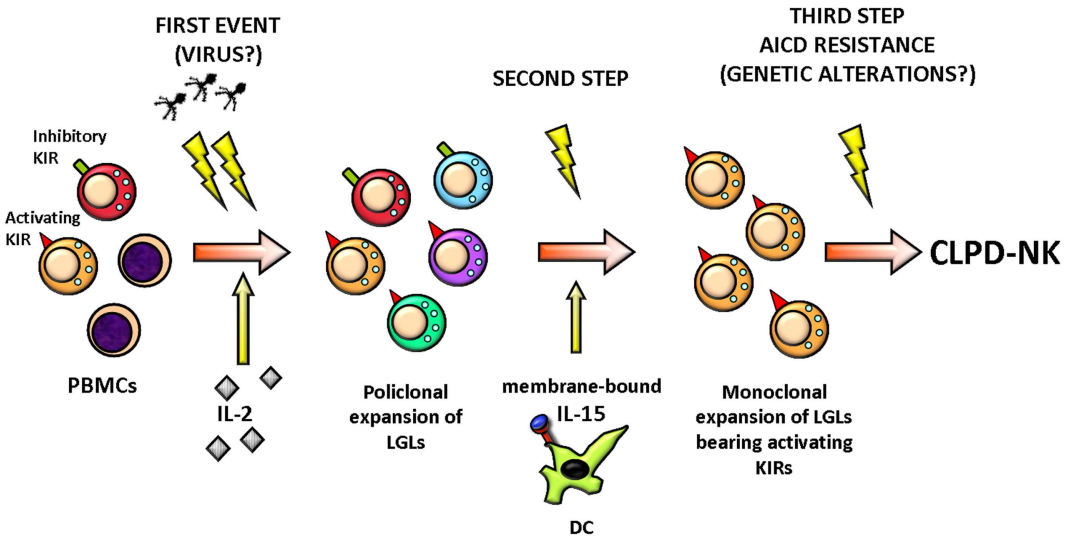

FIGURE 2 | Hypothetical pathogenic events leading to CLPD-NK. A stimulation by exogenous antigen(s), such as virus, represents the initial stimulus inducing the activation and clonal expansion of NK cells, selecting preferentially NK cells equipped with activating KIR, probably because these NK cells are more responsive. LGL expansion at the beginning could be polyclonal but then it becomes monoclonal and chronic, sustained by the action of cytokines, like IL-2 and IL-15. Finally, the clonal LGL expansion does not encounter a resolution for a third event establishing the activation induced cell death (AICD) resistance, probably due to a genetic alteration. All these events result in LGL aberrant outliving and number increase. PBMCs, peripheral blood mononuclear cells; KIR, killer immunoglobulin-like receptor; LGLs, large granular lymphocytes; DC, dendritic cell; AICD, activation induced cell death; CLPD-NK, chronic lymphoproliferative disease of natural killer cells.

It is believed that bone marrow, which is frequently involved in CLPD-NK patients, represents the setting where the putative inciting antigen could reside and dendritic cells (DCs) have been suggested to represent the target of infection in these patients (17). Interestingly, analysis of bone marrow biopsies of patients demonstrated a topographic distribution of DCs and NK cells that indicates a close contact between the two cell types (17). DCs are also likely to represent the source of IL-15, which is crucial in the mechanisms sustaining the maintenance of NK proliferation. IL-15 has been found to mediate its activity by altering Bcl-2 family members, and more specifically by modulating Bid expression (18).

ACTIVATING KIR AND VIRAL PROTECTION: IS IT TRUE IN CLPD-NK?

Epidemiologic studies link activating KIR genes to resistance against numerous virus infections (32). Beziat et al. showed that infection with human CMV induce expansion and differentiation of KIR-expressing NK cells, causing as stable imprints in the repertoire (33). Interestingly, these authors showed that NK education by inhibitory killer cell immunoglobulin-like receptors (KIRs) was associated with a unique contribution of activating KIRs (KIR2DS4, KIR2DS2, or KIR3DS1), in addition to NKG2C, in the expansion of human NK cells. Interestingly, CMV-associated factors have been suggested to specifically influence KIR gene expression by regulating epigenetic expression of KIR genes (34). In addition, in allogeneic bone marrow transplantation setting, donor KIR2DS1 has been reported to protect against human CMV reactivation (35). KIR3DS1 in conjunction with HLA-Bw4 with an isoleucine at position 80 has been reported to be associated with slower progression of HIV infection to AIDS (36). Pelac et al. demonstrated that NK cells from individuals with multiple copies of KIR3DL1, in the presence of KIR3DS1 and the appropriate ligands inhibit HIV-1 replication (37). This is frequently associated with a significant expansion of KIR3DS1+, but not KIR3DL1+, NK cells in HIV positive patients' peripheral blood. Epidemiological studies have indicated a protective effect for the activating *KIR2DS1* and *KIR3DS1 genes* also in patients with EBV-associated Hodgkin's lymphoma, although there is as yet no direct evidence for the involvement of these receptors in the recognition of EBV-transformed cells (38). *KIR3DS1* genotype has been shown to exert a positive effect on HCV viral clearance during the first weeks of treatment in HCV/HIV infected patients (39).

Although many of the above reported viral infections are at least in part controlled by activating KIRs, a direct proof of the role of these NK receptors in controlling a putative viral infection in patients with CLPD is not available. This is likely due to the fact that not a single, specific agent is responsible for the NK proliferation, which perhaps represents the expression of an abnormal processing of different foreign antigens.

ARE CLPD-NK CELLS LICENSED?

It is well known that NK functions are regulated by the integration of signals received from activating and inhibitory receptors. To prevent inadvertent activation against normal tissues, NK must be educated to tolerate self, this process, termed "licensing," acting through an MHC-dependent mechanism requiring interaction between inhibitory KIR and cognate MHC class I ligands (40). Up to date, two models have been proposed to describe NK cell

education (41): "arming" model and "disarming" model. In "arming" model, which is the best-known model proposed by Raulet and Vance (42), NK cells acquire functional competence after ligation of inhibitory receptors by self MHC class I molecules, while NK cells lacking these inhibitory receptors for self MHC enter in an "anergy" state. In this way, engagement of inhibitory receptor by self MHC during NK cell maturation guarantees NK cells to acquire "license to kill" and to become fully functional competent.

Natural killer licensing involves not only inhibitory KIRs but also activating KIRs have probably an underestimated role in this process, which is complementary but with different outcome, since it has been reported that education via activating KIRs decreases NK cells response (43). However, "unlicensed" NK are not so "anergic" but seem to have a crucial role in discrete settings. As an example, in murine model, unlicensed NK cells were the main mediators of NK cell-mediated control of mouse cytomegalovirus infection *in vivo*. In fact, depletion of unlicensed NK cells impaired control of viral titers, but depletion of licensed NK cells did not. Furthermore, the transfer of unlicensed NK cells was more protective than the transfer of licensed NK cells, indicating that unlicensed NK cells are critical for protection against viral infection (44).

As stated above, in CLPD-NK, proliferating NK cells are characterized by skewed KIR expression and selection and expansion of NK subset expressing activating KIRs, instead of the more common NK cells expressing inhibitory KIRs, represent a crucial step in the development of this disorder (10). Based on the recent insights in NK cell education and function, in CLPD-NK, we can suppose that expansion of long-lived NK cells involves "unlicensed" NK equipped with activating KIRs, which have a predominant role during viral infections, in accordance with the hypothesis that viral stimulation may be the starting trigger of the disorder. According with this suggestion, we performed HLA genotyping analysis in 29 CLPD-NK patients showing that in 93% of cases, a KIR/HLA-I mismatch was present indicating that NK cell proliferation in CLPD is mostly represented by unlicensed cells (21). Considering that in healthy individuals, KIR/HLA-I genetic mismatch has been detected in nearly 50% of cases, our results point to a role of the KIR/HLA-I mismatch in the pathogenesis of the disease.

CONCLUSIVE REMARKS

Chronic lymphoproliferative disorders of NK cells are characterized by the expression of a restricted pattern of activating KIR receptors on proliferating NK cells. It is believed from indirect evidence that exogenous agent(s), likely of viral origin, might contribute to the initial steps of disease. Recent knowledge on the mechanisms of NK cell function and different contribution of NK cell receptors against viral infections might help in the comprehension of the mechanisms leading to selection of a discrete NK cell population. A possible inciting role of the putative antigen within DCs in the bone marrow can be suggested. The inability of antigen clearance and/or the occurrence of new events might contribute to the persistence of NK clone. In this way, the identification that STAT3 SH2 somatic mutations (45, 46), which can be found in a fraction of CLPD-NK patients, might indicate that an acquired genetic mutation could contribute to the immortalization of NK proliferation.

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Activating killer cell Ig-like receptors in health and disease

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Expression of non-rearranged HLA class I-binding receptors characterizes human and mouse NK cells. The postulation of the missing-self hypothesis some 30 years ago triggered the subsequent search and discovery of inhibitory MHC-receptors, both in humans and mice. These receptors have two functions: (i) to control the threshold for NK cell activation, a process termed "licensing" or "education," and (ii) to inhibit NK cell activation during interactions with healthy HLA class I-expressing cells. The discovery of activating forms of KIRs (aKIR) challenged the concept of NK cell tolerance in steady state, as well as during immune challenge: what is the biological role of the activating KIR, in particular when NK cells express aKIRs in the absence of inhibitory receptors? Recently it was shown that aKIRs also participate in the education of NK cells. However, instead of lowering the threshold of activation like iKIRs, the expression of aKIRs has the opposite effect, i.e., rendering NK cells hyporesponsive. These findings may have consequences during NK cell response to viral infection, in cancer development, and in the initial stages of pregnancy. Here we review the current knowledge of activating KIRs, including the biological concept of aKIR-dependent NK cell education, and their impact in health and disease.

Keywords: NK cells, KIR, HLA, education, infection diseases, cancer, pregnancy, autoimmune diseases

INTRODUCTION

The history of non-rearranged MHC class I-binding receptors has been a passionate story for three decades. Shortly after the discovery of NK cells in the mid-1970s (1, 2) MHC class I was suspected to be important for regulating NK cell responses (3–5). The subsequent postulation of the "missing-self" hypothesis (1, 2, 6), predicted that NK cells would express inhibitory MHC class I-binding receptors. In 1992, the discovery of inhibitory MHC class I-binding Ly49 receptors in mice transformed the "missing-self" hypothesis into a now well-admitted dogma (3–5, 7). Shortly after, human inhibitory receptors binding to HLA-C (KIR2DL1/P58.1/EB6 and KIR2DL3/P58.2/GL183) (8–10) and HLA-B (NKB1/KIR3DL1) (11), were identified as functional analogs to the murine inhibitory Ly49 receptors. Moretta and Colleagues later discovered that the monoclonal antibody clone EB6 also could recognize a second receptor p50 (KIR2DS1), and that this receptor transmitted activating signals upon recognition of HLA-Cw4 targets (12). The genes coding for these receptors were later cloned and were confirmed to be specific for HLA-B and -C molecules (13). Since the initial discovery of the first inhibitory and activating KIRs in humans (referred to iKIRs and aKIRs respectively in the rest of the manuscript), a number of additional KIRs have been identified. The KIR family now includes seven iKIR and six aKIR, in addition to KIR2DL4, which has both inhibitory and activating function. Adding to the complexity, each KIR gene is highly polymorphic, and the product of different alleles can interact more or less strongly with different HLA class I alleles (**Table 1**).

All KIR genes are encoded in the leukocyte receptor complex (LRC) in the chromosome 19 (19q14.3). Thus, the LRC includes the 15 genes of the KIR family. Importantly, for some KIRs, there is extreme allele variability, whereas some KIR genes are highly conserved and exist only as few alleles. The variability of KIR alleles can be nicely visualized[1]. Interestingly, compared to iKIR, aKIR display rather limited allele variability compared to iKIR. For instance there are more than 75 alleles coding for KIR3DL1 and only 17 coding for KIR3DS1 (32), and *KIR2DS1* gene encompassed only 16 alleles in contrast to *KIR2DL1* gene, which include more than 40 alleles[2].

In addition to allele polymorphism, there is strong haplotype variability due to the number of KIR genes present in the LRC. Two types of KIR haplotypes, A and B, have been defined pending on gene content. Group B haplotypes are defined by the presence of at least one of the following KIR genes: *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS5*, and *KIR3DS1*. Conversely, group A haplotypes are characterized by the absence of all these genes. All information about KIR alleles and haplotypes has been collected in the KIR web-based database (see text footnote 1). In addition, another repository has been created for association between KIR and diseases[3] (33).

Currently, the aKIR include KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, and KIR3DS1 (**Table 1**). The specificity of

[1] http://www.allelefrequencies.net/kir6008a.asp
[2] http://www.ebi.ac.uk/ipd/kir/
[3] http://www.allelefrequencies.net/diseases/default.asp

Table 1 | Activating and inhibitory KIRs and ligands.

Activating KIR	Ligand	Detectable by FACS	Reference
2DS1	HLA-C2 (weaker than 2DL1)	Yes	(14, 15)
2DS2	HLA-C1 (weak), HLA-A*11:01	Yes	(16–18)
2DS3	Unknown	? (Retained intracellularly?)	(19)
2DS4	HLA-C*05:01, A*11:02, C*16:01	Yes	(20)
2DS5	Unknown	Yes	(21–23)
3DS1	Unknown	Yes	(24)
Inhibitory KIR	**Ligand**	**Detectable by FACS**	
2DL1	HLA-C2 (N77/K80)	Yes	(8)
2DL2	HLA-C1 (S77/N80), HLA-C2, HLA-B*46:01 and HLA-B*73:01 (C1 epitope)	Yes	(9, 25)
2DL3	HLA-C1 (S77/N80), HLA-C2, HLA-B*46:01 and HLA-B*73:01 (C1 epitope)	Yes	(9, 25)
2DL4	HLA-G (intracellular interaction?)	Yes (not shown *in vivo*)	(26)
2DL5A/B	Unknown	Yes	(27)
3DL1	HLA-A (with Bw4 motif), HLA-Bw4	Yes	(11, 28)
3DL2	HLA-A3/A11	Yes	(29, 30)
3DL3	Unknown	? (Methylated promoter)	(31)

each receptor as well as their cell surface expression has been elusive for many years, despite the fact that the extracellular domains of these molecules are extremely similar to their inhibitory counterparts, in terms of sequence and 3D structure. Studies implicating aKIRs in diseases are therefore almost exclusively based on genetic studies. More recently, new staining protocols and monoclonal antibodies have allowed identification of cells expressing KIR2DS1, KIR2DS2, KIR3DS1, and KIR2DS5 (15, 16, 22–24, 34–38). The possibility to identify the specific expression of distinct aKIRs has made it possible to determine how these receptors regulate NK cell function at the single level, and in concert with other activating and inhibitory HLA class I-binding receptors.

KIR2DL4 remains a particular KIR because of its structure, expression, and functions (39). Hence, this receptor is expressed by all NK cells, in contrast to other KIRs, which are clonally expressed. *KIR2DL4* has also a lower diversity but its allele diversity influences the surface expression and function in NK cells (40, 41). Hence, the 9A allele of KIR2DL4 is not stable at the plasma membrane and is rapidly recycled. Consequently, intracellular KIR2DL4, engagement with its ligand HLA-G in endocytosis vesicles resulted in production of cytokines such as IFN-γ but also other cytokines such as IL-1β, TNF-α, and IL-8, etc., [nicely reviewed by Rajagopalan and Long (39)].

THE ELUSIVE NATURE OF ACTIVATING KIR LIGANDS

Despite the high degree of sequence homology between activating and inhibitory KIRs, the specificities of most aKIRs remain elusive. In particular, this is true for KIR2DS1, KIR2DS2, and KIR3DS1. KIR2DS1, the first described, and most well-studied activating KIR, binds to HLA-C molecules within the C2 group (with the N77/K80 motif) (12). KIR2DS2 has been shown to recognize group 1 HLA-C and induce a KIR–HLA-dependent NK cell activation (42, 43). However, many other studies have failed to identify KIR2DS2–HLA-C1 interaction [nicely reviewed by Moesta and Parham (44)]. Unexpectedly, a recent publication revealed that KIR2DS2 recognizes HLA-A*11 (18). With respect to KIR3DS1, while there are indications that KIR3DS1 interacts with HLA-Bw4^{80I}, there have been no studies demonstrating a direct interaction between these two molecules (24). In contrast to the above-mentioned aKIRs, KIR2DS3 and KIR2DS5 have no inhibitory counterparts. Although detection at the cell surface of NK cells has been recently proven possible (19, 21), the identification of their respective ligand(s) has not been successful. KIR2DS4 however has been shown to interact with HLA-A*11:02, HLA-C*05:01, and HLA-C*16:01 (20).

It is possible that the aKIRs have HLA class I as their major ligands. However, it also remains possible that these receptors could recognize altered HLA class I complexes, e.g. specific HLA/peptide complexes, or complexes of HLA class I together with viral proteins, or non-HLA class I ligands altogether (45). For example, KIR2DS4 has been suggested to bind to an unidentified protein expressed on melanoma-derived tumor cells, independently of HLA class I (46). In mice, the activating Ly49H receptor directly recognizes the CMV-encoded protein m157 (47, 48), and thereby allows NK cells to control CMV infection. Similarly, mouse CMV m04 together with the MHC class I molecule H-2D^{k} allows recognition of CMV-infected cells by NK cells expressing the activating receptor Ly49P (49). It thus remains possible that the aKIRs could directly, or indirectly, recognize virally encoded ligands, although such ligands have yet to be identified in humans.

Several iKIRs display a certain degree of peptide selectivity, where the nature of the HLA class I presented peptide affects the binding to KIRs (43, 45, 50, 51). Similarly, KIR2DS1 displays a certain degree of peptide selectivity in its binding to HLA-Cw4 (43), indicating that also aKIRs can be modulated by the nature of the presented peptide. Noteworthy the recent crystal structure of KIR2DS2–HLA-A*11:01 was obtained in complex with a vaccinia viral peptide (18). Moreover, the authors showed that peptide sequence affects the binding of KIR–HLA. Additionally, HLA

allele themselves can display a different affinity for their attributed KIR (42).

In addition to alterations in peptide content during, e.g., viral infections, the overall cell surface pattern of MHC class I can be altered. Interestingly, it was recently shown in mouse, that peptide-specific clusters of HLA class I/peptide complexes are formed on the cell surface of infected cells, depending on the nature of the peptide presented (52). These peptide-specific clusters create a high level of expression of a given MHC class I/peptide complex on the cell surface of infected cells, which allow a more efficient recognition by cognate T cell receptors (52). It is tempting to speculate that in humans, the clustering of specific HLA class I/peptide complexes during infections could be one way for aKIRs to recognize virus-infected cells. Noteworthy, most studies on aKIRs have evidenced a weaker affinity for their ligands compared to their inhibitory counterparts (43, 44).

EDUCATION OF NK CELLS BY ACTIVATING KIR

Activating KIRs convey their signals through the adaptor KARAP/DAP12 (53, 54). Upon ligation with antibodies or ligands, Src kinase-dependent phosphorylation of DAP12 allows the recruitment of SYK and the triggering of LAT-dependent signaling pathways (PI3K–AKT, NKFAT, and MAPK) (55).

As mentioned above, the ligands for most aKIRs remain elusive and KIR2DS1 is the activating receptor with the best described ligand (HLA-C2) (43, 56, 57). Several studies have shown that KIR2DS1$^+$ NK cells efficiently kill HLA-Cw4$^+$ target cells (12, 14, 15, 43, 45, 58). However, it has been hard to reconcile the fact that NK cells can express activating receptors whose ligand is expressed on healthy cells, as this interaction potentially could lead to autoreactivity. To cope with potential autoreactivity, it is likely that NK cells, much like T cells, have evolved a system for tolerance also for aKIRs. One possibility is negative selection of NK cells expressing self-HLA class I-binding aKIRs in the absence of self-specific inhibitory KIRs. However, recent data refuted this hypothesis, as NK cells expressing KIR2DS1 in the absence of self-specific inhibitory KIRs or CD94/NKG2A can be detected in HLA-C2$^+$ donors (15, 36, 37, 59).

An alternative hypothesis is that NK cells expressing aKIRs are not deleted, but rather rendered hyporesponsive if the ligand is present in the host. Indeed, in HLA-C2 homozygous donors NK cells expressing KIR2DS1 in the absence of self-HLA class I-specific KIRs (KIR2DS1sp) were hyporesponsive to target cell stimulation, whereas no such hyporesponsiveness was observed in HLA-C1 homozygous donors (15), indicating that the responsiveness of KIR2DS1$^+$ NK cells is tuned down in HLA-C2 homozygous donors (15, 59). Similarly, IL-2 activated KIR2DS1sp NK cell lines and clones from HLA-C2 homozygous donors have been reported to have lower responses to HLA-C2$^+$ target cells (14, 59).

This newly identified NK cell education complements education via inhibitory HLA class I-binding receptors (iKIR and NKG2A/CD94), since it renders otherwise potentially autoreactive NK cells self-tolerant. The system of iKIR- and aKIR-mediated education can in some ways be compared to the positive and negative selection of T cells; where T cells are positively selected based on having TCRs that recognize self-MHC class I, and negatively selected if they express a TCR that recognize MHC class I in complex with self-peptides. In comparison with the T cells, education by iKIR would be the analog of positive selection, and education via aKIRs would be the analog of negative selection, with the major difference that T cell selection results in deletion or survival, whereas for NK cells it sets the threshold for activation, without affecting survival (60, 61).

Interestingly, although the mechanisms have not yet been identified, education by aKIRs shares features with the hyporesponsiveness induced by chronic stimulation of other activating receptors expressed by NK cells. For example, chronic exposure to NKG2D ligands in mice renders NK cells hyporesponsive to target cells (62). Similarly, when the ligand (m157) for the activating Ly49H is constitutively expressed, mouse Ly49H$^+$ NK cells become hyporesponsive (63). Finally, NK cells in NKp46-deficient mice are more responsive, suggesting that NKp46 hampers the reactivity of NK cells via an unidentified constitutively expressed ligand (64). Thus, the mechanism ensuring tolerance by self-HLA class I-binding aKIRs might be similar to that of these activating receptors. Other examples of activating receptor-dependent NK cell tolerance exist but the molecular mechanisms are likely to be different. For instance, mouse 2B4 may be associated with different adaptors that transmit inhibitory or activating signals (65). Interestingly, the tuning of NK cells expressing self-HLA class I-binding aKIRs seems to be restricted to target cell recognition, as it does not affect the response to cytokine stimulation (15). Furthermore, based on murine studies, education of NK cells appears to be a dynamic and reversible process, both via activating and inhibitory receptors (66, 67). This suggests that NK cells expressing self-HLA class I-binding receptors may be silenced under normal conditions, but once an appropriate signal is given to the cells, they may become an important component of an efficient immune response. For example, sudden changes, in contrast to chronic long-term changes, might provide such a signal, a concept that was recently proposed in the "discontinuity theory for immunity" (68). Compiling all relevant literature, the authors noted that innate immune cells, but also to some extent adaptive immune cells, are tolerant to "self," irrespective of how aberrant the "self" is, until an "unexpected" event disrupts the equilibrium that is maintaining tolerance, thus awakening the immune effectors.

Overall, the iKIR- and aKIR-dependent education of NK cells by HLA class I molecules, together with the polymorphic nature of KIRs and HLA class I, and the variegated expression of KIRs on NK cells, results in a highly complex system of NK cell regulation. The effects of this complex system are only just starting to be investigated in different diseases, and most available data thus comes from genetic studies.

ACTIVATING KIRs IN VIRAL INFECTIONS

Over the past 10 years, a number of studies have aimed at elucidating a potential role of aKIRs in virus infections, including HIV, HCV, and CMV. There is now compelling evidence, although mostly at the genetic level, that aKIRs can affect the outcome of different infections. The large degree of linkage disequilibrium between different *KIR* genes, e.g., *KIR2DL2* and *KIR2DS2*, and *KIR2DS1* and *KIR3DS1*, however makes it hard to pinpoint a specific KIR/HLA class I interaction at the genetic level, as responsible for protection or susceptibility to a given infection. In addition,

the lack of well-characterized ligands for several of the aKIRs, e.g., KIR3DS1, KIR2DS2, and KIR2DS3, as well as the lack of specific monoclonal antibodies to these aKIRs have severely hampered investigations of the functional role of these receptors in viral infections. The identification of ligands and the development of new antibodies specific for aKIRs are thus required to move the field forward toward understanding how these receptors regulate NK cell function in different infectious disease settings.

KIR3DS1 is the by far most well-studied aKIR in the setting of infectious diseases [also nicely reviewed by Körner and Altfeld (69)]. The first evidence for a role of KIR3DS1 in viral infections came from genetic studies in HIV-infected individuals where individuals homozygous for *KIR3DS1* progressed slower to AIDS, but only if they also carried *HLA-Bw4* with an isoleucine at position 80 (*HLA-Bw4*^{80I}) (70). Interestingly, donors homozygous for *KIR3DS1*, but lacking *HLA-Bw4*^{80I} progressed faster to AIDS (70). Furthermore, *HLA-Bw4*$^{80I+}$ individuals with increasing number of copies of *KIR3DS1* due to copy number variation have been shown to have lower viral load set points (71). The frequency of *KIR3DS1* positive individuals has also been shown to be higher in HIV-exposed seronegative individuals, compared to HIV seropositive and HIV negative individuals, indicating that KIR3DS1 might also be providing protection against infection (72). In addition, the frequency of KIR3DS1^{+}KIR3DL1^{-} NK cells is increased in *HLA-Bw4*$^{80I+}$ individuals, both at acute and chronic stages of HIV infection compared to healthy controls (73). The protective effect of KIR3DS1 in HIV infection was further strengthened by evidence that individuals carrying both *KIR3DS1* and *HLA-Bw4*^{80I} suppress HIV replication *in vitro* to a greater extent than NK cells from individuals carrying *KIR3DS1* in the absence of *HLA-Bw4*^{80I}, or vice versa, carrying *HLA-Bw4*^{80I} in the absence of *KIR3DS1* (56). In addition, purified KIR3DS1^{+}KIR3DL1^{-} NK cells from *HLA-Bw4*$^{80I+}$ individuals suppressed HIV replication *in vitro* to a much greater extent than KIR3DS1^{-}KIR3DL1^{+} and KIR3DS1^{-}KIR3DL1^{-} NK cells (56), indicating that the effect of KIR3DS1 is likely to be mediated by KIR3DS1^{+} NK cells, and not by KIR3DS1^{+} T cells. The same group subsequently demonstrated that the effect of KIR3DS1 on viral inhibition *in vitro* was highest in individuals carrying *KIR3DS1*, *HLA-Bw4*^{80I}, and at least one copy of *KIR3DL1* (71). Surprisingly, NK cells from *HLA-Bw4*$^{80I+}$ individuals homozygous for *KIR3DS1*, but lacking *KIR3DL1*, did not suppress HIV replication *in vitro* to a greater extent than NK cells from *HLA-Bw6* homozygous individuals, indicating that the effect of KIR3DS1 is dependent on both KIR3DL1 and HLA-Bw4^{80I}. Interestingly, the expression of KIR3DS1, both at the mRNA and protein level, was elevated in individuals carrying *KIR3DS1* in the presence of two copies of *KIR3DL1*, compared to individuals carrying *KIR3DS1* in the presence of one copy of *KIR3DL1*. However, that study did not compare the expression levels of KIR3DS1 to individuals carrying two copies of *KIR3DS1* in the absence of *KIR3DL1*, making it hard to determine whether increased expression levels of KIR3DS1 alone could explain the increase in viral inhibition, as suggested by the authors. An alternative explanation for the increased suppression of HIV replication by *KIR3DS1/KIR3DL1* heterozygous individuals, compared to *KIR3DS1* homozygous individuals could be an increased efficiency of NK cells co-expressing KIR3DL1 and KIR3DS1, potentially allowing NK cells to sense a down regulation of HLA-Bw4 via KIR3DL1, and at the same time activate them via KIR3DS1-mediated recognition of HLA-Bw4 with, e.g., an altered peptide repertoire. However, sorted KIR3DL1^{+} NK cells from *KIR3DS1/KIR3DL1* heterozygous individuals, that could contain NK cells co-expressing KIR3DL1 and KIR3DS1, did not efficiently suppress HIV replication *in vitro*, compared to NK cells expressing only KIR3DS1 (56). This indicates that co-expression of KIR3DL1 and KIR3DS1 is not required for KIR3DS1-mediated NK cell suppression of HIV replication *in vitro*. It is also noteworthy that very little is known about co-expression of other activating and inhibitory KIRs and other HLA class I-binding receptors (e.g., NKG2A, NKG2C, and LIR-1) on KIR3DS1^{+} NK cells, both in healthy and in HIV-infected individuals.

The complexity of the effects of KIR3DS1 in HIV infection is further highlighted by reports that KIR3DS1 is associated with an increased progression to AIDS, but when restricted to *HLA-Bw4*$^{+}$ individuals, *KIR3DS1* presence is associated with a slower progression (74). Other reports have suggested an HLA-Bw4-independent effect of KIR3DS1 in HIV-infected individuals, at least when measuring CD8 T cell activation, which is strongly associated with HIV disease progression (75). Taken together, the data collected to date indicate a role for KIR3DS1 in HIV disease and progression to AIDS, but it is also clear that more efforts are needed to explain the complex nature of KIR3DS1-mediated protection in HIV-infected individuals, including the identification of a ligand for this receptor.

In contrast to the role of KIR3DS1 in HIV infection, much less is known about the role of other aKIRs in viral infections. Similar to studies of aKIR and HIV, most of the studies have been performed at the genetic level, and commonly in smaller cohorts of patients. Nevertheless, a number of studies have implicated a role for aKIRs in other viral infections. For example, the frequency of *KIR2DS3* positive individuals is higher in patients with chronic HCV infection, compared to individuals that have resolved HCV infection (76). Interestingly, the detrimental effect of *KIR2DS3* was only observed in *HLA-C2*$^{+}$ individuals, which is not believed to be a ligand for KIR2DS3 (76). An increase in *KIR2DS3*, as well as *KIR2DS2* positive individuals has also been reported in a Brazilian cohort of chronically HCV-infected patients (77). Whether the association between *KIR2DS3* and chronic HCV infection reflects a direct interaction between HCV-infected cells and KIR2DS3^{+} NK cell remains unknown since there is neither a known ligand, nor specific antibodies for KIR2DS3. A likely explanation proposed by Dring et al. is that KIR2DS3 is instead a marker of a particular haplotype that is associated with the disease progression (76).

In addition to a role for KIR3DS1 in HIV infection, *KIR2DS2* has been associated with faster progression to AIDS (74). A similar association was found in a West African cohort of HIV-infected women, where haplotype B KIRs, including *KIR2DS2*, *KIR2DS3*, and *KIR3DS1*, was associated with lower CD4 T cell counts (78). Interestingly, homozygosity for *HLA-C1*, a putative ligand for KIR2DS2 and KIR2DS3, decreased the association between lower CD4 T cell counts and the aKIRs. However, there is no evidence for a direct functional role of KIR2DS2 or KIR2DS3 in recognition of HIV-infected cells. Because HLA-C is not downregulated by HIV-infected cells (79), it is tempting to speculate that NK

cells expressing HLA-C-binding aKIRs in the absence of HLA-C-binding iKIRs, could mediate recognition of HIV-infected cells. For example, KIR2DS1$^+$KIR2DL1$^-$ NK cells, which are activated by interaction with HLA-C2$^+$ target cells, could potentially recognize HIV-infected cells in HLA-C2$^+$ individuals. The lack of effects of *KIR2DS1/HLA-C2* in HIV infection could potentially be explained by the education of NK cells via aKIRs, where KIR2DS1$^+$ NK cells in HLA-C2$^+$ individuals are hyporesponsive.

In addition to the associations between activating KIRs and viral infections described above, *KIR2DS1* and *KIR2DS3* have also been associated with fatal outcome in Ebola infection. The frequencies carriers of both these genes were increased in patient cohorts with fatal outcome of Ebola infections, compared to survivors, contacts, and healthy controls (80). However, as the study investigated a rather small cohort, no further dissection of interactions between these aKIRs and HLA-C was possible. Expansions of KIR2DS2$^+$, KIR2DS4$^+$, and KIR3DS1$^+$ NK cells have also been reported to occur in a subset of CMV seropositive individuals (17). However, direct evidence for a role of these aKIRs in the recognition of CMV-infected cells is still lacking.

In summary, although there are a fairly large number of studies associating *KIR* gene content with outcome of viral infections, more direct functional evidence for a role of aKIRs in recognition of virus-infected cells is lacking. The identification of novel, possibly virus-encoded or virus-induced, ligands for activating KIRs, as well as specific monoclonal antibodies, are required to understand the role of these receptors in viral infections.

ACTIVATING KIR IN CANCER

A number of studies have investigated the associations between inhibitory and activating KIR and cancer prognosis. However, similar to the role of aKIRs in infections, the evidence for a role for aKIRs in cancer has largely been at the genetic level due to the limitations in available specific monoclonal antibodies. The education of NK cells via aKIR/HLA class I may play a significant role in controlling NK cell responses to transformed cells, as NK cells expressing aKIRs are hyporesponsive in the presence of self-HLA class I ligands. NK cells expressing aKIR in individuals where the ligand is present will be hyporesponsive, and thus may not be able to mount an efficient response against tumor cells in the same individual. Conversely, NK cells expressing aKIRs in individuals where the ligand is not expressed will not be able to recognize the tumor cells due to the lack of the ligand. However, it remains possible that there are hitherto unknown ligands for aKIRs expressed on tumor cells (46), potentially providing a role for aKIRs in various cancers. A recent study evidenced that CCR7 expressed by dendritic cells or lymphoblasts could be transferred to NK cells in a KIR2DS1/HLA-C2-dependent manner. Consequently, CCR7$^+$ NK cells would migrate to secondary lymphoid organs and further improve anti-leukemic effect of NK cells, notably after hematopoietic stem cell transplantation (haplo-HSCT) (81).

The role of activating KIRs in cancer seems to vary with type of cancer, therapy and clinical measurement, and evidence for both protective and detrimental effects exist. For example, *KIR2DS1* gene frequency was found to be higher in breast cancer patients compared to healthy controls, and in contrast to *KIR2DL1* was suggested to promote cancer progression (82). Similarly, individuals with chronic myeloid leukemia carrying the *KIR2DS1* gene had a lower response to antibody-treatment with Imatinib (Glivec) (83). In addition, multiple myeloma patients carrying *KIR3DS1* had shorter progression-free survival (PFS), compared to those that lacked *KIR3DS1*, after autologous stem cell transplantation (84). However, among the *KIR3DS1* positive individuals in that study, patients carrying *HLA-Bw4* had longer PFS compared to those lacking *HLA-Bw4*. In contrast, Karabon et al. recently reported that *KIR3DS1* positive individuals with B cell lymphocytic leukemia (B-CLL) had a trend toward longer progression-free survival compared to KIR3DS1 negative individuals. Interestingly however, in the same study co-carriage of *KIR3DS1* among *HLA-Bw4*$^+$ patients, as well as co-carriage of *KIR2DS1* in *HLA-C2*$^+$ patients were associated with longer PFS (85).

Allogeneic stem cell transplantation to treat leukemia is one setting where NK cells expressing aKIRs may play a significant role, in particular when using KIR ligand mismatched donor/recipient pairs. In this setting, aKIR$^+$ NK cells from donors lacking expression of ligands for the aKIRs could potentially recognize the recipients' leukemia blasts, e.g., when transplanting a *HLA-C2* positive recipient with cells from a *KIR2DS1* positive but *HLA-C2* negative donor. Indeed, it was recently shown in acute myeloid leukemia that patients receiving allografts from *KIR2DS1* positive donors had a lower probability of relapse, but only if the donor was not homozygous for *HLA-C2* (86). Interestingly, there was no difference in relapse between *HLA-C2* homozygous patients receiving a *KIR2DS1* positive allograft, compared to those receiving a *KIR2DS1* negative allograft. In contrast, there was a lower probability of relapse in *HLA-C1/C1* and *HLA-C1/C2* patients receiving a *KIR2DS1* positive allograft, compared to those receiving a *KIR2DS1* negative allograft (86). Based on their data, the authors concluded that it would be beneficial to select *KIR2DS1* positive *HLA-C1* positive donors for transplantation into *HLA-C1* positive donors, and suggested that tolerance-induction via KIR2DS1/HLA-C2 could potentially explain the lack of effects when the allograft was derived from KIR2DS1 positive HLA-C2/C2 donors. The data are also compatible with KIR2DS1$^+$ donor NK cell education via interactions with HLA-C2 in the recipient, since there was no effect of donor *KIR2DS1* in *HLA-C2/C2* recipients. However, it is hard to reconcile how KIR2DS1$^+$ NK cells could mediate an anti-leukemic effect when transplanted into *HLA-C1* positive donors, as there would be no ligand for KIR2DS1 expressed by the recipient's leukemia blasts. One possible explanation, which is still compatible with aKIR-mediated education, is that an unknown non-HLA class I ligand is expressed by leukemic blasts, and as such provides a mechanism by which KIR2DS1$^+$ NK cells could recognize the leukemia blasts, but only if the donor-derived KIR2DS1$^+$ NK cells are not rendered tolerant by the donors or recipient's HLA-C2. However, no such ligands been identified to date. In addition to the study by Venstrom et al. (86), several other studies have indirectly indicated an effect of activating KIR in hematopoietic cell transplantation. In a study with over 1000 AML patients, receiving grafts from KIR haplotype B donors, in particular KIR cen-B homozygous donors, was associated with a higher relapse-free survival, compared to those receiving grafts from KIR haplotype A/A donors, which lack most aKIRs (87, 88). Interestingly, no effect of donor KIR haplotype B

was observed on outcome of ALL in the same study. In contrast to these studies, McQueen et al. reported that KIR haplotype A/A patients with AML/MDS receiving KIR haplotype B grafts (containing aKIR) had a lower survival together with a higher relapse and GvHD rates, compared to KIR haplotype A/A donors receiving a KIR haplotype A/A graft (89). It should however be noted that the number of patients with KIR haplotype A/A receiving a KIR haplotype B graft in that study was low (8–11 patients), making it hard to draw firm conclusions.

More direct evidence for a role of aKIRs in tumor recognition was provided by Pende et al. in patients receiving haploidentical haplo-HSCT from KIR ligand-mismatched donors. Donor-derived KIR2DS1$^+$ NK cells were shown to efficiently kill HLA-C2$^+$ leukemia blasts, indicating that aKIRs could mediate important anti-leukemic effects in the setting of bone marrow transplantation (90).

Overall, although a number of studies have analyzed the co-carriage of *KIR* genes and their ligands, general conclusions regarding the role of aKIR in cancer and cancer therapy cannot be drawn, as it is likely that the role of aKIRs will vary depending on the type of cancer and treatment. Given the linkage disequilibrium of activating KIRs, large cohorts of well-defined patients are needed to pinpoint the role of specific aKIRs, as well as the presence of their ligands. In addition, functional data investigating the role of aKIRs, and education via these receptors, is largely lacking. More studies monitoring aKIR-dependent NK cell functions are thus warranted in order to decipher the impact of aKIR-mediated education on cancer incidence and progression.

ACTIVATING KIR IN HUMAN REPRODUCTION

More and more studies reveal the presence of NK cells in tissue and in particular uterine NK (uNK) cells have been found highly interesting with regard to their regulation by aKIRs. uNK cells represent the dominant leukocyte subset in the decidua (uterine endometrium in pregnancy) during the initial period of pregnancy (91). The uNK cells have been linked to the particular physiological changes that the implantation of the embryo to the uterine wall entails [elegantly reviewed by Mofett and Loke (92)]. Briefly, during the initial stages of pregnancy, fetal extravillous trophoblasts (EVT) implant the embryo by invading into the maternal uterine wall and remodel uterine spiral arteries to ensure the supply of oxygen and nutrients to the growing placenta and fetus. EVTs are peculiar in that they express no HLA-A and -B, but do express HLA-C, -E, and -G. Interestingly, KIR2DL1/S1 and KIR2DL2/3/S2 expression is more frequent on uNK cells than on matched peripheral blood NK cells (93), suggesting a tissue-specific role for KIRs on the uNK cells (which also express KIR2DL4, NKG2A, and LILRB1). The invading EVT reside in the same anatomical location as uNK cells and it has been hypothesized that KIR/KIR ligand combinations thus affect implantation and pregnancy success. In support of this, mothers lacking activating *KIR genes* (haplotype A/A), who consequently express only inhibitory KIR2DL1, in combination with a HLA-C2$^+$ fetus, were at higher risk for preeclampsia and IUGR (94). In contrast, presence of the activating *KIR2DS1* gene was protective, which indicated that activation of uNK cells via aKIR might be important in the interaction with EVT. Indeed, the same group recently showed that KIR2DS1 is functional on uNK cells (95). KIR2DS1 had an educating effect on uNK cells in HLA-C2 donors with regard to target cell induced degranulation, similar to the effect described for peripheral blood NK cells (15). However, signaling via KIR2DS1 on uNK cells also led to production of GM-CSF that could be used to attract trophoblasts *in vitro*. Together this provides mechanistic insight into the *in vivo* importance for activating KIR on uNK cells, in their potential interaction with EVT. Regarding other aKIRs on uNK cells, KIR2DS4 is also expressed on uNK cells, and using clonally expanded uNK cells, KIR2DS4 has been shown also to be functional (96). Future experiments will reveal whether KIR2DS4 plays a similar role as KIR2DS1 in the regulation of uNK cell function. HLA-G is expressed by trophoblasts and KIR2DL4 is also expressed by uNK cells. Expression of soluble HLA-G during pregnancy has been documented (92) and correlates with successful pregnancy (97). Consequently, intracellular KIR2DL4, engagement with its ligand HLA-G in endocytosis vesicles would result in production of various cytokines, which may have vascular remodeling consequences similar to KIR2DS1 activation (98).

ACTIVATING KIR IN AUTOIMMUNE OF INFLAMMATORY DISEASE

The relationships between KIR expression and autoimmune or inflammatory disorders remain largely unknown. Similarly to other diseases, the role of interaction between aKIRs and HLA class I has only been extrapolated from genetic studies looking both at *KIR* and *HLA* gene expression. *KIR2DS1* gene was suggested to be less common in patients with atopic dermatitis compared to healthy controls (99), suggesting that that KIR2DS1 might have a protective effect. In contrast, *KIR2DS1* was found more often in patients with systemic lupus erythematous (100), and the same was true for *KIR3DS1* in multiple sclerosis (MS) (101). Psoriasis vulgaris is also characterized by a clear association between *KIR2DS1* and HLA-Cw6, whose co-carriage was over-represented in patients (102, 103). In this setting, Łuszczek et al. hypothesized that interactions between KIR2DS1 and its ligand would contribute to pathogenesis. Given that KIR2DS1$^+$ NK cells are normally hyporesponsive in HLA-C2 positive donors, these studies indicate that there is a potential break of tolerance leading to psoriatic lesions. In contrast, a former study on psoriatic arthritis showed that *KIR2DS1* and *KIR2DS2* were associated with susceptibility to disease, but only in the absence of their cognate HLA ligands (104). Although responsiveness of KIR2DS1$^+$ NK cells must be assessed, it is possible that an aKIRs education-dependent higher responsiveness would render KIR2DS1$^+$ NK cells able to mediate autoimmune reactions, in the absence of HLA-C2, thus recognizing an unknown ligand.

CONCLUDING REMARKS

Although significant progress has been made in the understanding of aKIRs and their interactions with HLA class I, the role of aKIRs in disease and health still remains largely unexplored. The studies of aKIR/HLA class I associations indicate that aKIRs are likely to play a role in a number of different diseases, including infectious diseases, cancer, and autoimmunity. However, the lack of specific monoclonal antibodies to analyze expression patterns of aKIRs, together with their polymorphic nature and linkage

disequilibrium with iKIRs have hampered detailed studies of aKIRs both in disease and in health. The development of specific antibodies, as well as the identification of ligands for aKIRs, potentially including non-HLA class I molecules, would greatly advance our understanding of aKIR biology. Finally, it is important to keep in mind that KIR expression is not restricted to NK cells but expression has also been documented on αβ and γδ T cells (105, 106). Particularly for αβ T cells and infection or autoimmune diseases where polymorphism of HLA is important, the expression of aKIR might shed light on these genetic associations.

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The DNA damage response: a common pathway in the regulation of NKG2D and DNAM-1 ligand expression in normal, infected, and cancer cells

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NKG2D and DNAM-1 are two activating receptors, present on the surface of NK cells and other cells of the immune system. Their ligands – MICA, MICB, ULBP1-6 for NKG2D, PVR/CD155 and Nectin-2/CD112 for DNAM-1 – can be constitutively expressed at low levels in some normal cells, but they are more often defined as "stress-induced," since different stimuli can positively regulate their expression. In this review, we describe the molecular mechanisms involved in the up-regulation of NKG2D and DNAM-1 ligands under different physiological and pathological "stress" conditions, including mitosis, viral infections, and cancer. We will focus on the DNA damage response, as recent advances in the field have uncovered its important role as a common signaling pathway in the regulation of both NKG2D and DNAM-1 ligand expression in response to very diverse conditions and stimuli.

Keywords: NK cells, stress, DNA damage response, NKG2D ligands, DNAM-1 ligands

INTRODUCTION

The immune system is tasked with protecting the organism from pathogen attack, but also with patrolling cells and tissues that have been dysregulated by non-microbial challenges, such as ultraviolet radiation, heat shock, oxidative stress, or tumor transformation. From a certain point of view, all these responses are not completely unrelated. In fact, one prominent consequence common to different types of stressors is the up-regulation of the MHC class I-like proteins MICA, MICB, ULBP1-6, which are present at low to undetectable levels in normal cells, but can be induced both by infectious agents and by sterile stresses, including cell division and/or tumor transformation (1–4). These molecules are the ligands of the activating receptor NKG2D, a member of the C-type lectin-like superfamily of innate receptors, able – alone or in combination with other receptors – to activate the effector functions of NK cells, $CD8^+$ T cells, γδ T cells, and a subset of $CD4^+$ T cells (1). Though less characterized, DNAM-1 is another activating receptor expressed by cytotoxic lymphocytes, and its ligands PVR and Nectin-2, two adhesion molecules belonging to the Ig-like superfamily, are similarly induced by cellular stresses (5–9). Thus, expression of ligands for activating NK cell receptors appears to be a critical mechanism of immunosurveillance against stressed cells (10). In addition, recent studies demonstrated that another shared aspect of stress responses consists in the activation of the DNA damage response (DDR), a major signaling pathway implicated in the up-regulation of ligand expression (11).

DNA must be protected from damage produced spontaneously during DNA replication or from endogenously generated reactive oxygen species (ROS) that are a byproduct of normal metabolic processes. In addition, a plethora of external stimuli, such as ultraviolet light, ionizing radiation, and viral infections can cause DNA lesions (both in a ROS-dependent and -independent manner) that can block genome replication and transcription (12). Therefore, the general term DDR is related to a complex series of cellular stress-induced pathways that detect DNA damage and that are involved in the maintenance of genome integrity and avoidance of mutated DNA duplication (13). Three members of the phosphatidylinositol 3-kinase-like serine/threonine protein family are central to this response: ATM, ATR, and the DNA-dependent protein kinase (DNA-PK) (14, 15). Both ATM and DNA-PK are known to be recruited to and activated by double-stranded DNA breaks, while ATR is activated by stalled replication forks and subsequently single-stranded DNA breaks (16–18). Following the recognition of DNA lesions by sensor proteins, these kinases activate many downstream mediators, such as the serine/threonine kinases Chk1 and Chk2, able to phosphorylate many effector proteins that induce either cell-cycle arrest and DNA repair or, if unsuccessful, initiation of programs instructing the cell to undergo apoptosis or enter terminal differentiation through senescence (12–14).

NORMAL CELLS

There is a substantial body of evidence showing the involvement of DDR in many physiological processes, such as mitosis (19), insulin response (20), V(D)J recombination (21, 22), or after lipopolysaccharide stimulation in macrophages (23). In addition,

the self-renewal capacity of hematopoietic stem cells was found to depend on an ATM-mediated modulation of the response to oxidative stress (24). Enhanced phosphorylation of either ATM or one of its substrates, the histone H2AX, as well as the increase of ATM protein levels were observed on T cells upon activation in response to a plethora of stimuli (8, 25–27).

In relation to activating ligands, studies performed in our own and other laboratories have shown that MIC, ULBP, and PVR molecules are induced on antigen-activated T cells (8, 27–29) (**Table 1**). Interestingly, both oxidative stress (mainly mediated by a macrophage-dependent production of ROS) and DDR were implicated in the induction of MICA and PVR on activated T cells (8, 27), suggesting that signaling via ATM/ATR kinases and DDR could represent a common pathway regulating the expression of NKG2D and DNAM-1 ligands on T lymphocytes (**Figure 1**). Of note, PVR and NKG2D ligand expression on T cells was mainly associated with progression to the S and G_2/M phases of the cell (8) (and our unpublished observations). Since ATM/ATR are known to be regulators of cell division, the increased cellular proliferation upon antigenic stimulation could be the crucial signal resulting in NKG2D and DNAM-1 ligand expression on healthy cells. In fact, a correlation of either NKG2D ligand or PVR expression with cell proliferation has been documented in several studies. Expression of MICA has been shown in fast dividing tissues including the gut epithelium (30) and in highly proliferating cell lines (31). Indeed, high surface MICA expression was detected in fibroblasts during the stage of rapid growth and was strongly down-regulated following cell–cell contact (32). Similarly, PVR expression in epithelial cells was tightly regulated by changes in cell density (33). Groh and colleagues have also demonstrated that the presence of MIC molecules on rheumatoid arthritis synoviocytes was strongly associated with the expression of the nuclear Ki-67 proliferation marker (31). A recent study indicated that MICA expression levels on endothelial cells were substantially increased by the induction of cell proliferation mediated by FGF-2 or wound healing (34). These *in vitro* observations were further supported by *in vivo* studies performed in rodents. Using bromodeoxyuridine incorporation in murine bone marrow grafts, RAE-1 (the murine orthologs of ULBP proteins) was detected on a large fraction of donor proliferating progenitor cells in the spleen of the transplant recipients rather than on the long-term hematopoietic stem cells (35), and in relation to DNAM-1 ligands, a study in the rat showed that the presence of PVR in the liver was confined to proliferating hepatocytes during liver regeneration (33). When the transcriptional regulation of activating ligands was analyzed in normal proliferating cells, their expression was reported to depend on NF-kB, Sp1, and the E2F family of transcription factors (27, 36–38).

The biological significance of an increased expression of both NKG2D and DNAM-1 ligands on the surface of dividing cells could be to alert the immune system of a potentially dangerous cell-cycle progression. Indeed, Davis's group reported that human NK cells bound to cells in mitosis more efficiently than the same cells in other stages of the cell cycle (39) and our studies further demonstrated that proliferating T cells become more susceptible to NK cell-mediated recognition and killing (8) (**Figure 1**). Thus, NK cell restriction of T cell responses might be relevant in the maintenance of lymphocyte homeostasis as well as in the context of autoimmunity or graft-versus-host disease (3).

VIRALLY-INFECTED CELLS

Infection by several viruses, including herpesviruses, adenoviruses, papillomaviruses, and retroviruses, is sufficient to activate some or all of the DDR-mediated repair pathways. Simplistically, this was perceived as recognition by the host cell of the incoming genetic material as its own damaged DNA, but it is now considered to be, at least in part, an anti-viral response aimed at combating the pathogen by posing a threat to viral genome integrity and replication (40). However, viruses have evolved a complex relationship with the DDR pathway being able to either inhibit or exploit DDR components in order to favor their own replication process, with some viruses using both strategies in a spatially

Table 1 | DDR-dependent up-regulation of NKG2D and DNAM-1 ligand expression.

Activator	Cell model	Activating ligand	Reference
ATM, ATR	T lymphocytes	MICA	(27)
ATM, ATR	T lymphocytes	PVR	(8)
ATM	LPS-stimulated macrophages	MICA	(23)
ATR	HIV-1 infected T cells	NKG2D ligands	(46, 47)
ATR	HIV-1 infected T cells	PVR	(50)
ATM, ATR, Chk1	Hepatoma	MICA/B	(69)
ATM	Hepatoma	MICB	(68)
ATM, ATR	Cervical and colon carcinoma, T cell leukemia	ULBP2	(67)
ATM, Chk2	Multiple myeloma	MICA	(65)
ATM/ATR	Multiple myeloma	MICA/B, ULBP1-3	(7)
ATM/ATR	Ewing sarcoma	MICB	(70)
ATM, Chk2	Colon cancer cells	MICA/B, ULBP1-3	(71)
p53	Lung cancer	ULBP1-2	(73)
p53	Colon/breast cancer	ULBP2	(74)
ATM, Chk1	Murine ovarian tumor cells	RAE-1	(11)
ATM	Murine B cell leukemia	PVR	(66)

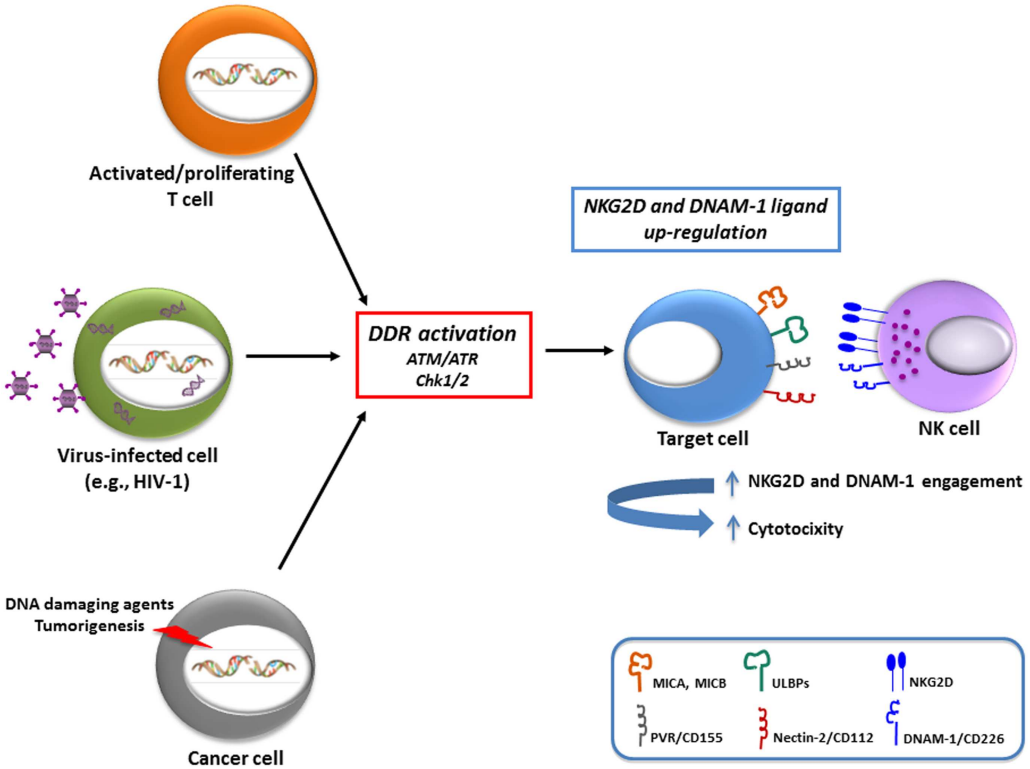

FIGURE 1 | Schematic representation of the variety of stimuli that can up-regulate NKG2D and DNAM-1 ligands. There is evidence that both in normal cells (e.g., antigen-activated T lymphocytes), as well as in pathological conditions, including virally-infected cells (in particular with HIV-1) and cancer cells, a major regulatory pathway involved in ligand up-regulation is the DNA damage response (DDR), activated by different stimuli. The increased expression of activating ligands has been shown to be implicated in the recognition and elimination of "stressed" cells by NK cells, and presumably also by other cytotoxic cells (i.e., γδ T cells and $CD8^+$ T cells).

and temporally orchestrated manner (41, 42). From a theoretical point of view, the viral-induced activation of DDR and the consequent up-regulation of the ligands for activating receptors could render infected cells susceptible to the recognition and elimination by cytotoxic lymphocytes, thus contributing to the anti-viral response. In humans, up-regulation of NKG2D and/or DNAM-1 ligands was indeed observed following infection by several viruses (e.g., HCMV, HCV, EBV, HIV-1) (43, 44), but the link between this phenomenon and DDR activation has been investigated only for HIV-1. Studies performed in our own and other laboratories have shown that HIV-1 infection of $CD4^+$ T lymphocytes up-regulates both MIC and ULBP proteins, especially ULBP2, as well as PVR, and thus exposes infected cells to recognition and lysis by NK cells (45–49) (**Figure 1**). Recently, the HIV-1 Vpr protein was identified as the key viral factor responsible for the up-regulation of both NKG2D ligands and PVR in infected $CD4^+$ T cells (46, 47, 50) (**Table 1**). The stimulatory effect of Vpr on ligand expression relies on its capacity to recruit a cullin-ring E3 ubiquitin ligase (DDB1-CUL4A) and to activate ATR (46, 51). The same Vpr interactions ultimately lead infected cells to arrest in G_2, a phase of the cell cycle that allows efficient virus production (52, 53), therefore it is possible that ligand up-regulation is secondary to G_2 arrest. Apparently, the effects of Vpr on ULBP2 and PVR expression operate at different levels, since ULBP2 but not PVR transcripts accumulate in Vpr-expressing cells (46, 47, 50). Thus, additional work is clearly needed to understand how Vpr up-regulates each ligand.

As a countermeasure for ligand up-regulation, HIV-1 as well as many other viruses, have developed the capacity to inhibit cell-surface ligand expression. For HIV-1, this activity is mediated by the viral proteins Nef, Vif, and Vpu that down-regulate NKG2D ligands and/or PVR, and, as a consequence, decrease the susceptibility of HIV-infected cells to NK-cell-mediated lysis (45, 48, 54). Interestingly, T cells infected with a mutated virus defective for the expression of the two proteins, Vpr and Nef, that exert opposite effects on NKG2D ligand and PVR expression, display higher ligand levels compared to uninfected cells (50) (and our unpublished data), suggesting the existence of an additional Vpr-independent mechanism of ligand up-regulation. This mechanism may be related to the previously reported triggering of ATM during HIV-1 DNA integration (55).

In sum, a picture is emerging in which HIV-1 hijacked some cellular DDR effector molecules that are required for efficient viral replication and, at the same time, has developed means to contrast the effect of DDR activation on NKG2D and DNAM-1 ligand expression that is dangerous for the virus itself. The fact that also several other viruses (e.g., HCMV, KSHV, HCV, HAdV, HHV, HCV) have evolved the capacity to down-regulate NKG2D and DNAM-1 ligands, suggests that activating NK cell receptors

and host immune responses mediated by NKG2D and DNAM-1 represent a serious threat that a virus must circumvent. Interestingly, these viruses are known to interact at some point of their life cycle with at least one component of the DDR machinery to aid their own replication. Therefore, a better understanding of the dual (pro- and anti-viral) role of DDR in the life cycle of HIV-1 and of other viruses may lead to new strategies aimed at suppressing viral replication while maintaining and, possibly, reinforcing anti-viral immune responses.

CANCER CELLS

The relevance of NKG2D in tumor surveillance has been demonstrated by *in vivo* experiments showing that overexpression of NKG2D ligands in cancer cells causes tumor rejection in mice (56, 57), and that NKG2D-deficient animals are defective in tumor surveillance in models of spontaneous malignancy (58). In humans, it has been shown that many tumors up-regulate NKG2D ligands, probably as a result of the oncogenic process itself, and this renders them more sensitive to recognition by NK and cytotoxic T cells (59–61). In relation to DNAM-1, *in vitro* studies have shown that this activating receptor triggers NK cell-mediated killing of a range of tumor cells expressing PVR and/or Nectin-2. Moreover, DNAM-1-deficient mice show an impaired clearance of PVR-expressing tumor cells and develop more tumors in response to chemical carcinogens (62).

In cancer cells, stress signals, and in particular those associated with DDR, induce both NKG2D and DNAM-1 ligand expression (7, 11) (**Table 1**). In fact, cells exposed to chemotherapeutic agents, genotoxic stimuli, or stalled DNA replication cycles, up-regulate NKG2D ligands through the activation of the DDR, suggesting that ATM, ATR, and Chk1 may be predominantly responsible for NKG2D ligand expression maintenance (11). These findings provided for the first time a link between the constitutive activation of DDR in tumors and the frequent up-regulation of NKG2D ligands in transformed cells, suggesting that constitutive ligand expression could be maintained by persistent genotoxic stress in tumor cell lines. Moreover, many evidences support the idea that DDR can be frequently activated in early neoplastic lesions, and probably NKG2D and DNAM-1 ligand induction by DNA damage represents a tumor surveillance mechanism operating at the very early stages of tumorigenesis, possibly increasing the sensitivity of damaged cells to NK- and/or T cell-mediated lysis (11, 63–66) (**Figure 1**). In particular, Croxford and colleagues have very recently provided the first *in vivo* evidence that T and NK cells play a critical role in the regression of B cell lymphomas in Eμ-myc mice, by showing that spontaneous rejection requires the expression of PVR on tumor cells, which is regulated by an ATM-initiated DDR (66). Studies from our laboratory have also contributed to better delineate the link between activation of DDR and regulation of NKG2D and DNAM-1 ligands. In particular, we demonstrated that genotoxic drugs, when used at doses that do not affect cell viability, induce the up-regulation of NKG2D and DNAM-1 ligand expression on several multiple myeloma cell lines and primary malignant plasma cells, and consequently enhance NK cell degranulation toward drug-treated tumor cells. This effect is dependent on the activity of ATM/ATR kinases, and occurs in combination with the establishment of a chemotherapy-induced senescent phenotype (7). Our observations in multiple myeloma are consistent with a number of other studies describing NKG2D ligand regulation by several DNA-damaging conditions (11, 65, 67–70) (**Table 1**). Moreover, Leung and colleagues have recently reported the up-regulation of NKG2D ligands by the aldosterone antagonist spironolactone through the DNA damage-independent activation of ATM-Chk2 in multiple colorectal cancer cells. The drug-mediated effect requires the activation of retinoid X receptor γ (RXRγ), probably capable of initiating chromatin remodeling, and results in activation of the ATM-Chk2 DNA repair checkpoint pathway that enhances NKG2D ligand expression (71). These observations demonstrate a key role for the protein kinases mediating DDR activation in the promotion of NKG2D ligand expression, and suggest that DNA lesions are not a prerequisite necessary to these effects.

One of the most extensively studied component of DDR is the tumor suppressor protein p53, and DNA damage leads to enhanced stability and activity of p53 upon its ATM-mediated phosphorylation (72). Conflicting results have been reported about the involvement of p53 in the regulation of NKG2D ligands, with data showing positive, negative, or no effect. Gasser and colleagues ruled out p53 from the mechanisms at the basis of genotoxic drug-induced NKG2D ligand up-regulation in mice and human cell lines, since the lack of p53 had no effect on NKG2D ligand expression after genotoxic stress (11). By contrast, other studies showed that ULBP1 and ULBP2 are direct p53 target genes in human cell lines and, accordingly, treatment of certain cancer cells with RITA, a small molecular compound that reactivates wild-type p53, resulted in the up-regulation of ULBP2 expression (73, 74). On the contrary, it has been recently shown that ULBP2 gene can be repressed via the p53-mediated increase in cellular miR-34 levels (75). Thus, the outcome of p53 activation on ULBP2, and possibly other NKG2D ligands, might depend on the context of its activation, pointing to a complex role of p53 that awaits further investigation.

A novel perspective in the regulation of MICA expression has been recently demonstrated by a study showing that up-regulation of MICA by genotoxic stress was enhanced by inhibiting STAT3 activity in both cancer and non-malignant cells (76). In agreement with this observation, studies conducted by our group have demonstrated that inhibition of STAT3 – obtained by using GSK3 kinase activity inhibitors – can enhance the expression of MICA induced by the chemotherapeutic drug melphalan in multiple myeloma cells (77). Therefore, these results add an additional layer of complexity in the molecular mechanisms regulating the expression of MICA and likely of other NK cell activating ligands.

CONCLUSION

As shown in less of 10 years of intense research, NK cell activating receptors and their ligands represent an important warning system alerting cytotoxic lymphocytes of danger and stress signals. Since the expression of NKG2D and DNAM-1 ligands is rarely seen in normal cells, this means that small changes in their cell-surface levels may significantly influence the susceptibility of the target cell to NK cell recognition. Their expression appears to be regulated at different levels (epigenetic, transcriptional,

post-transcriptional), but in this review we have summarized the current literature and highlighted the importance of the DDR in promoting NKG2D and DNAM-1 ligand expression, both at protein and mRNA levels, though the precise molecular mechanisms mediating these effects and the possible cooperation/regulation with upstream and downstream additional signaling pathways remain to be further clarified. However, DDR may represent a crucial point of convergence for ligand up-regulation, triggered by a big variety of circumstances and stressful stimuli.

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Molecular bases for the regulation of NKG2D ligands in cancer

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NKG2D is an activating receptor expressed by NK and T cells primarily involved in the elimination of transformed and infected cells. NKG2D ligands are self-proteins restrictedly expressed in healthy tissues, but induced in response to signaling pathways commonly associated with transformation. Proliferative, tumor suppressor, and stress signaling pathways linked to the tumorigenic process induce the expression of NKG2D ligands, initiating an immune response against the incipient tumor. Nevertheless, the activity of NKG2D ligands is counter-regulated *in vivo* by the immunoediting of cancer cells, resulting in the expression of multiple mechanisms of immune evasion in advanced tumors. The redundancy of NKG2D ligands, besides increasing the complexity of their regulation, may impair the generation of these immune evasion mechanisms. In this review, we attempt to integrate the mechanisms and pathways involved in the regulation of NKG2D ligand expression in cancer.

Keywords: NKG2D, MICA, MICB, ULBP, NK cell, T cells, signaling pathways, regulation

HUMAN NKG2D AND ITS LIGANDS

NKG2D is a type-II transmembrane-anchored glycoprotein constitutively expressed on the surface of NK, CD8 T, and γδ T cells (1). At the cell membrane, human NKG2D associates with DAP10, while mouse NKG2D may associate with both DAP10 and DAP12, which activate downstream signaling pathways resulting in the activation of NK cells and co-stimulation of CD8 T cells (2). Cross-linking of NKG2D receptor alone on freshly isolated NK cells does not seem to trigger a significant cell-mediated cytotoxic response; and simultaneous cross-linking of other activating receptors or stimulation of NK cells with IL-15 or high dose of IL-2 is rather necessary to kill tumor target cells (3).

NKG2D is a receptor for multiple ligands (NKG2DLs), which are distant members of the MHC class I family. In humans, NKG2D is a receptor for MICA and MICB (MICA/B) and ULBP1–6 molecules (4–8) (**Figure 1**). NKG2DLs are restrictedly expressed in healthy cells avoiding autoimmunity, but they are frequently overexpressed in infected and transformed cells, acting as a danger signal that favors the perforin-mediated elimination of transformed cells, thus acting as an extrinsic mechanism of cancer surveillance that complements the intrinsic processes that control growth and proliferation of tumor cells.

NKG2D IS INVOLVED IN THE IMMUNE SURVEILLANCE OF CANCERS

NKG2D-deficient mice are more prone to develop cancer (9), and neutralization of NKG2D increases the sensitivity of mice to carcinogen-induced sarcomas (10). Conversely, murine NK cells may efficiently eliminate cancer cells expressing NKG2DLs *in vivo*, highlighting the relevant role of this receptor in the cancer immune surveillance in mice (11–13). In humans, NKG2DLs are restrictedly expressed in healthy cells, but a broad expression of these proteins is observed in transformed cells, rendering them more susceptible to NK cell-mediated killing (1, 14–16). Moreover, cell-associated NKG2DL expression correlates with the outcome in colorectal (16, 17), breast, (18) and pancreatic cancers (19) and hepatocellular carcinoma (20). However, it is noteworthy that high expression of ULBP2 and ULBP4 has been paradoxically associated with poor prognosis in ovarian cancer, and it does not appear to be related to an increase of the shedding of these molecules (21, 22). Further, the development of immune evasion mechanisms by cancer cells has been associated with poor prognosis in different types of cancer (see below). Thus, the serum level of soluble MICA (sMICA) is an independent prognostic factor for multiple myeloma (23) and advanced hepatocellular carcinoma (24). sMICA and soluble MICB (sMICB) serum levels were correlated with disease stage and survival rates in oral squamous cell carcinoma patients (25), whereas soluble ULBP2 (sULBP2) was associated with poor prognosis in melanoma (26), chronic lymphocytic leukemia (27), and lung cancer (28).

Albeit these data clearly indicate an important role for the NKG2D response in the immune surveillance of human cancers, the molecular bases underlying the regulation of NKG2DL expression have not been fully elucidated. Nevertheless, increasing evidence indicates that the activation of oncogenic pathways commonly associated with the tumorigenic process may be responsible for the induction of NKG2DL expression and the activation of the NKG2D response (29) (**Table 1**).

DNA DAMAGE AND NKG2D RESPONSE

DNA damage potentially represents an initiating event for carcinogenesis as it is frequently observed in precancerous lesions. As a result of the DNA damage response (DDR), cell cycle checkpoints are activated, stopping the cell cycle to give the cell time to repair

the damage, and, if such damage is too great, to trigger apoptosis. DNA damage checkpoint activation is controlled by two kinases: ATM (ataxia telangiectasia, mutated), which responds to DNA double-strand breaks and disruptions in chromatin structure; and ATR (ATM- and Rad3-related), which primarily responds to stalled replication forks. Activated ATM or ATR initiates a protein kinase cascade that includes the activation of the checkpoint kinases CHK1 and CHK2, and p53. In a seminal paper, it was shown that DNA damage also activates the immune response as an additional extrinsic control mechanism that favors the elimination of damaged and cancer cells through the induction of NKG2DL expression (30). It is worth-mentioning that the up-regulation of NKG2DLs occurs, at least in mice, in a p53-independent manner, which is particularly relevant given that a p53 expression is lost in a majority of clinical aggressive and invasive cancers. However, the downstream regulators of the DDR involved in NKG2DL expression remain ill-defined.

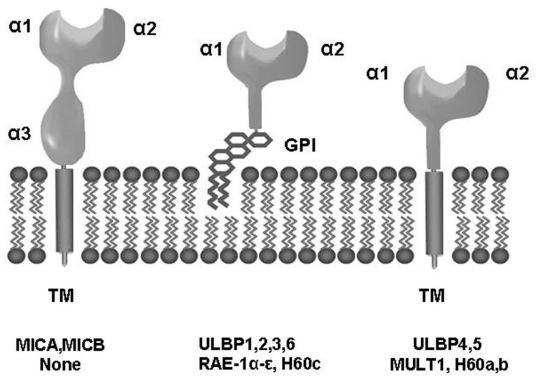

FIGURE 1 | Biochemical and structural properties of human and mouse NKG2D ligands. TM, transmembrane; GPI, glycosylphosphatidylinositol.

DNA damage response is activated in response to ionizing radiation and chemotherapy. Consequently, the immune response may contribute to the efficacy of these conventional cancer treatments. Many viruses with distinct replication strategies, such as DNA viruses and retroviruses, may also activate the DDR, which may potentially activate the NKG2D response. The replication of viral DNA in the nucleus has the potential to trigger the DDR if the ends of viral genomes are exposed and recognized as double stranded breaks. Retroviral DNA integration creates a discontinuity in the host cell chromatin, also activating the DDR. Likewise, induction of NKG2DLs by two retroviral infections, caused by Abelson murine leukemia virus and human immunodeficiency virus (HIV), supports a role of DDR in the NKG2D-mediated anti-viral response (48, 49). A more universal role of this pathway in the elimination of virus-infected cells remains to be established.

NKG2DL expression may also be induced as a result of DNA replication stress and activation of ATM/ATR in human T cells that proliferate in response to mitogens, antigens, and superantigens (50). This has been proposed to limit undesired immune responses; however, the underlying mechanisms and the biological significance of NKG2DL expression in T cells and other healthy cells are poorly understood.

OTHER TYPES OF CELLULAR STRESS REGULATE NKG2D RESPONSE

Different forms of cellular stress frequently associated with the tumorigenic process are involved in the regulation of NKG2DL expression. Early reports showed that heat shock was able to induce *MICA* and *MICB* expression (32). This induction of *MICA/B* transcription was lately described as the result of the binding of heat shock factor 1 (HSF1) to the promoters of *MICA* and *MICB* genes (33). Several studies showed that oxidative stress also induced *MICA/B* gene and protein expression in several tumor cell lines (34, 51). In normal human bronchial epithelial cells, oxidative stress induced MICA and ULBPs expression through the

Table 1 | Main signaling pathways that regulate NKG2D ligand expression.

Signaling pathway	Cell model	NKG2D ligand	Reference
STRESS PATHWAYS			
DNA damage response	Non-tumor cell lines	Mouse and human ligands	(30)
	Myeloma		(31)
Thermal stress	Epithelial cell lines	MICA/B	(32, 33)
Oxidative stress	Epithelial cell lines/epithelial bronchial cells	MICA/B, ULBP1–4	(34, 35)
PROLIFERATIVE/ONCOGENE PATHWAYS			
c-Myc	Primary lymphoma	Rae-1ε	(36)
H-RasV12	Mice and human cell lines	Rae-1α Rae-1β, ULBP1–3	(37)
HER2/HER3	Breast cancer cell lines	MICA/B	(38)
BCR–ABL	CML cells	MICA/B	(39–41)
PI3K	Multiple	Mouse and human ligands	(37, 38, 42, 43)
TUMOR SUPPRESSOR PATHWAYS			
P53	Epithelial cell lines	ULBP1,2	(44–46)
TUMOR PROGRESSION AND METASTASIS			
EMT	Epithelial cell lines	MICA/B, ULBP1–3	(47)

CML, chronic myelogenous leukemia; EMT, epithelial-to-mesenchymal transition.

activation of ERK (35). Hypoxia has also been shown to down-regulate the surface expression of MICA in osteosarcoma cell lines through the induction of hypoxia-inducible factor 1α (HIF-1α), but independently of nitric oxide (NO) production (52).

PROLIFERATIVE SIGNALS INDUCE NKG2D RESPONSE

Early reports showed that *MICA/B* mRNA and protein expression are mostly limited to proliferating epithelial cells, but they are scarcely expressed in quiescent cells (32, 33, 50). Recently, proliferation has been associated with the induction of the expression of murine RAE-1 family of NKG2DLs in mouse fibroblasts, and of MICA/B and ULBP2 in HCT116 colorectal cancer cells (42). The induction of NKG2DL expression was independent of stress pathways. Instead, *Raet1* genes, but not MULT1 and H60b, were found to be direct targets of E2F family of transcription factors, which plays a major role during the G1/S transition and cell cycle reentry. This provides a mechanistic link between proliferation and NKG2D response. Moreover, murine RAE-1ε induction in primary fibroblast cultures was blocked by inhibiting proliferative pathways such as cyclin-dependent kinases, PI3K–mTOR, and MAPK pathways (42).

Unrestrained proliferation is a common characteristic of cancer cells. Albeit it has not always been mechanistically linked to cell proliferation, it has been reported that inappropriate over-expression of several oncogenes induced NKG2DL expression independently of DDR. However, the expression of certain oncogenes (*K-ras* and *c-myc* or *Akt* and c-*myc*) was not sufficient to induce NKG2DL expression in primary ovarian epithelial cells (30). This probably reflects that transformation is a multistep process, and changes in many pathways, such as tumor suppressor pathways, are required for both transforming a normal cell into a cancer cell and for activating the NKG2D response. In fact, it has been reported that several oncogenic pathways up-regulate NKG2DLs. For instance, NKG2DLs are induced on spontaneously arising tumors in an Eμ-myc murine model of lymphoma (36). The induction of NKG2DLs involves the activity of c-Myc, which is a master regulator of proliferation. Similarly, the oncogenic H-RasV12 protein up-regulates the expression of Rae-1α and Rae-1β in mouse and of ULBP1–3 in human cells (37). H-RasV12 mainly regulated the expression of Rae-1 by post-transcriptional mechanisms via Raf–MAPK/MEK and PI3K pathways. Ras signals also contribute to the induction of NKG2DL expression in primary fibroblast cultures in response to the serum, which may be blocked by epidermal growth factor receptor (EGFR) inhibitors (37). EGFR comprises a family of four closely related transmembrane tyrosine kinase receptors (also known as HER1–4). Amplification or over-expression of the HER2/HER3 dimer is involved in the progression of different cancers, particularly in certain types of aggressive breast cancer. It has been shown that over-expression of HER2/HER3 activates the expression of MICA/B, mainly through the PI3K/AKT pathway (38). Philadelphia chromosome is a specific chromosomal translocation associated with chronic myelogenous leukemia (CML). It results in BCR–ABL fusion protein, with a continuously activated tyrosine kinase activity, thus promoting the unrestrained cell division of leukemia cells. BCR–ABL kinase activity was also linked to NKG2DL expression and the activity of NK cells (39, 40). Thus, BCR/ABL induces the MICA surface expression on CML leukemia cells, whereas it was absent on healthy hematopoietic cells (41).

Induction of host cell proliferation is a common viral strategy that favors viral survival and replication. However, it may also induce NKG2DL expression, resulting in an NKG2D-dependent elimination of the infected cell. Thus, the activation of PI3K pathway by the murine cytomegalovirus results in the expression of RAE-1 family of NKG2DLs (43). Similarly, adenovirus serotype 5 (Ad5) E1A oncogene up-regulates RAE-1 expression, but not murine ULBP-like transcript 1 (53).

Overall, these data suggest that proliferative signals generated by oncogene activation and virus infection and, possibly by other physiological situations, may alert the immune surveillance response through the induction of the NKG2D response.

TUMOR SUPPRESSION AND SENESCENCE MAY INDUCE NKG2DL EXPRESSION

The deregulation of the activity of oncogene proteins is counter-regulated by tumor suppressor proteins. These proteins repress cellular proliferation and the loss of their function is an additional characteristic associated with tumorigenesis. For instance, in the Eμ-myc murine model of lymphoma, sustained over-expression of Myc signaling induces $p19^{Arf}$ expression, resulting in apoptosis through the inhibition of Mdm2 and stabilization of the p53 tumor suppressor protein. Thus, a combination of deregulated activity of Myc and the loss of a tumor suppressor protein (either $p19^{Arf}$ or p53) is involved in both tumor progression and in the induction of Rae-1 in lymphoma cells (36). Thus, despite the existence of few experimental data, the current knowledge about tumor biology suggests that the combination of oncogenes and tumor suppressors may be involved in the regulation of NKG2DL expression.

Accordingly, the tumor suppressor p53 has been associated with the regulation of NKG2D response. Over-expression of wild-type p53 in cancer cell lines strongly up-regulated the expression of *ULBP1* and *ULBP2*, but not of other NKG2DLs, upon binding of p53 to response elements located in the intronic regions of these genes (44, 45). Moreover, the small molecule inhibitor Nutlin-3a decreased ULBP2 levels in a p53-dependent manner, through the increase in cellular levels of the suppressive miR-34 (46).

Additionally to growth arrest and apoptosis, p53 may induce senescence in response to diverse forms of cellular stress, including telomere shortening, DNA damage, oncogene activation, oxidative stress, and chemotherapeutic drug administration. The senescence program activates p53 and $p16^{INK4a}$–pRb tumor suppressor pathways, which establish an irreversible form of cell cycle arrest. The most fully described function of senescence *in vivo* is the ability to establish a potent barrier to tumorigenesis in response to oncogene activation or DNA damage. A typical characteristic of senescent cells, known as "senescence-associated secretory phenotype," is the secretion of inflammatory cytokines and chemokines that may recruit and activate distinct adaptive and innate immune cells subsets, including NK and T cells.

It has been shown that chemotherapeutic drug treatment of myeloma cells induces DDRs and activates the expression of NKG2DLs, mainly in senescent cells, resulting in their elimination by NK cells through the recognition by NKG2D and DNAM-1

receptors (31). NKG2DLs are also up-regulated on activated senescent hepatic stellate cells (54). Further, a recent study in a mouse model of senescence generated by induction of p53 expression also showed that NK cells participate in the elimination of senescent cells in an NKG2D-dependent manner (55). Nevertheless, induction of p53 did not increase NKG2DL expression, but, instead, induced senescent cell to secrete chemokines that recruit NK cells.

NKG2DL EXPRESSION DURING TUMOR PROGRESSION

It has been well established that NKG2D may protect the host from tumor initiation (9–13); however, less information exists regarding the role of NKG2D in tumor progression and metastasis. Early reports showed the loss of MICA/B expression in metastatic melanoma suggesting the immune selection of MICA/B negative tumors (56). Similarly, we have recently reported that epithelial-to-mesenchymal transition (EMT) is an immunological checkpoint that controls tumor progression through NKG2D-mediated immune responses (47, 57). EMT is one of the first steps of the metastatic cascade, which results in the loss of epithelial characteristics and the gain of mesenchymal properties by malignant cells, such as migration and invasiveness. EMT induction by Snail1 over-expression, GSK-3 inhibition, or TGF-β stimulation up-regulated NKG2DL expression in epithelial cells, rendering EMT cells more susceptible to NK cell-mediated killing. Sp1 and Sp3 transcription factors are key regulators of the basal transcription of *MICA/B* and *ULBP1–3* (33, 58, 59); and induction of Sp1 activity in colorectal tumor cells was involved in the up-regulation of NKG2DL expression during the EMT process. *In vivo*, MICA/B and ULBP1 proteins are expressed in healthy colon mucosa cells, displaying a polarized apical distribution; and no interaction with NKG2D-bearing immune cells was observed (47, 57). The polarized expression of MICA and ULBP1 was also observed in malignant cells retaining epithelial characteristics, whereas a loss of the polarization of NKG2DL expression was observed in malignant cells that suffer EMT. Moreover, a dramatic increase in the number of NKG2D-bearing tumor-infiltrating T lymphocytes was observed in specimens lacking MICA expression, suggesting that the loss of the epithelial integrity and polarity, characteristics of the EMT process, may allow the diffusion of MICA/B proteins along the membrane of mesenchymal cells, resulting in their elimination by NKG2D-bearing immune cells. Overall, these data suggest that tumor progression and metastasis, a key characteristic of malignant cancers, may also be under the control of the NKG2D response.

TUMOR EVASION OF NKG2D RESPONSE

NKG2D-deficient mice expressed higher amounts of NKG2DLs in cancer cells than similar tumors in wild-type mice, which suggests that NKG2DL expression in a host may be counter-regulated by the immunoediting of cancer cells (9). This is in agreement with the description of a plethora of immune evasion mechanisms that impair the NKG2D-mediated response. Repression of NKG2DL expression is frequently observed in advanced tumors and metastases compared with primary tumors, and this may be regulated by epigenetic mechanisms. In advanced tumors, histone deacetylases (HDACs) may interact with the promoter region of NKG2DL genes, regulating the chromatin structure and impairing the access of transcription factors to their promoters. Thus, HDAC1 may inhibit the transcription of *MICA/B* on leukemia cells (60), whereas HDAC3 may repress the transcription of *ULBP1–3* in epithelial tumors (59). Consequently, HDAC inhibitors stimulate the expression of NKG2DLs on tumor cells (60–63). Other mechanisms, such as the over-expression of suppressive microRNAs in tumors, may also account for the repression of NKG2DL expression (46, 64–66). Contrarily, sustained NKG2DL expression and prolonged interaction with NKG2D receptor also leads to a strong down-regulation of NK cell cytotoxic activity (67, 68).

NKG2DLs may be also shed as soluble proteins (69, 70), which may cause the endocytosis and degradation of NKG2D receptor in immune cells (2). Interaction of MICA on the surface of tumor cells with the chaperon molecule ERp5 plays a relevant role in sMICA shedding (71). ERp5 may form a transitory disulfide bond with MICA, which induces a conformational change, allowing the proteolytic cleavage of MICA by proteases. Likewise, membrane ERp5 was functionally associated with sMICA shedding in chronic lymphocytic leukemia patients (72); and sMICA serum levels have been associated with ERp5 expression in multiple myeloma (73) and Hodgkin lymphoma (74). sULBP2 may also be released from tumor cells by proteolytic cleavage (75). However, engagement of sULBP2 not necessarily down-regulates NKG2D receptor. sULBP4 may be shed from tumor cells by alternative splicing mechanisms (76). Additionally, cancer cells may release MICA, MICB, and ULBPs into exosomes (77, 78). This event appears to be ligand-dependent, since ULBP2 is mainly shed by metalloproteinases, whereas ULBP3 is abundantly released as part of exosomes. Moreover, in the case of MICA, it may be allele-dependent (79). Exosomes containing NKG2DLs selectively down-regulate NKG2D expression on CD8 T and NK cells.

CONCLUDING REMARKS

As discussed above, relevant biological pathways associated with tumorigenesis, including proliferative, tumor suppressor, and stress pathways, are key signals involved in the induction of the NKG2D response in cancer. Many of these pathways are frequently activated in viral infections and in other pathological and physiological situations, providing a common link in the regulation of NKG2DLs between these pathologies and cancer. Nevertheless, little information regarding to the regulation of NKG2DLs in non-cancer-conditions is available, and future research in this field is needed.

However, the activity of NKG2DLs is counter-regulated *in vivo* by the immunoediting of cancer cells and for the development of immune evasion mechanisms in virus. Likewise, advanced tumors develop multiple mechanisms of immune evasion that frequently modify NKG2DL expression, thus making difficult to differentiate the intrinsic mechanisms of NKG2DL regulation from the consequences of the immune selection. As described above, distinct NKG2DLs are differentially regulated by specific oncogenic pathways. Thus, the redundancy of NKG2DLs presumably increases the range of different pathological situations in which NKG2D response may be activated and may also impair the generation of immune evasion mechanisms.

AUTHOR CONTRIBUTIONS

All authors participated in the preparation, discussion, and writing of this manuscript.

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Natural killer cells and neuroblastoma: tumor recognition, escape mechanisms, and possible novel immunotherapeutic approaches

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Neuroblastoma (NB) is the most common extra-cranial solid tumor of childhood and arises from developing sympathetic nervous system. Most primary tumors localize in the abdomen, the adrenal gland, or lumbar sympathetic ganglia. Amplification in tumor cells of MYCN, the major oncogenic driver, patients' age over 18 months, and the presence at diagnosis of a metastatic disease (stage IV, M) identify NB at high risk of treatment failure. Conventional therapies did not significantly improve the overall survival of these patients. Moreover, the limited landscape of somatic mutations detected in NB is hampering the development of novel pharmacological approaches. Major efforts aim to identify novel NB-associated surface molecules that activate immune responses and/or direct drugs to tumor cells and tumor-associated vessels. PVR (Poliovirus Receptor) and B7-H3 are promising targets, since they are expressed by most high-risk NB, are upregulated in tumor vasculature and are essential for tumor survival/invasiveness. PVR is a ligand of DNAM-1 activating receptor that triggers the cytolytic activity of natural killer (NK) cells against NB. In animal models, targeting of PVR with an attenuated oncolytic poliovirus induced tumor regression and elimination. Also B7-H3 was successfully targeted in preclinical studies and is now being tested in phase I/II clinical trials. B7-H3 down-regulates NK cytotoxicity, providing NB with a mechanism of escape from immune response. The immunosuppressive potential of NB can be enhanced by the release of soluble factors that impair NK cell function and/or recruitment. Among these, TGF-β1 modulates the cytotoxicity receptors and the chemokine receptor repertoire of NK cells. Here, we summarize the current knowledge on the main cell surface molecules and soluble mediators that modulate the function of NK cells in NB, considering the pros and cons that must be taken into account in the design of novel NK cell-based immunotherapeutic approaches.

Keywords: neuroblastoma, natural killer cells, PVR, B7-H3,TGF-beta, tumor escape mechanisms, immunotherapeutic approaches, chemokine receptors

NEUROBLASTOMA, WHERE ARE WE?

Neuroblastoma is a very heterogeneous disease (1) that includes rare familial (<2%) and sporadic forms. It presents as a locoregional (Stage 1, 2, and 3 or L1, L1/L2, and L2) or progressing metastatic disease involving bone, skin, liver, brain, and bone marrow (BM), the latter being frequently refractory to standard therapy (Stage 4 or M). Moreover, a rare metastatic form of spontaneously regressing/maturating disease (Stage 4S or MS) may occur in children below the age of 18 months (2–5). Over the years, major efforts have been focused to unveil the genetic and biological features of the different forms of NB in order to identify novel prognostic factors and druggable targets. To date, stage, patient's age, and presence or absence of the amplification of MYCN, a transcription factor crucial for central nervous system (CNS) development (6), are considered major predictors of patients' clinical outcome. Moreover, risk-group stratification is based upon additional parameters such as loss of chromosome 11q, tumor histology, and ploidy (3, 5). MYCN amplification strongly associates with loss of heterozygosity at chromosome 1p and occurs in approximately 15% of children affected by NB, with increased frequency in stage 4 patients (approximately 50%). A more accurate evaluation of the risk of treatment failure according to these prognostic factors allowed overtime a significant reduction of mortality in patients that at diagnosis presented with localized non-MYCN-amplified tumors (low-risk) and non-MYCN-amplified stage 4 under 18 months of age (intermediated-risk), who received less aggressive surgical and chemotherapeutic treatments. In particular, Rubie and coworkers have recently demonstrated that low-dose chemotherapy improved 5 years survival of 9% in infants with low-risk NB without threatening symptoms compared to cases treated

with high dose chemotherapy due to resistance to the low-dose regimen or presence of one threatening symptom (7). A more dramatic scenario exists in children with high-risk NB at diagnosis, which includes stage 4 patients <18 months with MYCN amplification or >18 months with or without MYCN amplification and rare patients with MYCN-amplified localized tumors (3, 5). These patients have a 5-year survival rate <50% although receiving aggressive combination therapies that include intensive chemotherapy, surgery, radiotherapy, autologous stem cell transplantation, and the administration of Retinoids (13-*cis*-retinoic acid) (3, 5), which represent adjuvants for high-risk NB therapy (8) due to their capability of driving neuronal differentiation *in vitro* (9).

Over the years, several studies focused on the identification of new molecular targets. However, although different experimental approaches have been used, including whole-genome sequencing, few somatic mutations in druggable pathways have been identified. Mutations in the anaplastic lymphoma receptor tyrosine kinase (*ALK*) are infrequent (<10% of sporadic NB). Mutations in the a-thalassemia/mental retardation syndrome X-linked (*ATRX*) gene are most common. However, they have not been identified in MYCN-amplified NB and the ATRX molecule does not appear to drive tumorigenesis (10, 11). Recurrent genetic alterations that, however, require further investigations, have been reported in the RAC–RHO pathway and in chromatin-remodeling genes At-rich interactive domain 1A (ARID1A) and 1B (ARID1B) (11–13). Due to the difficulty at targeting directly the MYCN transcription factor, the major oncogenic driver identified so far (14), new strategies currently aim to neutralize molecules involved in apoptosis, angiogenesis, invasion, or metastasis (5). Interestingly, the antibody-mediated targeting of the oncofetal differentiation antigen GD2 in combination with GM-CSF and IL-2 resulted in an improved outcome of patients and has been recently included in the standard care of high-risk NB (15). Unfortunately, pain, the major side effect of this therapy (16), as well as cytokine release syndrome and iridoplegia, limits its use; moreover, to date, only patients with minimal residual disease can benefit of this therapeutic approach (17). The success of the anti-GD2/GM-CSF/IL-2 therapy might depend on the FcγR-mediated activation of granulocytes (18) as well of other immune cell types such as macrophages and natural killer (NK) lymphocytes.

Among cytotoxic lymphocytes, NK cells represent the most potent anti-tumor effectors and represent promising weapons against aggressive tumors such as NB (19, 20). In addition, as recently demonstrated, they may also attack cancer stem cells (CSC), i.e. tumor cells with stem cell properties (21–23). Thus, in the past decade, several studies have been focused on the identification of the molecular mechanisms involved in the interaction between NB and NK cells in order to establish a convincing biological starting point for a novel NK cell-based immunotherapeutic approach.

NATURAL KILLER CELLS AND NEUROBLASTOMA RECOGNITION

Tumor cells express surface molecules that either switch off or switch on NK cell-mediated cytotoxicity. HLA class I molecules on tumors negatively regulate NK cell function by engaging immunoreceptor tyrosine-based inhibition motifs (ITIM)-bearing receptors (24) that include the inhibitory killer Ig-like receptors (KIRs, CD158), highly polymorphic clonally distributed receptors able to distinguish among different HLA-A, -B, and -C allotypes (25), and CD94/NKG2A heterodimers, specific for non-classical HLA-E (26). KIR and CD94/NKG2A are differently expressed in $CD56^{bright}$ $CD16^{-}$ and $CD56^{dull}$ $CD16^{+}$ NK cell subsets, which represent sequential stages of maturation (27, 28). While high numbers of $CD56^{bright}$ $CD16^{-}$, KIR^{-}, IFN-γ producing cells are found in non-reactive lymph nodes and tumor sites, the majority of NK cells circulating in the blood are mature $CD56^{dull}$ $CD16^{+}$, KIR^{+}, cytolytic ($perforin^{high}$) cells (29). $CD56^{bright}$ can progress to $CD56^{dull}$ cells, which upon activation increase the cytotoxic and IFN-γ producing capabilities (28, 30).

Tumor cells switch on NK cell function by expressing at the cell surface non-MHC class I, "danger" molecules that are recognized by an array of activating NK receptors. These include both the FcγRIII (CD16) that mediates the antibody-dependent cytotoxicity (ADCC) and receptors that function in the absence of antibodies. These latter molecules mainly consist of the NKp46, NKp30, and NKp44 receptors, collectively termed natural cytotoxicity receptors (NCR), which are mostly NK-restricted, and NKG2D and DNAM-1 that are also expressed by T cell subsets (31). In the last decade, several groups dedicated many efforts to unveil the nature of the surface tumor ligands specifically recognized by the activating receptors. NKp46 still remains a receptor with "orphan ligand," while NKp44 has been shown to recognize a novel isoform of the mixed-lineage leukemia (MLL5) protein (32) and NKp30 binds to B7-H6 molecules (33). The NKG2D and DNAM-1 (CD226) receptors recognize MICA/B and ULBPs or, PVR (CD155) and Nectin-2 (CD112), respectively (31). DNAM-1 shares the capability of recognizing PVR with tactile (CD96) (34) and TIGIT (35). All the ligands identified so far represent *de novo* expressed danger signals or molecules that are expressed in healthy cells and over-expressed upon tumor transformation. In most cases, the expression on tumors of multiple ligands leads to the engagement of different activating NK receptors that cooperate in triggering the anti-tumor cytotoxicity (31).

When both inhibitory and activating receptors are engaged by the specific ligands on potential targets, the function of ITIM-bearing inhibitory receptors dominates over activation. Thus, in an autologous setting NK lymphocytes spare healthy cells that express high, "protective" levels of HLA class I molecules while they kill tumors, such as NB, in which HLA class I expression is downregulated (36, 37). Moreover, NK cells kill allogeneic targets that express non-self MHC class I alleles. This might occur when two individuals are "KIR/KIR-ligand mismatched," i.e., individual A is characterized by fully functional, educated NK cell subset(s) expressing KIR specific for HLA class I alleles that are missing in individual B. In the context of HLA-haploidentical hematopoietic stem cell transplantation (haplo-HSCT), the presence of a KIR/KIR-ligand mismatch in the donor versus recipient direction allows donor's NK cell subsets to kill recipient's (HLA class I^{high}) residual leukemic blasts thus resulting into a graft-versus-leukemia effect (GvL), as well as mature DC and activated T lymphocytes, thus preventing graft-versus-host-disease (GvHD) and graft-rejection, respectively (38).

In NB, initial data were obtained by the use, as target cells, of *in vitro* established NB cell lines (39). NK cells displayed strong cytolytic activity against different NB cells and NCR were involved in the mechanisms leading to killing (39). The susceptibility of human NB cell lines to NK-mediated killing was validated in the context of a metastatic model set up in NOD/SCID mice. In this experimental setting, repeated infusions of IL-2 or IL-15 activated NK cells resulted in both an increased mean survival time of HTLA-230-bearing mice and reduced BM infiltration (40). Considering that long term cultured cell lines might be poorly representative of original tumors, more interesting data originated from *in vitro* studies that use neuroblasts, freshly purified from BM aspirate of stage 4 patients (36) (**Figures 1** and **2**). In this study, allogeneic activated NK cells killed neuroblasts isolated from patients although at a lesser extent as compared to the NB cell lines used as control. According to the absence or negligible expression of HLA class I molecules, the NK-mediated lysis of neuroblasts did not increase in the presence of anti-HLA class I mAb. Different molecular mechanisms responsible for the reduced HLA class I expression in NB cells have been elucidated. The immunohistochemical analysis of high-risk human NB showed different abnormalities in the antigen processing machinery, which include defects in the expression of immunoproteasomal subunits LMP2 and LMP7 and of transporters of antigen processing (TAP) (37). *In vitro* treatment of NB cells with IFN-γ induced up-regulation of HLA class I expression (37). Although decreasing their susceptibility to autologous NK cells, this up-regulation of HLA class I molecules could enhance T cell-mediated recognition. In this context, a restoration of killing mediated by antigen specific (MAGE3) cytolytic T cells was observed upon cotransfection of NB cell lines with IRF1 and NF-kB p65, HLA class I transcriptional activators that are also induced by INFγ and TNFα, respectively (41). It cannot be excluded that also *in vivo* neuroblasts could acquire/upregulate HLA class I expression. For example, this might occur in the context of transplantation or following the anti-GD2 antibody-mediated therapy that engages $Fc\gamma R^+$ immune cells such as NK cells, which are capable of releasing high amounts of IFN-γ upon activation (**Figure 1**). This phenomenon was observed in a murine NB model, where recurrent tumors developed after an NK-dependent anti-tumor response induced by a humanized IL-2 immunocytokine targeted to GD2. In these mice, NB cells showed markedly enhanced MHC class I expression as compared with tumors growing in controls (42). The possible *in vivo* increase of HLA class I expression in NB cells could explain the benefit of a KIR/KIR-L mismatch in the NK versus NB direction (43–45).

Bone marrow-purified neuroblasts from different patients, although expressing in all instances low levels of HLA class I, displayed a great heterogeneity in terms of susceptibility to lysis by NK cells. The good or poor susceptibility to NK-mediated killing correlated with the presence or the absence of PVR expression on neuroblasts (**Figures 1** and **2A**)(36). Interestingly, neuroblasts lacking PVR expression were from patients in relapse, whereas those expressing PVR were from children at the onset of the disease, suggesting a possible correlation between PVR expression and NB status. The predominant role of DNAM-1/PVR interaction in NK-mediated killing is striking considering that, in most cases, killing of human tumors depends on the cooperation between various activating receptors (31). In this context, it is of note that none of the BM-purified neuroblast fractions expressed major adhesion molecules such as ICAM1 or LFA-3 (36). Thus, in NB the presence of PVR, originally described as an adhesion molecule, could become critical in rendering tumor cells susceptible to NK-mediated killing. Although PVR and nectin-2 are closely related molecules of the Nectin family, the presence/absence of nectin-2 on NB cells did not influence the susceptibility to NK-mediated killing. Nectin-2, which displays binding affinity to DNAM-1 similar to PVR (46), also shows homophilic recognition. This, by hampering its recognition by DNAM-1, may force the receptor to preferentially bind PVR (46). In line with the increased killing of PVR^+ neuroblasts, mAb mediated masking of DNAM-1 significantly reduced the NK-mediated lysis of neuroblasts showing a pivotal role of DNAM-1 in NB cell killing. While a minor contribution of NKp46 and NKp30 activating receptors could be appreciated (36), consistent with the low expression of the specific ligands, NKG2D did not play a significant role in killing of BM-purified neuroblasts (**Figure 1**). The expression of NKG2D ligands has been also investigated in human primary NB and cell lines (47). *MICA, MICB*, and *ULBPs* transcripts were found in most tumors and cell lines. However, MICA surface expression was absent in primary tumors and present only in some NB cell lines. Importantly, the soluble form of MICA (sMICA) was detected in sera of most patients and in the culture supernatant of some cell lines and was shown to downregulate NKG2D surface expression

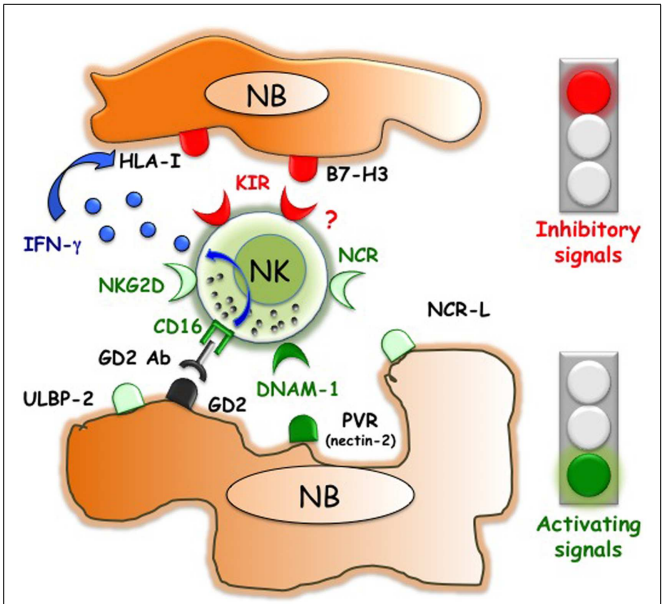

FIGURE 1 | Inhibitory and activating interactions between human NK cells and neuroblasts. NB express ligands that are recognized by NK receptors with activating or inhibitory function. While DNAM-1/PVR interactions play a pivotal role in triggering NK cell-mediated killing, B7-H3 dampens NK cell function. NB usually lack or express low, non-protective levels of HLA class I molecules. However, therapeutic approaches such as the *in vivo* administration of anti-GD2 Abs could induce not only ADCC of NB but also the release of INF-γ, which upregulates the expression of the ligands for inhibitory KIR.

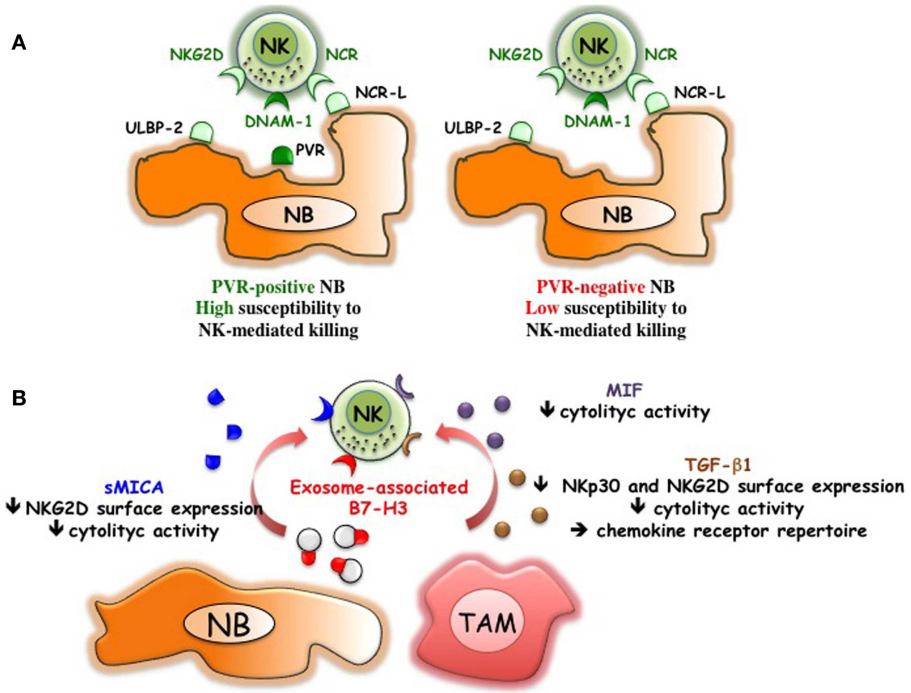

FIGURE 2 | NB-mediated mechanisms of escape from the NK-mediated immunosurveillance. **(A)** Downregulation of PVR, ligand of DNAM-1 activating receptor, renders NB cells poorly susceptible to NK-mediated killing. **(B)** NB can release soluble MICA, exosome-associated B7-H3, immunomodulatory factors such as MIF and TGF-β1. TGF-β1 might be also released by tumor-associated macrophages (TAM) that display M2-like functional properties.

in peripheral-blood T cells and decrease NK cell-mediated killing of MICA$^+$ NB cells (47) (**Figure 2B**). MICB was detected exclusively in the cytosol of NB cells, either primary tumors or cell lines. ULBP-1 was rarely detected, ULBP-2 was expressed by approximately 50% of primary tumors and cell lines, and ULBP-3 was absent in primary tumors but was expressed in most NB cell lines (47).

A crucial role of the DNAM-1 receptor has also been reported in myeloid and lymphoblastic leukemia (48), ovarian tumors (49), and different murine tumor models (50, 51). In particular, it has been shown that in response to chemical carcinogens, DNAM-1-deficient mice develop more DNAM-1 ligand$^+$ fibrosarcoma and papilloma cells than wt mice. Thus, the study of the molecular mechanisms responsible for the modulation of DNAM-1 ligands appears to be highly relevant. In this context, it has been shown that malignant RAJI lymphoma cells present hypermethylated PVR promoter-associated CpG islands. The epigenetic status was partially reversed by hypomethylating agents that led to upregulation of both transcription and surface expression of PVR (52). PVR up-regulation has been shown to be dependent on DNA-damage response (DDR)-dependent pathways that are activated by oxidative stress (53). Moreover, modulation of PVR surface expression might occur during infections. Different pathogen-derived stimuli such as LPS, poly I:C, and flagellin upregulated the surface expression of PVR (and nectin-2) in human DC (54) and in murine antigen-presenting cells (55) via the MYD88 and TRIF pathways. It has also been shown that expression of the human immunodeficiency virus type 1 (HIV-1) Vpr protein increases PVR levels in Jurkat T cells. This is due to activation of ATR kinase that triggers the DDR pathway and G2 arrest. Moreover, Vpr induces a PVR upregulation in HIV-infected CD4$^+$ T lymphocytes that overcomes the PVR downregulation induced by the HIV Nef protein (56). A negative regulation of PVR surface expression has been observed during cytomegalovirus (CMV) infection resulting in ineffective NK cell-mediated killing of infected cells (57). The UL141 viral glycoprotein is responsible for PVR downregulation, since it retains the immature form of PVR in the endoplasmic reticulum (57). Recently, it has been shown that UL141 binds to PVR with high affinity (58). Considering the role of PVR in determining high susceptibility to the NK-mediated attack, up-regulation of PVR surface expression might represent an appealing strategy to strengthen the immune responses against tumors. However, the dark side of PVR function cannot be disregarded. Indeed, although data in NB are missing, PVR has been described as a positive regulator of cancer progression, since it is capable of enhancing tumor cell invasiveness and migration (59). Thus, further studies are required to understand whether the immunological favorable effects of PVR up-regulation might balance the induction of a more aggressive tumor phenotype.

A tumor-promoting role has been shown also for B7-H3, a surface molecule originally identified on NB thanks to the generation of a specific monoclonal antibody (M5B14) (60). Studies on different tumor histotype indicate that B7-H3 might drive tumor cell development by different molecular mechanisms. These

include the capability of B7-H3 of reducing the effect of the chemotherapeutic-induced apoptosis in breast (61) and pancreatic carcinoma (62), as well as that of promoting migration and invasiveness (63, 64). The latter properties have been confirmed *in vivo* in an orthotopic model that showed that xenografted B7-H3 silenced glioma cells invaded significantly less into the surrounding brain tissue as compared to wt tumors. Moreover, B7-H3 expressed on glioblastoma cells negatively regulated NK cell-mediated cytotoxicity (64). These observations are consistent with previous data demonstrating that B7-H3 expression on BM-purified neuroblasts decreased NK cell-mediated killing (60) (**Figure 1**). Thus, different experimental approaches demonstrated that, in humans, B7-H3 might block the NK-mediated attack by interacting with a still unknown inhibitory NK receptor. A soluble form of B7-H3 (sB7-H3) that results from MMPs cleavage of the surface protein was detected in sera of tumor patients and appeared to correlate with tumor burden (65, 66). Moreover, B7-H3 is present in NB cell lines-derived exosomes (67). The exosome-associated form could inhibit NK cell function by engaging the B7-H3-specific inhibitory receptor (**Figure 2B**). Thus, B7-H3 is an interesting NB-associated molecule that combines immune-evading and tumor progression properties. This correlates with the negative prognostic value associated to high expression of B7-H3 in several tumor types (64, 66, 68–72). In particular, in primary NB (73), high B7-H3 surface intensity and percentage of positive cells correlated with poor event-free survival. Interestingly, the differences in event-free survival were observed also in patients with localized disease (stage 1–3), suggesting the need of a more careful follow-up/aggressive treatment of B7-H3$^+$ low-risk patients. It should be mentioned that, in the mouse system, B7-H3 has been described as a "friend" in tumor immunology (74). In particular, intratumoral injection of an expression plasmid encoding mouse B7-H3 led to complete regression of 50% of tumors, which was mediated by NK and CD8$^+$ T cells (75). It is to note that the mouse *B7-H3* gene codes for a molecule characterized by two Ig-like domains (2Ig-B7-H3) in the order IgV and IgC, while the predominant isoform in human tissues and cell lines is a longer molecule (4Ig-B7-H3) characterized by four Ig-like domains (IgV–IgC–IgV–IgC), which results from duplication of the exons encoding the IgV and IgC domains (76). The mouse B7-H3 has been shown to bind the TREM-like transcript 2 (TREML2, TLT-2) triggering receptor, which is expressed by myeloid cells, CD8$^+$ T cells, and activated CD4$^+$ T cells (77, 78). In humans, TREML2 does not appear to be a receptor of B7-H3 (79) and the predominant inhibitory role of B7-H3 strongly suggests the existence of a (still undefined) receptor that down-regulates NK cell function. However, the existence in the human system of specific receptors with opposite functions cannot be excluded. Indeed, human B7-H3 belongs to the B7 family that includes members such as B7–1 (CD80), B7–2 (CD86), B7-H2 (ICOS-L), B7-H1 (PD-L1), B7-DC (PD-L2), B7-H4 (B7S1, B7x), and BT3 (80), some of which represent specific ligands of receptors with either activating or inhibitory function. The *scenario* should become even more complex when considering that the B7-H3 inhibitory and activating receptors might be co-expressed in immune cells rather than expressed in different cell types/subsets.

TUMOR ESCAPE MECHANISMS IN NEUROBLASTOMA AND POSSIBLE NOVEL NK CELL-BASED IMMUNOTHERAPEUTIC APPROACHES

In principle, downregulation of HLA class I surface expression allows NB to evade T cell-mediated attack operated by the host immune system (36, 37), while rendering tumor cells susceptible to NK cell-mediated recognition and killing (31). However, both the fast tumor progression in high-risk NB patients and the *in vitro* data highlighted the existence of additional escape mechanisms that dampen the NK cell-mediated anti-tumor activity as well. As discussed above, the lack of ligands for activating receptors such as PVR (36) (**Figure 2A**), MICA/ULBPs (36, 37), or adhesion molecules (36, 81) represents a strategy allowing NB cells to strongly reduce their susceptibility to NK-mediated killing. Thus, one could set up immunotherapeutic interventions aimed at upregulating the expression of these ligands. However, the role of PVR in tumor progression (59), as well as its expression in normal cells, should not be underestimated. In particular, DNAM-1 co-operates with NKp30 or NKp46 in the NK-mediated killing of autologous immature DC and unpolarized (M0) or M2-polarized macrophages, which express low, "non-protective" amounts of HLA class I molecules. Conversely, mature DC and M1-polarized macrophages express high levels of HLA class I molecules and are protected by the NK cell-mediated attack (54, 82). The NK-mediated selection of DC and macrophages with optimal antigen-presentation properties has been named "immunoediting" and results from the fine-tuning of inhibitory and activating signals provided by the ligands physiologically expressed by DC and macrophages. The forced over-expression of ligands for activating receptors, such as PVR, might dangerously alter the immunoediting process. Moreover, it should be taken into account that PVR is expressed in normal endothelium, where it plays a role in leukocyte extravasation (83, 84). Also in this case, up-regulation of PVR expression might overcome the HLA class I-mediated inhibitory signals and result in loss of self-tolerance toward autologous endothelium. On the other hand, it has been shown in primary glioblastoma that endothelial cells of proliferating tumor vessels express PVR at higher surface densities as compared to normal vessels (22). Moreover, several studied indicated that most tumor cells including CSC constitutively express levels of PVR higher than the normal counterparts (21). Although further studies are needed to dissect the possible side effects, therapeutic approaches focused on PVR might represent a chance in the treatment of highly aggressive tumors such as NB. Up-regulation of PVR in NB patients might increase the susceptibility to NK-mediated lysis of PVR$^+$ NB and restored that of PVR$^-$ NB. In this context, it is of note that the lack of PVR expression was detected in given BM metastatic neuroblasts (36) while no data are available on PVR expression in primary tumors. Thus, it cannot be excluded that PVR$^-$ NB cells might originate because of the selection pressure occurring during the dissemination process and that primary tumors could contain PVR$^+$ targetable neuroblasts. If so, a larger cohort of patients might benefit from PVR up-regulation therapies. Importantly, PVR might represent a powerful therapeutic target on both neuroblasts and NB-associated vessels. PVR has been originally identified as the receptor for the poliovirus, a highly contagious

virus that only affects humans. As already mentioned in a study performed in a poliovirus-susceptible animal model, the oncolytic treatment by a novel attenuated poliovirus eradicated PVR^+ NB cells without signs of paralysis (85).

B7-H3 might represent an additional attractive molecular target in NB. Beside its tumor-promoting properties, human B7-H3 represents a shield protecting HLA class $I^{low/-}$ neuroblasts from the NK cell-mediated attack (60, 64) (**Figure 1**). B7-H3 is expressed in normal endothelium, but is strongly upregulated in tumor-associated vasculature (86). Encouraging results derived from the first in-human intrathecal injection of radioiodinated anti-B7-H3 Ab (following surgery, craniospinal irradiation, and chemotherapy) in 21 NB patients with recurrent CNS metastasis (87). Seventeen patients remained CNS disease free and had a median survival time (33 months) significantly better than patients treated with standard protocols (6.6 months) (88). Anti-B7-H3 antibodies, possibly delivered using lipid-based (liposome) formulations (89–91), might represent a suitable option to target tumoricidal compounds to NB and to increase immune responses (**Figure 3**). Indeed, acting on different cell types, it might simultaneously block invasiveness/migration of tumor cells, destroy tumor-associated vessels, and strength the activity of NK cells. Moreover, anti-B7-H3 antibodies might induce NK cell-mediated ADCC against NB and destroy the B7-H3/inhibitory receptor interactions. An alternative approach to trigger NK cell function might consist in the use of bi-specific antibodies reacting with B7-H3 (on tumor cells) and activating receptors (on NK cells). In addition B7-H3 might be considered as a molecule alternative to GD2 in CAR-based immunotherapy (**Figure 3**). CAR are chimeric antigen receptors, formed by tumor-specific Ab single chain Fv fragments (scFvs) genetically fused through a transmembrane domain to the CD3ζ chain, which are transfected into cytolytic lymphocytes (92, 93). The use of CD19-specific CARs resulted in a significant clinical benefit in two children with B-cell precursor acute lymphoblastic leukemia (94). The NK92 cell line engineered to express the GD2-specific CAR showed increased cytolytic activity against NB cell lines and primary NB cells (95). Moreover, T lymphocytes equipped with GD2-specific CAR are being used in preclinical and phase I studies with clinical benefit in NB patients (96, 97). Essential requirement in CAR-based immunotherapy is the selection of an adequate tumor surface antigen with a documented tumor-promoting role (92). B7-H3 satisfies this criterion, thus representing a suitable candidate in CAR-based immunotherapy. Moreover, while GD2-specific CAR target a molecule that is easily shed by the NB cell surface (98), B7-H3-specific CAR would target a stable type I transmembrane surface molecule expressed by NB cells and NB-associated vessels. Infusion of engineered and/or activated (by cytokine or DC) (99) NK cells might be helpful in adoptive immunotherapeutic approaches of high-risk NB patients receiving autologous (5) or haplo-HSCT. Indeed, a gap of several weeks exists between the infusion of $CD34^+$ stem cells and the appearance in peripheral blood of fully "armed" mature NK cells (38). During the early post-transplant period, lack of NK-mediated immunosurveillance could favor re-growth of residual NB cells escaping the preparative regimen to the allograft, this causing tumor relapses. Haplo-HSCT, which represents a new standard therapy in the cure of adult and pediatric leukemic

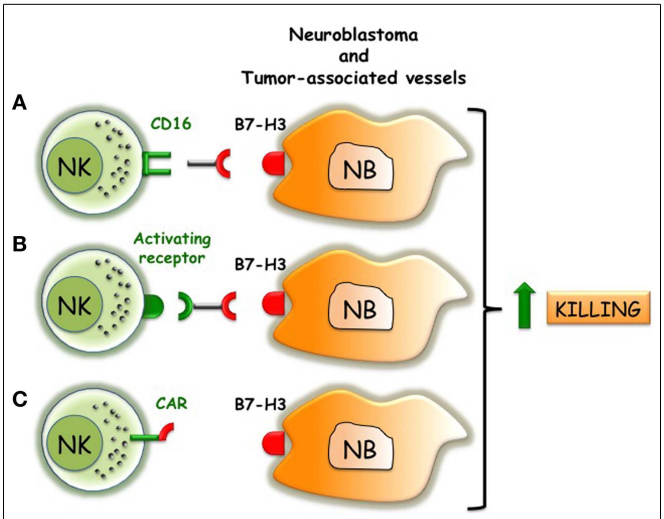

FIGURE 3 | Possible immunotherapeutic approaches that target B7-H3 and trigger NK cell function. **(A)** Anti-B7-H3 antibodies induce ADCC of NK cells against NB. **(B)** Bi-specific antibodies that react with B7-H3 on NB and trigger activating receptors on NK cells. **(C)** NK cells engineered with B7-H3-specific CAR that transduce activating signals via CD3 chain.

patients, could also increase the event-free survival of NB patients (45). Recently, a novel transplant strategy based on negative depletion of both α/β T lymphocytes and of CD19+ B-cells has been proposed for pediatric hematological disorders. This approach leaves in the graft an array of immune cells with anti-tumor properties, including mature armed donor-derived alloreactive NK cells and γ/δ T cells (38). Also in this type of transplant that preserves donor mature NK cells in the graft, engineered NK cells might support the anti-tumor responses. In the perspective of *in vivo* NK-based adoptive immunotherapies as adjuvant in different transplant strategies, recent studies optimized the *in vitro* expansion and activation of NK cells that preserved the anti-NB activity (100–103).

In addition to the escape mechanisms described above (47, 60, 104), NB cells produce soluble factors such as MIF (105, 106) and TGF-β (107) capable of dampening NK cell activity (108, 109) (**Figure 2B**). Initial data showed that *in vitro* conditioning of NK cells with rTGF-β1 resulted in downregulation of NKp30 and NKG2D activating receptors and in significant reduction of NK-mediated killing (109). Different studies confirmed *in vivo* the role of TGF-β in regulating NKG2D expression and function in NK cells (110, 111). Although knockout mice lacking TGF-β1 or TGF-β2 showed distinct phenotypes suggesting that each isoform could also have specific, non-overlapping functions (112), *in vitro* high concentrations of both rTGF-β1 and rTGF-β2 (>10 ng/ml) showed similar capability of inhibiting the expression of the activating NK receptors (113). It has been shown that NB cell lines released low/medium amounts of TGF-β1 (<3 ng/ml), which were unable to significantly downregulate NKp30 and NKG2D expression while clearly modulated the chemokine receptor repertoire of NK cells (113). Interestingly, non-MYCN-amplified NB cells produced amounts of TGF-β1 (and several other soluble factors)

significantly higher than MYCN amplified cells (113). NB-derived TGF-β1 upregulated the expression of CXCR4 and CXCR3 in all NK cells and downregulated that of CX3CR1 in the $CD56^{dim}$ subset. Notably, a similar altered chemokine receptor repertoire was observed in peripheral-blood NK cells of stage 4 NB patients resulting in the appearance of a $CX3CR1^{low}$ $CD56^{dim}$ NK cell population. In patients, also a tendency to CXCR3 upregulation on $CD56^{bright}$ NK cells was appreciated. No significant differences in CXCR4 expression were detected in NK cells from NB patients as compared to healthy donors. This might be due to a general difficulty in detecting *in vivo* CXCR4 expression, which in particular in the BM, might be fully occupied by the CXCL12 (SDF1) ligand (114). In NK cells from NB patients, also a significant CXCR1 down-modulation was observed, that, however, was TGF-β1-independent (113). Thus, *in vivo*, NB cells can release TGF-β1 and other, still unknown soluble factor(s) that profoundly affect the expression of a number of chemokine receptors that play pivotal roles in NK cell BM homing, egress, interaction with endothelium, and recruitment into peripheral tissues. High concentrations of TGF-β1 might locally exert a paracrine effect on tumor-associated NK cells that might decrease their anti-tumor activity by downregulating NKp30 and NKG2D expression. In this context, it has been shown that $CD56^{bright}$, immature, poor cytolytic NK cells represent the major subset present in tumor tissues (115, 116). The above data suggest that TGF-β1 antagonists and/or proinflammatory cytokines capable of overcoming the modulatory effect of TGF-β might represent a reasonable adjuvant therapy in the cure of different tumors, including NB. In this context, mouse models showed that use of IL-2 targeted to GD2 is associated with increased infiltration of NK (and CD8+ T cells) in subcutaneous NB (117).

Novel immunotherapeutic approaches should also consider the complexity of tumor microenvironment that is populated by different immune cell types that could be endowed with immunomodulatory functions. These cells include fibroblasts (118), regulatory T cells (Treg), myeloid derived suppressor cells (MDSC) (119), and tumor-associated macrophages (TAM) (120). Different NB mouse models demonstrated the immunosuppressive and tumor-promoting role of Treg (120) and MDSC (121). To date, however, data on their presence within the human NB tumor and on their clinical relevance are still scarce. A recent study showed that metastatic NB have higher infiltration of TAM than locoregional tumors, and that metastatic tumors in patients $\geq$18 months have higher expression of inflammation-related genes than those in patients <18 months (122). TAM are characterized by an "M2-like" functional phenotype and exert tumor-promoting and immunoregulatory properties, including the release of TGF-β1 (120) (**Figure 2**). M2-polarized macrophages still present a functional plasticity and they can be recommitted toward an M1-like tumor suppressing, immunostimulatory phenotype. In particular, it has been shown that microbial products such as LPS or BCG reverted their functional phenotype toward M1, which released immunostimulatory cytokines (IL-12, IL-18) and induced strong activation of autologous NK cells. NK cells upregulated CD69 and CD25 (that associates with CD122 to form the high-affinity receptor for IL-2), expressed CCR7 (a chemokine receptor involved in homing of NK cells to secondary lymphoid organs), increased the anti-tumor cytolytic activity, and released high amounts of IFN-γ (82). Interestingly, it has been shown that a subset (30–40%) of M2 express a membrane-bound form of IL-18 (mIL-18) that is released upon TLR stimulation (123, 124). This soluble form of IL-18 (sIL-18) by acting in close cell-to-cell contact is crucial for both IFN-γ release and expression of CCR7 by NK cells. These data suggest that reconverting the immunosuppressive TAM phenotype using apathogenic TLR ligands might represent an additional immunotherapeutic approach to fully activate immature tumor-associated NK cells.

CONCLUSION

Progress in the understanding of NB cell biology will allow a more accurate stratification of patients, thus reducing toxic side effects of aggressive therapy in low-risk patients. High-risk patients who currently have a dismal prognosis could benefit from multidisciplinary therapeutic protocols that include novel NK cell-based immunotherapeutic strategies. The latter will take advantage of our knowledge about the presence/absence of NB-associated ligands interacting with activating/inhibitory receptors expressed by NK cells. Moreover, it should also take into account the multiple immunomodulatory strategies set up by NB and various immune cell types to impair the recruitment and activation of NK cells in the tumor microenvironment. The encouraging results emerged from haploidentical hematopoietic cell transplantation in pediatric hematological malignancies, might strongly motivate a re-evaluation of transplant approaches in the therapy of high-risk NB patients.

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Natural killer cells modulation in hematological malignancies

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Hematological malignancies (HM) treatment improved over the last years resulting in increased achievement of complete or partial remission, but unfortunately high relapse rates are still observed, due to remaining minimal residual disease. Therefore, sustainment of long-term remission is crucial, using either drug maintenance treatment or by boosting or prolonging an immune response. Immune system has a key role in tumor surveillance. Nonetheless, tumor-cells evade the specific T-lymphocyte mediated immune surveillance using many mechanisms but especially by the down-regulation of the expression of HLA class I antigens. In theory, these tumor-cells lacking normal expression of HLA class I molecules should be destroyed by natural killer (NK) cells, according to the missing-self hypothesis. NK cells, at the frontier of innate and adaptive immune system, have a central role in tumor-cells surveillance as demonstrated in the setting of allogenic stem cell transplantation. Nevertheless, tumors develop various mechanisms to escape from NK innate immune pressure. Abnormal NK cytolytic functions have been described in many HM. We present here various mechanisms involved in the escape of HM from NK-cell surveillance, i.e., NK-cells quantitative and qualitative abnormalities.

Keywords: hematological malignancies, natural killer cells, natural cytotoxicity receptors, immune escape, immunotherapy

INTRODUCTION

The natural killer (NK) cells are central players in innate immunity particularly regarding the surveillance against malignant tumors (1, 2). NK role in tumor-cells clearance is proved by allogenic stem cells transplantation, since a better engraftment and a low relapse rate are observed when the graft NK inhibitory receptors mismatch with recipient HLA molecules (3) (**Figure 1**). The triggering event of NK-cells activation and killing of target cells results from a balance between activating and inhibitory signals sent by membrane receptors that either enhance or block the NK-mediated cytotoxicity (4). Inhibitory signals arise from interaction between HLA-specific inhibitory receptors, as the killer immunoglobulin-like receptors (KIR), NK group protein 2A (NKG2A), or Immunoglobulin-like transcript 2 (ILT-2) with HLA class I molecules, whereas the absence or abnormal expression of the later molecules induces NK-cells cytotoxicity (5). The down-regulation of HLA class I molecules is an immune escape mechanism frequently used by tumor cells (6) that, accordingly, should not be recognized by the T-lymphocyte receptor (TCR). The absence of normal HLA class I molecule on tumor cells should lead to NK-cells activation, more efficiently when co-stimulatory molecules and ligands for NK activating receptor are present at tumor cell surface. The natural cytotoxicity receptors (NCR) NKp46/NCR1, NKp30/NCR3, and NKp44/NCR2 (7), NKG2D, DNAM-1 and also co-receptors such as 2B4/CD244 and NTBA, play a central role in NK activation. Once activated, NK lymphocytes kill tumor cells via FcgRIIIA (CD16) which can trigger antibody-dependent cellular cytotoxicity (ADCC) on encountering target cells opsonized with IgG, via the Fas/Fas-L pathway and via cytotoxic granules (perforin/granzyme) secretion (1, 8).

Defects in NK-cell cytotoxicity have been described in most hematological malignancies (HM) (9–12) (**Table 1**). Interestingly, tumor-cells develop various escape mechanisms to NK-cell surveillance and contribute to the dysfunction of NK-cell cytotoxicity (4).

NK-CELLS DYSFUNCTIONS IN HEMATOLOGICAL MALIGNANCIES

QUANTITATIVE ABNORMALITIES

The first mechanism explaining tumor escape is the quantitative abnormalities of NK cells. In myelodysplastic syndrome (MDS) patients, decreased NK-cell cytolytic functions correlate with a low number of circulating NK cells and a high level of sIL-2R (13, 14). In chronic myelogenous leukemia (CML), functional NK-cell deficiency can be reversed *in vitro* by IL-2 (15), but this effect is progressively lost while a progressive decrease in NK-cell number is observed (16). In Philadelphia (Ph1)-negative myeloproliferative syndrome (MPS), NK cytotoxic activity is decreased, mostly in idiopathic myelofibrosis (IMF) patients. The percentage of NK cells is decreased in IMF and increased in polycythemia vera (PV) (17). We have confirmed that the percentage and absolute number of NK cells are significantly increased in PV, but we failed to detect any abnormalities in the expression of activating NK-cell receptors or cytotoxic functions (personal data, C. Sanchez). An

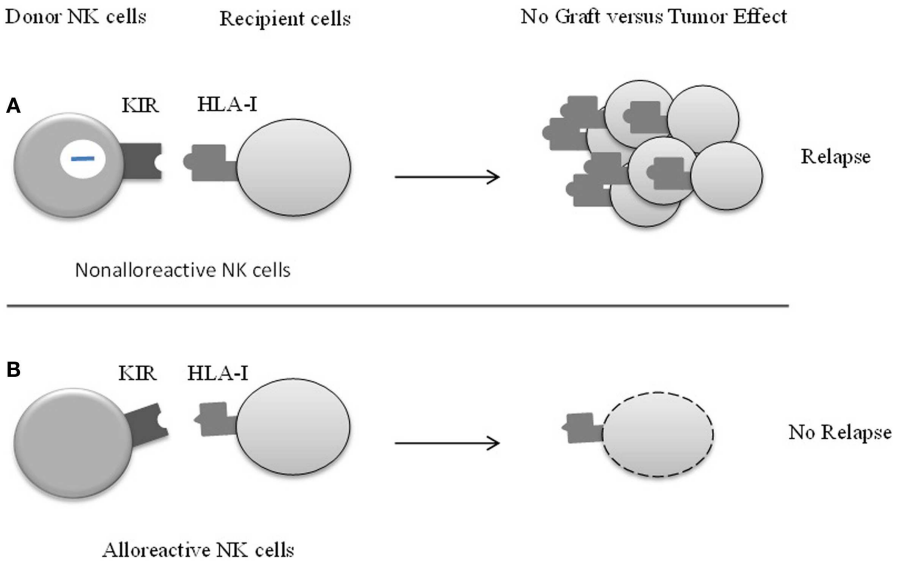

FIGURE 1 | The effect of KIR/HLA class I mismatch in patients undergoing allogenic stem cells transplantation (HSTC). The main challenge in HSTC is to keep the balance between conserving the graft versus leukemia (GvL) effect on the one hand and preventing risk of relapse on the other. **(A)** Killer cell immunoglobulin-like receptor (KIR) of donors NK cells is engaged by corresponding HLA ligand, which inhibits NK-cell function. Donor NK cells are non-alloreactive and do not kill recipient's blasts, which leads to the relapse of patient. **(B)** The concept and recognition of KIR-ligand incompatibility (also known as KIR-ligand mismatch) has important implications. KIR-mismatch is an independent predictor of survival in patients. Graft versus tumor NK-cell alloreactivity reduces the risk of leukemia relapse, and markedly improves event-free survival.

increase in the total number of NK cells in the peripheral blood has also been described in chronic lymphocytic leukemia (CLL) but still associated with defective cytolytic functions (18).

ALTERED ACTIVATING RECEPTORS PROFILES

In acute myeloid leukemia (AML) the down-regulation of activating receptors NKp30/NCR3 and NKp46/NCR1 correlates with defective NK-cell cytotoxicity and poor leukemia prognosis (9, 19). In patients attaining complete remission (CR) after chemotherapy, NKp46/NCR1 expression returns to normal levels while patients who do not achieve CR or who relapse maintain abnormal NCR expression (9, 19). The defect in NCRs expression could be potentiated by the low expression of NCR and NKG2D ligands by leukemic cells (20–22). Down-regulation of the NK activating receptors/co-receptor DNAM-1, 2B4/CD244, and CD94/NKG2C have also been reported in AML (23, 24). Leukemic blasts that express DNAM-1 ligands induce DNAM-1 down-regulation at the NK-cell surface (25), thus impending NK-cell functions.

In acute lymphoblastic leukemia (ALL), expression of the NKG2D activating receptor ligands MICA/B was only observed in NK sensitive T-ALL cell line, while NK-resistant B-ALLs did not express detectable amounts of MICA/B (26). Deficient engagement of other activating receptors may also contribute to ALL resistance to NK lysis, since B-ALL cells lose or express low levels of several other NK activating ligands such as UL-16 binding proteins (ULBPs), PVR (polio virus receptor, CD155), Nectin-2 (CD112), or CD48 (27).

In MDS, a pre-leukemic stage, Epling-Brunette et al. (13) have shown that expression of NKp30/NCR3 and NKG2D was decreased, in contrast with the data of Kiladjian et al. (28); this discrepancy could be related to the heterogeneity of MDS patients.

In CML patients, Boissel et al. (29) reported high serum sMICA levels and weak NKG2D expression on NK cells, that correlate with low NK-cell cytotoxicity capacities. Imatinib mesylate, the first inhibitor of tyrosine kinase used in CML, increases NKG2D expression and decreases MICA protein production and release, thus contributing to normal NK cytotoxicity through the restoration of a functional NKG2D signaling (29).

Monoclonal gammopathy of undetermined significance (MGUS) is a common disorder of aging and a precursor lesion to multiple myeloma (MM). In MGUS, tumor-cells express high levels of MICA, whereas low levels of sMICA are detected in peripheral blood (30). This explains the capacities of NK cells to kill MGUS tumor cells by interaction between MICA and NKG2D. Conversely, MM patients present high plasma level of sMICA while tumor-cells express low level of MICA, thus impending NK stimulation via NKG2D (31). This reveals that the alterations in the NKG2D pathway signaling are associated with the progression from MGUS to MM (30, 32). In peripheral blood from patients with MM a normal expression of the NCRs and NKG2D is observed, while 2B4/CD244 and the low-affinity Ig- Fc receptor CD16 display significantly weaker expression in comparison with healthy donors (31). Nonetheless, when NK are studied at the site of tumor location, i.e., bone marrow, (33) a drastic down-regulation of three major activating NK receptors (NKp30/NCR3, NKG2D, and 2B4/CD244) is observed in comparison with bone marrow from healthy donors (34). This suggests that some NK abnormalities may be underestimated if only peripheral blood is studied.

Table 1 | Mechanisms of immune escape and drugs used with their effects on NK cells in hematological malignancies.

Hematological malignancies	Principal mechanisms of escape	References	Principal class of drugs used	Effects of drugs on NK activation	References
MDS	NK-cell quantitative deficiency Elevated TNF	(13, 14, 49)	Demethylating agents IMIDs	Up-regulation of KIR and NKG2D ligands NKp46 upregulation	(76, 81)
AML	Upregulation of HLA-I Decrease expression of NKp30, 2B4/CD244 Production of ROS	(9, 19, 39, 40, 54)	HDACIs Histamine dihydrochloride All-trans retinoic acid Monoclonal antibody (IPH2101)	Upregulation of MICA/B expression Suppress ROS production Promote NK-cells cytotoxicity	(65, 67, 72, 73, 84, 85)
CLL	Upregulation of HLA-I low level of MICA or ULBP	(18, 37)	Monoclonal antibody	Mediate NK-cells cytotoxicity	(88)
MM	Production of sMICA/sMICB Weak expression of CD16	(30, 31)	IMIDs Proteasome inhibitor IPH2101	NKp46 upregulation NKG2D ligand upregulation	(79, 82–84, 86)
CML	Production of sMICA and weak expression of NKG2D	(29)	Tyrosine kinase inhibitor	sMICA down-regulation and NKG2D upregulation	(29)
ALL	Low production of MICA/B and ULBP Down regulation of HLA-A and HLA-Bw6	(26, 27, 41)			

MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; s/MICA/B, soluble/stress-induced molecules human class I-like molecules A and B; ULBPs, UL-16 binding proteins; ROS, reactive oxygen species; KIR, killer immunoglobulin-like receptors; PDGF, platelet-derived growth factor; HDC, histamine dihydrochloride; IMIDs, immune-modulatory drugs; HDACIs, Histone deacetylase inhibitors.

In CLL, NK cells have weak cytotoxic functions which can be restored by stimulation with recombinant IFN-γ or IL-2 (35). We failed to detect a difference in NCR expression between patients and age-matched healthy donors (36), but we found a correlation with abnormal activating molecule expression and poor prognosis factors. In CLL decreased NK functions could be explained in part by absent or low level of MICA or ULBP (18, 37) at CLL lymphocyte cell surface, thus impeding NKG2D engagement.

ABNORMAL KIRs PHENOTYPE AND INHIBITORY MOLECULES

Killer immunoglobulin-like receptor-mismatch in allogenic stem cells transplantation improves the disease-free survival in AML (38). In fact, NK-cell cytotoxicity is down-regulated by the engagement of HLA-specific inhibitory receptors, including KIRs and CD94 and NKG2A/B heterodimers. The analysis of KIR phenotype in AML patients shows that the frequency of particular inhibitory KIRs in association with their putative HLA class I ligands is significantly increased compared to healthy donors (10, 39, 40). This supports the hypothesis that AML blasts escape from immune surveillance according to the dominance of inhibitory over activating KIR signals. In ALL, the resistance of B-cell precursors to cytotoxicity is explained by the interaction between HLA-G and KIR2DL4 (26). Demanet et al. (41) have observed in ALL and CLL a selective down-regulation of HLA-A and HLA-Bw6 associated with HLA-Bw4 preservation, which provided an escape mechanism from NK-cell immune surveillance. Maki et al. (18) reported in CLL cells an increased expression of HLA-G1, a class I molecule that engages NK-cell inhibitory molecules and which has for ligand p49/KIR2DL4/CD158d (expressed on NK cells and a fraction of T cells), ILT-2 [expressed on NK, T, B cells, dendritic cells (DC), and monocytes], and ILT-4 [expressed on antigen-presenting cells (APC)] (42).

TUMOR ENVIRONMENT AND ROLE OF CYTOKINES

One of the strategies used by tumor cells having an effect on NK-cell function is the production of inhibitory molecules, which decrease NK-cells number and inhibit NK-cell activation. Increasing evidence supports the role of the tumor microenvironment in conferring drug resistance, a major cause of relapse and incurability of cancers. Tumor microenvironment includes tumor-cells contact and interaction, but also production of soluble factors, which provide signals for tumor growth and survival or inhibition of NK.

ROLE OF CYTOKINES AND CELLULAR LIGANDS

Several cytokines decrease NK-cell activation and cytotoxicity, such as the Transforming Growth Factor beta (TGF-β). High circulating TGF-β level correlates with poor prognosis in acute leukemia (43) and is linked to reduced NK-cell activity with reduced expression of NKp30/NCR3 and NKG2D (44). TGF-β antagonizes IL-15, a cytokine that induces NK-cell proliferation and activation. Thus, TGF-β inhibits the expression of both NK-cell activation

receptor molecules and components of the cytotoxic apparatus (45, 46). Several studies have also underlined that low INF-γ producing capacity of NK cells was correlated with loss of NK-cell cytotoxicity (47, 48).

A physiologic concentration of platelet-derived growth factor (PDGF) significantly inhibits human NK-cell cytotoxicity. Patients suffering from IMF and essential thrombocythemia have significantly elevated circulating levels of plasma PDGF. Pretreatment of normal NK cells with concentrated PDGF-containing platelet-poor plasma from patients with these diseases significantly inhibits NK cytotoxicity. This inhibitory effect is reversed by neutralization of plasma PDGF with anti-PDGF (17, 49). All these data suggest that PDGF is probably a key factor of NK functional deficiency in MPS.

Interaction between tumor cells and NK impairs NK-cell-mediated cytotoxicity and thus induces tolerance to tumor invasion. Up-regulation of the immunosuppressive cell surface glycoprotein CD200 and of soluble GITRL (glucocorticoid-induced TNFR related protein ligand) on AML cells specifically compromises NK-cell anti-tumor responses (50, 51) and is a poor prognosis factor. AML cells exert direct immunosuppressive effects on NK cells mediated by immunosuppressive ligands or soluble factors and induce regulatory T lymphocytes (Treg) that weaken NK-cell responses (52). NK cells can also interact with DC leading to activation of Treg and inhibition of NK cells (53). Recently, ligand of NKp44/NCR2 (NKp44L) was identified as an isoform of mixed-lineage leukemia-5 (MLL5) (54). This ligand is not detectable in the normal tissues but is present in hematopoietic, non-hematopoietic tumor and transformed cells. The expression of MLL5 is a good prognosis factor in AML (55). Thus we can speculate that the prognostic value of MLL5 expression is linked to its capacity to activate anti-leukemia NK cells.

REACTIVE OXYGEN SPECIES

Non-malignant phagocytic cells down-modulate lymphocyte functions by producing and releasing NADPH oxidase-derived reactive oxygen species (ROS) (56). Monocytic and myelo-monocytic (French-American-British classification M4/M5 subtypes) AML cells, but not cells from myeloblastic (FAB class M2) or immature (FAB class M1) AML, produce ROS via the NADPH oxidase component gp91phox, and trigger extensive apoptosis of NK cells *via* a poly-[ADPribose] polymerase-1 dependent pathway, together with a down-regulation of NKp46/NCR1. This suggests a novel mechanism of immune evasion in myelo-monocytic and monocytic AML (57).

MATERIAL TRANSFER DURING CELL–CELL CONTACT

Tumor membrane-derived microvesicles (tMV) are important mediators of cell-to-cell communication. These circular membrane fragments are enriched in various bioactive molecules and directly stimulate cells as a kind of "signaling complex." An important mode of communication between carcinoma cells and immune cells involves tMV, also known as exosomes, ectosomes, or microparticles. These microvesicles carry lipids, proteins, mRNAs, and microRNAs and travel short or long distances to deliver undegraded and undiluted material to other cells (58). Microvesicles present in AML patients' sera contain TGF-β that down-regulates the expression of NKG2D and thus interfere with NK-cell activation. Nonetheless, IL-15 protects NK cells from adverse effects of tMV and could thus contribute to maintain their anti-tumor response (59). Baj-Krzyworzeka et al. (60) have observed that tMV carry mRNA of tumor cells and transfer some of them to monocytes and modify their activity. This type of mRNA transfer could participate to NK inactivation and tumor escape to innate immunity.

IMMUNOTHERAPY APPROACHES

Modulation of NK-cell function by down-regulation of receptors and/or ligand corresponds to an immune escape mechanism for tumors. Restoring the expression of activating receptors on NK cells, or corresponding ligands on cancer cells, is an effective approach to cancer immunotherapy in order to improve disease-free survival after therapy. Currently, in most AML patients, the induction treatment leads to CR, defined as microscopic disappearance of leukemic disease along with the return of normal hematopoiesis. However, many patients in CR relapse with poor prospects of long-term survival. One of developing immunotherapy that enhances NK-cell ability to kill tumor cells is the allogenic transplantation after chemotherapy. In AML, a recipient of haploidentical allogenic transplant with a NK HLA-specific receptor-mismatch is associated with a favorable prognosis because this increases the anti-leukemic graft reactivity (61, 62). Rapid recovery of NK cells after hematopoietic stem cell transplantation has been associated with a reduction in the rates of relapse and acute graft-versus-host disease (GvHD) (63). The transfer of NK cell from haploidentical origins into AML recipients is a potent immunotherapy intervention that is, unfortunately, associated with a significant transplant-related morbidity and mortality that limit its use (64).

The use of cytokines is another therapeutic approach to enhance NK-cell cytotoxicity. IL-2, IL-12, Il-15 (65), and IL-18 have been used in culture to increase cell cytotoxic prior to the injection of NK cells in cancer patients (66). Another strategy consists in restoring normal NCR expression since these molecules are pivotal for the anti-leukemia response. A phase III study in 320 AML patients has demonstrated that immunotherapy with histamine dihydrochloride (HDC) and IL-2 decreases and delays relapses in AML (67). HDC suppresses or inhibits ROS formation in mononuclear and polymorphonuclear myeloid cells. This prevents from oxygen radical-induced NK apoptosis, restores NK-cell capacity to respond to IL-2, and improves NK proliferation and production of immuno-stimulatory cytokines (56, 68–71). Moreover, in presence of HDC, cytotoxic functions of NK cells remain intact due to the preserved expression of the activating receptors NKG2D and NKp46/NCR1.

DNA methylation also has a key role in the control of gene activity in cancer cells. Two agents are currently used in MDS treatment: 5-azacytidine (Vidaza) and 5-aza-20-deoxycytidine (Decitabine) (72). These two hypomethylating agents up-regulate NKG2D ligands MICA/B leading to enhanced NK-cell cytotoxicity (73, 74). Conversely we have observed the down-regulation of 2B4/CD244 in NK from AML patients treated with 5-azacytidine, that could

have an opposite effect, i.e., cytotoxicity down-regulation (Leclerq et al. personal data).

The immune-modulatory drugs (IMIDs) such as thalidomide and lenalidomide are used in MM and MDS treatment and have anti-angiogenic and anti-inflammatory properties. They also act as IMIDs by cytokine release and activating effector cells by enhancing ADCC and NK-cell cytotoxicity thanks to the up-regulation of NKp46/NCR1 (75–77).

Bortezomib is a proteasome inhibitor used in MM treatment. Physiologically, proteasome is involved in protein degradation. Its inhibition by the drug interferes with tumor growth and with innate immunity. Bortezomib is involved in the down-regulation of HLA I molecules and in the up-regulation of NKG2D, TRAIL, and DNAM ligands, thus leading to increased NK-cell cytotoxicity against plasma cell (78, 79).

Another attractive therapeutic approach consists in blocking the NK inhibitory receptors. A phase 1 trial has tested the IPH2101, a fully humanized IgG4 anti-KIR monoclonal antibody, in patients with relapsed/refractory AML (clinical trial registration number NCT01256073) and MM (clinical trial registration number NCT00999830). IPH2101 promotes immune complex formation and NK-cell cytotoxicity specifically against MM cell targets but not normal cells. No evidence of autoimmunity was observed. These findings suggest that IPH2101 is safe and tolerable and that this approach warrants further development in MM and AML (80–82) as we are waiting for clinical results.

Several studies revealed ADCC as one major mode of action of antibody-based therapeutics and stimulated more interest in how to mobilize, expand, and activate NK cells in humans (83). In CLL, the ADCC pathway via the Fc receptor (FcgRIIIa) CD16 at surface of NK cells is pivotal in the clinical effect of mAbs such as rituximab or ofatumumab which mediate ADCC by NK cells (84). Second generation mAb are designed in order to maximize both direct apoptosis and ADCC. The role of ADCC is underlined by better clinical responses to rituximab when NK cells expressed the high-affinity form of the FcgRIII (85, 86).

Bi-specific NK-cell engagers (BiKE) simultaneously bind CD16α and c-Met (a receptor overexpressed in many tumors) and thus may increase NK-cell ADCC (87).

Another type of therapeutic strategy consists in taking advantage from anti-cancer drugs properties (**Table 1**). Chemotherapy drugs can be separated by their ability to inhibit (such as vinblastine, chlorambucil, docetaxel) or enhance (such as asparaginase, bleomycin, doxorubicin) NK-cell-mediated killing of target cells (88, 89). In cancer, epigenetic changes are also involved in dysregulating NK-cell ligand expression. Histone deacetylase inhibitors (HDACIs) such as trichostatin, are epigenetic anti-cancer agents that modulate innate immunity by the regulation of expression of NKG2D or DNAM-1 ligands. Indeed, HDACIs increase expression of ligands of these two activating receptors, MICA/B or PVR and Nectin-2 respectively, on acute leukemia cells (90, 91). This suggests that epigenetic drugs make tumor cells more sensitive to NK-cell-mediated lysis (92). However, it has also been demonstrated that HDACIs suppress NK-cell cytotoxic activity by down-regulation of NKp30/NCR3, NKp46/NCR1 (93). Thus, the same drug can have contradictory effects on NK cells.

CONCLUDING REMARKS

The more precise and exhaustive analysis of NK dysfunction in HM has opened the way to novel therapeutic strategies involving either specifically developed drugs/antibodies or innovative use of "old" drugs such as IMIDs. Due to the complexity of the immune response and the putative opposed effects of a drug on the various partners of the immune network, data obtained by *in vitro* experiments or *in vivo* in animal models have to be evaluated in clinically and biologically carefully monitored clinical trials.

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Improving the outcome of leukemia by natural killer cell-based immunotherapeutic strategies

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Blurring the boundary between innate and adaptive immune system, natural killer (NK) cells are widely recognized as potent anti-leukemia mediators. Alloreactive donor NK cells have been shown to improve the outcome of allogeneic stem-cell transplantation for leukemia. In addition, *in vivo* transfer of NK cells may soon reveal an important therapeutic tool for leukemia, if tolerance to NK-mediated anti-leukemia effects is overcome. This will require, at a minimum, the *ex vivo* generation of a clinically safe NK cell product containing adequate numbers of NK cells with robust anti-leukemia potential. Ideally, *ex vivo* generated NK cells should also have similar anti-leukemia potential in different patients, and be easy to obtain for convenient clinical scale-up. Moreover, optimal clinical protocols for NK therapy in leukemia and other cancers are still lacking. These and other issues are being currently addressed by multiple research groups. This review will first describe current laboratory NK cell expansion and differentiation techniques by separately addressing different NK cell sources. Subsequently, it will address the mechanisms known to be responsible for NK cell alloreactivity, as well as their clinical impact in the hematopoietic stem cells transplantation setting. Finally, it will briefly provide insight on past NK-based clinical trials.

Keywords: NK cells, NK-based immunotherapy, acute myeloid leukemia, NK cell expansion, hematopoietic stem cell transplantation

INTRODUCTION

Natural killer (NK) cells play a key role in the immune response to infections and malignancies by direct cytolysis of infected or transformed cells and by secretion of potent immune mediators. NK cell killing depends on the overall balance of inhibitory and activating signaling mediated by an array of surface receptors recognizing cognate ligands on putative targets. Besides their lytic activity, NK cells are cytokine-producing cells. Several reports highlight the fact that NK cells are also regulatory cells engaged in reciprocal interactions with dendritic cells, macrophages, T cells, and endothelial cells (1). NK cells have a central role in tumor-cell surveillance (2) as demonstrated in the setting of allogeneic hematopoietic stem cell transplantation (HSCT) (3–8).

Inhibitory NK receptors with specificity for HLA class I antigens are well described. Among these, killer Ig-like receptors (KIR) bind several HLA class I ligand groups (9), while the CD94–NKG2A/B heterodimer recognizes HLA-E (10). Several activating receptors are also described. Activating NKG2D is a major mediator of spontaneous cytotoxic activity. Natural cytotoxicity receptors (NCRs), including three molecules (i.e., NKp46, NKp30, and NKp44) specific for mostly unknown ligands (11, 12), mediate cell lysis of many cancer cells. Escape mechanisms from NK cell surveillance may involve NK cell quantitative deficiency and NK cell functional impairment.

Recent studies suggest that NK cell-based immunotherapy may continue to be an effective approach for patients with leukemia and emerging strategies are currently under investigation, initially based on adoptive transfer of NK cells.

This review will first describe current laboratory NK cell expansion and differentiation techniques by separately addressing different NK cell sources. Subsequently, it will address the mechanisms known to be responsible for NK cell alloreactivity.

NK CELL DIFFERENTIATION AND EXPANSION

NK CELL EXPANSION AND NK CELL GENERATION METHODS

Successful NK cell immunotherapies require an adequate number of NK cells. In addition to NK cell number, NK cell purity and function are key factors for a clinically efficacious NK cell product (13). Various methods of NK cell expansion have been explored (14). In general, NK cells can be produced from peripheral blood (PB), umbilical cord blood (UCB), bone marrow (BM), embryonic stem cells (hESC), or induced pluripotent stem cells (iPCS). While short-term NK cell culturing does not normally enhance the functional capabilities for *in vivo* transferred NK cells, long-term expansion methods may yield large numbers of functional NK cells, which may potentially benefit cancer patients (15). Several alternative protocols for NK cell *ex vivo* expansion for adoptive immunotherapy have been reported to date. However, only some

strategies have been developed under good manufacturing practice (GMP) conditions. In addition, substantial variability in NK cell expansion efficiency, phenotype, and function has been observed among different protocols and among individual donors (16–20).

EXPANSION OF NK CELLS FOR CLINICAL PURPOSES ISOLATED FROM PERIPHERAL BLOOD HUMAN

Several protocols for the expansion of PB NK cells are currently available, and others are under development. Various feeder cell-based systems have been used for NK cell expansion from peripheral blood mononuclear cells (PBMC), including third-party Epstein–Barr virus transformed lymphoblastoid B cell lines (EBV–BLCL), genetically modified K562 cells, or irradiated autologous cells (21–24). *Ex vivo* expansion of bulk peripheral NK cells using third-party EBV–BLCL feeders approximately yields a 180-fold NK cell expansion after 2 weeks of culture (22). Another expansion technique, yielding clinical valuable amounts of NK cells, is based upon K562 cell feeder double-transduced with IL-15 and 4-1BB (CD137) co-stimulatory ligand (K562–mb15–41BBL) (23). K562 cells transduced with IL-21 have also been used as feeder cells in NK co-culture systems (25). While K562–mb15–41BBL have been shown to expand and functionally enhance PB NK cells, K562 genetically engineered with membrane-bound IL-21 allow an even higher proliferation and cytotoxicity of expanded NK cells, which also display longer telomeres and less senescence (25). To expand CliniMACS-purified PB NK cells, autologous irradiated feeder cells have also been used as feeder cells in culturing systems containing human serum, IL-2, IL-15, and anti-CD3 antibody (21).

Many PB NK expansion strategies hold promise for NK-based immunotherapies. However, even using identical protocols, NK cell expansion yields and purity are typically inconsistent, and significant donor-to-donor variation is common. Moreover, complete absence of any residual viable tumor feeder in all final cell products is a critical requirement for large-scale NK cell therapy applications and their pharmaceutical translation.

The type of disposable cell culture systems for NK cell culturing also appears to influence the characteristics of the final cell product. Currently used disposable cell culture systems include flasks, bags, or WAVE® bioreactors. Compared to flasks, use of bioreactors allow a 10-fold higher NK cell expansion after 3 weeks of culture (26), at the expense of a reduced purity of the final product, which also contains T cells (CD3+/CD56−) as well as NKT cells (CD3+/CD56+). Presence of T cells limits the application of this cell product to the autologous setting in the absence of downstream T-cell depletion.

NK CELL GENERATION FROM UMBILICAL CORD BLOOD

Umbilical cord blood is thought to be an excellent source for cell therapy applications. Initial work on positively selected cord blood NK cells, cultured on a feeder layer of mesenchymal stromal cells using a combination of IL-2, IL-15, Flt-3L, and IL-3, resulted in a mere 60-fold median expansion (27). In consideration of the low starting NK cell number in standard cord blood units, this approach is not feasible to generate NK cell numbers needed for a therapeutic NK cell product. Additionally, NK cell differentiation from CD34+ hematopoietic stem cells (HSC) has been addressed (28). Initially, research in this field focused on the *ex vivo* generation of NK cells from BM CD34+ cells (29–33), but later also involved CD34+ cells derived from UCB (34–39), a particularly rich source of HSC. These studies used different combinations of growth factor and cytokine mixtures, BM stroma cells, and culture media with or without animal or human sera. These culture systems generally contain components of animal origin; moreover, they fail to yield significant numbers of mature NK cells. For these reasons, it is unlikely that they will be used for clinical applications. Interestingly, the system reported by Silva et al. contained human serum without stroma cells and was therefore favored for clinical scaling-up (40). This NK cell expansion method was later abandoned, once stroma cells were recognized as a potential critical component for the enhancement of NK cell proliferation (40). In 2007, Kao et al. showed that serum-free expanded CD34+ cells may be differentiated into a NK cell product with an average purity of 40–60% after 5–7 weeks of culture, with a mean expansion rate of 300-fold. Such expansion, however, was obtained in the presence of fetal bovine serum (41). Later on, Vitale et al. studied the effects of methylprednisolone on CD34+ precursor cells and their ability to differentiate toward NK cells (38). They performed direct NK cell differentiation – i.e., no upfront CD34+ expansion – using RPMI-1640 medium supplemented with human serum, fetal bovine serum, and a cytokine cocktail including IL-15 and IL-21. At day 25, they observed a 10-fold NK cell expansion, with an average purity of approximately 30%.

In 2010, a novel cell culture technology for the *ex vivo* expansion and NK differentiation of UCB-derived CD34+ cells was developed. This technique was based upon a clinical-grade serum-free culture medium and a mixture of heparin and cytokines, in order to mimic the extra-cellular matrix BM microenvironment in the absence of feeder cells (42). This method yielded up to 10^{10} CD34+ cell-derived NK cells, generated in static cell culture bags and automated bioreactors. Importantly, NK cell products were not found to be contaminated by T cells, and may therefore be safely used in the allogeneic setting (43). Additionally, NK cells expressed high levels of activating receptors (e.g., NKG2D and NCR), and are able to efficiently kill myeloid leukemia and melanoma cell lines, as well as primary leukemia blasts. Furthermore, mouse studies have shown that they expand *in vivo*, initially home to the lung, and later to the blood, lung, spleen, liver, and BM and prevent outgrowth of local tumors in the BM (44). A phase I trial in elderly acute myeloid leukemia (AML) patients using NK cell products based upon this expansion technique is currently in progress (CCMO nr. NL31699 and Dutch Trial Register nr. 2818) (43).

Definitely, producing NK cells from CD34+ hematopoietic precursors can be practically advantageous, since stem cells can be isolated and frozen, and can overcome several obstacles posed by purified PB NK cells (15).

NK CELL GENERATION FROM HUMAN EMBRYONIC STEM CELLS OR INDUCED PLURIPOTENT STEM CELLS

In contrast to the CD34+ UCB-derived NK cells, the generation of NK cells from embryonic stem cells or induced pluripotent stem cells is to date largely experimental (45). As previously mentioned, human NK cells can be differentiated from CD34+ HSC

under certain culture conditions (31, 35, 42). Currently, optimizing the generation of CD34+ HSC from hESC and iPSC remains a major challenge to rationally approach hESC- and iPSCs-based NK cell therapy (45–48). hESCs or iPSCs derived CD34+ cells are known to be enriched for hematopoietic progenitors defined by colony-forming cells, which could serve as a suitable source for cell therapy (47). Some years ago, a method for the efficient generation of functional NK cells from hESC using a two-step procedure was described. NK cell products contained cytotoxic cells, displayed a mature NK cell phenotype, including CD16 and KIR, CD94/NKG2A, NKp46, NKp44, NKG2D, TRAIL, and FasL (49).

Engineering CD34+ cells, hESCs, or iPSCs to express chimeric antigen receptors (CAR) specific for tumor-associated antigens is a potentially promising immunotherapeutic strategy (48).

NK CELL LINES FOR CLINICAL USE

Several authors have reported on the use of NK cell lines for therapeutic purposes. Unlike primary NK cells, whose *in vivo* life span is limited, NK cell lines are immortal. However, most NK lines are weakly cytotoxic to cancer targets. Conversely, the NK-92 cell line (Neukoplast, Conkwest, San Diego) has shown to mediate liable cytotoxicity and good expansion kinetics when cultured in bags or bioreactors. NK-92 has been well characterized in *in vitro* and shown to display remarkable anti-tumor activity in severe combined immunodeficiency (SCID) mouse xenotransplant models (50). Furthermore, it has been extensively tested in the clinical setting (51).

ALTERATION OF NK-MEDIATED CYTOTOXIC RESPONSE AGAINST LEUKEMIA

The escape of hematological malignancies from NK cell immunity can be explained by general mechanisms involving the quantitative deficiency of NK cells, their qualitative impairments caused by increased inhibition, decreased activation signaling, or by the negative influence of tumor microenvironment. It is widely recognized that leukemia cells may oppose a variety of immune escape strategies, including immune suppression and phenotypic mimicry, to elude NK-mediated killing.

The balance between activating and inhibitory signals received by NK cells will determine the NK cell-mediated elimination of leukemia cells. Decrease in ligands for activating receptors on leukemia cells could abolish NK cell dependent cytotoxicity. Furthermore, interaction of other ligands with cognate inhibitory receptors on NK cells surface could diminish NK cell killing activity, granule mobilization, and interferon production. Accumulating evidence indicates that chronic and acute leukemia cells could modulate NK cell activity by secreting soluble and exosomal ligands for NK cell receptors (52, 53). A key strategy used by leukemia cells to escape elimination by NK cells is related to apoptosis, and altered expression of molecules involved in different apoptosis signaling pathways may result in resistance to NK-mediated killing (54–56). In this setting, deregulation of the mitochondrial apoptotic machinery, functional blocks of caspase cascade, downregulation or inactivation of proapoptotic molecules, and upregulation of antiapoptotic molecules may all potentially favor leukemia chemoresistance or relapse. Finally, mechanism of NK cell resistance may also include drug resistance. For example, it has been shown that doxorubicin resistant cells increase expression of HLA class I on their surface (57).

CAPTURING NK CELL ALLOREACTIVITY IN HEMATOPOIETIC STEM CELL TRANSPLANTATION

In the last 15 years, growing knowledge of NK tolerance to self, cancer immunosurveillance, and functional licensing has been extensively applied to HCT, in an effort to identify donors protecting from leukemia relapse through NK-based alloreactivity. Most studies investigating NK effects on transplantation outcome are rationally based upon one of the following three models: the missing-self recognition paradigm, the missing ligand model, and the activating receptor-based NK cell alloreactivity. This section addresses the mechanisms known to be responsible for NK cell alloreactivity, as well as their clinical impact in the HSCT.

RECOGNITION OF HLA CLASS I ANTIGENS BY KILLER IMMUNOGLOBULIN-LIKE RECEPTORS

The KIR gene cluster is mapped on chromosome 19q13.4, within the 1 Mb leukocyte receptor complex (LRC). It contains four ubiquitous "framework" genes (one centromeric, *KIR3DL3*; one telomeric, *KIR3DL2*; and two central, *KIR3DP1* and *KIR2DL4*) that flank centromeric and telomeric gene motifs characterized by extensive variation in both gene content (type and number) and polymorphism (58–60). Based on *KIR* gene content, multiple KIR haplotypes are identified, and categorized into two distinct groups, A and B. Group A haplotypes contain genes exclusively encoding inhibitory receptors and the activating *KIR2DS4*, while group B haplotypes contain genes encoding both inhibitory and activating receptors. Both KIR A and B haplotypes possess centromeric and telomeric gene content motifs. Products of functional KIR genes are type I transmembrane receptors with two (KIR2D) or three (KIR3D) highly homologous, extra-cellular immunoglobulin domains (61, 62, 63). Due to their clonal distribution in the NK repertoire, an individual NK cell may express one or more KIR (64, 65).

Inhibitory KIR may recruit the SH2-domain-containing tyrosine phosphatase 1 protein (SHP1) (66–69) through a single or double immunoreceptor tyrosine-based inhibitory motif (ITIM) contained in their long cytoplasmic tail (denoted L, i.e., KIR2DL; KIR3DL). Of several known inhibitory KIR, 2DL1, 2DL2, 2DL3, and 3DL1 are particularly relevant for HLA class I recognition. KIR2DL1 is specific for HLA-C2 group antigens (sharing the Asn^{77}/Lys^{80} residues in the HLA-Cw heavy chain); KIR2DL2 and KIR2DL3 are specific for HLA-C1 group antigens (sharing the Ser^{77}/Asn^{80} in the HLA-Cw heavy chain) (70, 71); and KIR3DL1 is specific for HLA-Bw4 ligands, sharing a group of sequence motifs in residues 77–83 of the heavy chain of certain HLA-B and HLA-A alleles (72–74).

Killer Ig-like receptor mediated activating signaling has also been identified. Unlike inhibitory KIR, they possess truncated portions that transduce activating signals via tyrosine phosphorylation of DAP12 and other proteins (75–77). Couples of cognate activating and inhibitory KIR, sharing almost complete homology (95–99%) in their extra-cellular domains, are recognized (75). Thus, activating KIR2DS1, KIR2DS2, and KIR3DS1 are, respectively, cognate receptors for the HLA class I-specific inhibitory

KIR2DL1, KIR2DL2, and KIR3DL1. Counter-intuitively, the identification of natural ligands for activating receptors remains largely elusive. Ligands for activating KIR2DS2 and KIR3DS1 receptors have not been identified. While it cannot be excluded that these receptors recognize HLA class I/peptide complexes, current evidence indicates that they may not affect NK function by generating activating signaling when HLA-C1 and HLA-Bw4 are self-ligands. Unique among activating KIR, 2DS1 recognizes HLA-C2 group antigens, similar to its inhibitory homolog 2DL1.

THE MISSING-SELF RECOGNITION PARADIGM AND NK CELL ALLOREACTIVITY

Inhibitory KIR interactions with cognate HLA class I ligands play a critical role in NK cell education and tolerance to self. In normal individuals, NK cells commonly possess KIR repertoires including one or more KIR with ligand specificity for self-HLA class I ligands (65). It is generally believed that such NK cells are rendered functionally competent, or licensed, by continuous signaling generated by inhibitory KIR upon interaction with self-HLA class I antigens (78, 79). HLA class I is critical to maintain NK tolerance to self, and cells failing to express sufficient levels of HLA class I ligands are promptly cleared by NK-mediated cytotoxicity. This phenomenon, known as missing-self recognition, was first postulated in a report by Kärre et al. describing that lack of MHC class I (H2) antigen expression rendered mice lymphoma cells highly sensitive to NK-mediated rejection (80).

Natural killer cells from donor-derived hematopoietic progenitor cells quickly reconstitute in HCT recipients (81–83). In the HSCT setting, HLA class I ligands of donor origin are believed to drive functional licensing. Reconstituted NK cells expressing one KIR for HLA class I present in the donor display stronger *in vitro* responsiveness than NK cells expressing one KIR for HLA class I present in the recipient, but absent in the donor (84). Additionally, CD107a externalization and IFNg production of NK cells reconstituting in recipients of donor unrelated or UCB grafts is markedly increased if they express KIR for donor self (85). This donor HLA-based NK education model implies, that the size of licensed donor NK cell is shaped by the frequency of inhibitory KIR-positive NK cells combined with the presence of cognate HLA class I ligands in the donor. Thus, KIR2DL1$^+$ NK cells would acquire functional competence if donor is *HLA-C2*; KIR2DL2–3$^+$ NK cells if donor is *HLA-C1*; and KIR3DL1$^+$ NK cells if donor is positive for *HLA-A* or -*B* alleles possessing the Bw4 motif.

In HLA-mismatched HSCT, *HLA-C* allele groups (*C1* or *C2*), and/or the Bw4 epitope may be present in the donor and absent in the recipient. In this situation, the repertoire of licensed donor NK cells may include NK clones mediating missing-self allorecognition against host tissues. For example, KIR2DL1$^+$/KIR2DL2–3$^-$/KIR3DL1$^-$ clones from a *HLA-C2* positive donor may display allorecognition of missing self in a *HLA-C2* negative recipient. Recognition of missing self-HLA class I may improve the outcome of HSCT. T-cell-depleted haplotype-mismatched grafts from NK alloreactive donors mediate strong graft vs. leukemia (GvL) effects in AML recipients, allowing for lower risk of relapse and better survival (3, 4). While in haplotype-mismatched grafts, the effect of KIR-ligand incompatibility is well established, studies on mismatched unrelated HSCT reported conflicting results. In this setting, Giebel et al. showed that donor NK cell alloreactivity improves recipient survival (86). A protective effect of KIR-ligand incompatibility on post-transplantation relapse was later confirmed in myeloid malignancies (87) and multiple myeloma (88). However, most studies failed to show a beneficial effect of donor NK cell alloreactivity on the outcome of mismatched unrelated HSCT (89–92). Similarly, studies exploring the clinical impact of NK alloreactivity mediated by KIR-ligand mismatch in UCB grafts have yielded variable results (93, 94). These inconsistent observations are possibly influenced by complex variables, such as donor KIR genotype (95), disease category, type of conditioning T-cell depletion, post-transplantation immune suppression for graft vs. host disease (GvHD) prophylaxis. For example, T cells may dominate alloreactive phenomena in mismatched unrelated HSCT and counteract the clinical benefit of NK alloreactivity (91). Accordingly, *in vivo* T-cell depletion with anti-thymocyte globulin (ATG) has been shown (86, 88, 96) to enhance the favorable impact of NK cell alloreactivity on HSCT outcome.

THE MISSING LIGAND MODEL AND NK CELL ALLOREACTIVITY

Killer Ig-like receptors and HLA genes are mapped on different chromosomes, and segregate independently according to a Mendelian inheritance pattern. Therefore, certain individuals may have *KIR* genes but not the corresponding HLA/KIR-ligand groups (97). KIR receptors are clonally distributed on NK cell surface, allowing for the possibility, that subpopulations of NK cells exclusively express KIR with ligand specificity for non-self-HLA class I ligands. These NK cells are not classically licensed by self-HLA class I ligands during their development, and are believed to be hyporesponsive to stimulation in physiologic conditions. During post-transplant immune reconstitution, however, this non-licensed status may be transiently suspended during post-transplantation immune reconstitution, and effector functions could indeed be mediated by donor NK cells expressing KIR with ligand specificity for non-self-HLA class I. An important implication of the missing ligand model is that NK alloreactivity would be observed even in the absence of donor/recipient KIR-ligand mismatch, a necessary condition for missing self-mediated NK alloreactivity.

Several studies exploring the effect of the missing ligand model on HSCT outcome indicate, that donors possessing inhibitory KIR but not the corresponding HLA class I ligand do mediate beneficial NK effects in HLA-identical siblings or HLA-matched unrelated recipients (5, 98–100). Hsu et al. originally explored a cohort of 178 subjects receiving a T-cell-depleted graft from a HLA-identical sibling. In patients with AML and myelodysplastic syndromes (MDS), lack of one or more HLA ligand for donor KIR resulted in lower relapse and better survival (5). Following the identification of beneficial NK effects in the HLA-matched setting, the function of classically non-licensed NK cells has been directly explored in the context of post-transplantation reconstitution. In T-cell-depleted grafts from HLA-identical siblings, NK cells expressing KIR for non-self-HLA display strong IFNγ production and cytotoxicity to target stimulation during the first trimester post-transplantation (101). These findings have not been confirmed in a cohort of recipients of T cell-replete grafts from HLA-identical siblings. Here, reconstituted NK cells expressing KIR for non-self-HLA ligands displayed tolerance to self. Moreover, lack of self-HLA ligands

for donor inhibitory KIR was found to have no effect on HSCT outcome (102). Presence or absence of T cells in the graft may differentially affect self-tolerance of non-licensed donor NK cells post-transplantation. Regardless, the interpretation of these conflicting results demands further studies on tolerance to self of donor NK cells reconstituting in the HLA-identical host.

ACTIVATING RECEPTOR-BASED NK CELL ALLOREACTIVITY

Because ligands for most activating KIR are currently unknown, studies reporting associations between activating KIR and HSCT outcome are not generally supported by the identification of an underlying immunological background mechanistically explaining the observed NK-mediated alloreactivity. In a cohort of 65 graft recipients from HLA-identical siblings, donors with genotypes containing both *KIR2DS1* and *KIR2DS2* genes provided protection from relapse (103). Donor activating KIR was later found to control CMV reactivation post-transplantation. Recipients of T cell-replete grafts were found to have a remarkable reduction of the incidence of CMV reactivation, if donor possessed more than one activating *KIR* genes (104). Confirmation of the protective effect against CMV reactivation by donor activating *KIR* was concomitantly reported by another group (105).

In 2009, Cooley et al. investigated the effect of different donor KIR haplotypes in 448 AML recipients of unrelated T cell-replete HSCT. Recipients of KIR B/x grafts (i.e., homozygous or heterozygous for KIR B group haplotypes) displayed a higher 3-year-overall survival (6). In a cohort of 1086 AML recipients of unrelated grafts, the same group later compared the contribution to HSCT outcome of donor centromeric and telomeric group A and B KIR haplotypes. Donors homozygous for centromeric B gene content motifs (Cen B/B) most strongly associated with low risk of relapse and prolonged survival (7). Among activating KIR, activating KIR2DS2 is mapped on the centromeric region of several B group haplotypes, and may thus mediate the clinical benefit observed for Cen B/B donors through interaction with an unknown ligand expressed on leukemia cells (7).

Recent studies investigated the effect of telomeric activating *KIR3DS1* and *KIR2DS1* genes on transplantation outcome. Patients receiving unrelated grafts from *KIR3DS1* donors exhibited a lower risk for grade II–IV GvHD and mortality (8, 106). Activating KIR2DS1 is found in approximately 1/3 Caucasians, and commonly occurs in individuals positive for *HLA-C2* (*C1/C2*; *C2/C2*) (107–109). KIR2DS1 expression occurs in more than 20% NK cells (109), and 2DS1 single positive ($KIR2DS1^{SP}$) NK cells (i.e., lacking inhibitory KIR expression), may also be identified. In *HLA-C2* individuals, $KIR2DS1^{SP}$ NK cells may potentially display auto-reactivity to normal self-tissues. Compared with *HLA-C1* donors, $KIR2DS1^{SP}$ NK cells from *HLA-C2* homozygous individuals are hyporesponsive to a HLA-C2 positive target cell (108). Similarly, mice studies described hyporesponsiveness of activating receptor-positive NK cells resulting from *in vivo* chronic interaction with a viral ligand (110, 111). Recently, $KIR2DS1^{SP}$ NK clones displaying *in vitro* anti-HLA-C2 cytotoxicity have been identified in all *HLA-C* genotypes (112). In *C2:C2* individuals, these clones are significantly reduced in frequency. In contrast, anti-HLA-C2 reactive $KIR2DS1^{SP}$ clones from *C1:C2* individuals are common, and functionally indistinguishable to those obtained from *HLA-C2* negative (*C1:C1*) donors (112). These observations indicate that tolerance development is affected by HLA-C2 ligand expression density, and are consistent with the "functional NK plasticity" phenomenon described in mice studies (113, 114). The effect of donor *KIR2DS1* on HSCT outcome has been assessed in a cohort of 1277 AML recipients of unrelated HLA-matched or 9/10 mismatched HSCT. Patients receiving grafts from *KIR2DS1* donors displayed a decreased rate of leukemia relapse (8). In agreement with experimental findings (112), *KIR2DS1*-mediated protection was observed in *HLA-C1* (*C1:C1* and *C1:C2*) donors, but not in *HLA-C2* homozygous donors, presumably due to the strong tolerogenic effect on KIR2DS1-positive NK cells mediated by the *HLA-C2* homozygous genotype (8).

NK CELL THERAPY MODALITIES

Early studies by Miller et al. in 2005 have open the way to demonstrate the safety of adoptive transferred human NK cells in patients with AML. Human haplo-identical NK cells could be expanded *in vivo* and transferred in patients with poor prognosis AML. Patients had to be prepared with an immunosuppressive high dose alkylating and purine analog conditioning regimen they can promote recirculation of infused allogeneic NK cells. Further haplo-identical allogeneic NK cells infusions were applied in patients with poor prognosis Hodgkin lymphoma and various solid tumors, i.e., melanoma (115) and renal cell carcinoma. In order to promote NK cell expansion, a lympho-depletion preparative regimen is required for the patient in all studies reported. To date, adoptive immunotherapy with unstimulated or IL-2 activated NK donor NK cells infusion is used for patients undergoing haplo-identical hematopoietic progenitor cells transplantation to prevent from relapse. No serious and immediate adverse effects have been observed following allogeneic NK cells infusion.

Clinical scale protocols to collect, enrich, and expand purified NK cells are nowadays feasible from cord blood, human donor apheresis, and dedicated cell lines. These cell therapy procedures remain costly and time consuming. They need an experimental lab with GMP facilities together with the skills and allowance to perform the cell manipulation.

The currently defined cell product has optimized T-cell depletion to avoid GvHD and allogeneic NK cell expansion; but we are still lacking a clinical standardized procedure for the use of enriched and expanded NK cells (116).

The most common limitation of the procedure remains the inability of collected, enriched, activated, and expanded allogeneic NK cells to expand properly *in vivo*. At first, a huge loss of NK cells during the isolation procedure can be seen following T-cell depletion procedure. Then CD56+/CD3− NK cells may be rejected by cytotoxic T cells or suppressed by myeloid-derived suppressor cells or Tregs.

To date, ongoing clinical scale protocols aim to both select CD56+/CD3− NK cells and increase drastically the total number of NK cells. Another limitation comes from the use of high dose alkylating chemotherapy-based protocols prior to infusion of NK cells that can increase the toxicity and efficacy of NK cell-based immunotherapy. Infusion of allogeneic NK cells can

be performed following standard doses regimen together with immune modulation with steroids and IL-15.

In the field of BM transplantation, infusion of donor-derived stimulated and expanded CD56+/CD3− allogeneic NK cells may be used in repetitive injections to promote the GvL effect. Further studies have to be designed to enhance the NK cell therapy efficiency: improving the NK cell selection, processing, *ex vivo* expansion, better designing of lympho-depletion protocols for the patient and infusion rate of NK cells for a optimized control of residual leukemic cells.

CONCLUSION

Natural killer cells continue to attract a lot of interest in transplantation. However defects in NK cell cytotoxicity have been observed in all hematological malignancies and the escape of hematological malignancies from NK cell immunity can be explained by general mechanisms. The latter are common to all immune-effector cells involving the quantitative deficiency of NK cells, their qualitative impairments caused by increased inhibition, or decreased activation signaling.

Natural killer cell selection from leukapheretic products is a largely inadequate approach to obtain large numbers of cancer-reactive NK cells. Given the clonal and stochastic distribution of activating and inhibitory receptors on NK cell surface, pools of cancer-reactive NK cells generally represent a minority of the bulk $CD3^-$, $CD56^+$ population. Several methods for *ex vivo* NK cell expansion have been attempted to date. However, none of the described approaches have definitively proved able to effectively circumvent the issue of limited NK cell availability and efficiency. Although an important anti-tumor role for alloreactive NK cells has been shown in patients with AML either after stem-cell transplantation or adoptive transfer of haplo-identical NK cells, their clinical efficacy in human trials has been modest, presumably due to tumor escape by alteration of NK cell function and resistance to killing. In this respect, the ability to manipulate not only the balance of activating and inhibitory receptors on NK cells but also their cognate ligands, as well as the sensitivity of tumor cells to apoptosis, opens new perspectives in NK cell-based immunotherapy. Sensitization of tumor cells to activated cytotoxic lymphocytes by up-regulating either TNF death receptors or effector-activating ligands on tumor cells combined with immunotherapy has been pursued to overcome tumor-cell resistance and establishes an effective anti-tumor response. Another therapeutic approach to enhance NK cell cytotoxicity is the use of cytokines, such as IL-2, IL-12, and IL-18. Chemokine manipulation is also of interest as molecules may both attract NK to the tumor-cell microenvironment and stimulate their cytotoxic properties. Another attractive approach to enhance NK cytotoxicity is to use monoclonal antibodies (mAbs). IPH-2101, a fully human IgG4 anti-KIR mAb (developed by Innate Pharma) is currently being tested in phase I and II clinical trials in patients with AML and multiple myeloma (117). Its blockade of inhibition could allow NK cell activation when activating ligands are present on target cells. It should also be emphasized that bispecific mAbs directed against both the target cells and NK cells are also currently under investigation. Anti-CD20 mAbs that have enhanced affinity for CD16 have been also developed, and they are more effective at NK activation than rituximab (118).

One limitation of the use of NK cells is due to the great capacity of leukemic cells to escape to NK recognition and killing. This resistance remains indeed a drawback in immunotherapy of hematological malignancies. In this regard, adoptive transfer of polyclonal or clonal NK cells with mismatch NK inhibitory receptors and HLA class I ligands would produce GvL (GvHD) in the absence of GvHD. The simultaneous differentiation of the effective killer effectors, the boosting of NK function, their expansion, and the sensitization of leukemic targets will offer potential opportunities in the treatment of hematological malignancies. Therefore, how to expand UCB stem cells for optimal downstream generation of terminally differentiated NK cells potentially alloreactive to leukemic cells is at present very challenging.

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Cancer-induced alterations of NK-mediated target recognition: current and investigational pharmacological strategies aiming at restoring NK-mediated anti-tumor activity

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Despite evidence of cancer immune-surveillance, which plays a key role in tumor rejection, cancer cells can escape immune recognition through different mechanisms. Thus, evasion to Natural killer (NK) cell-mediated anti-tumor activity is commonly described and is mediated by various mechanisms, mainly cancer cell-induced down-regulation of NK-activating receptors (NCRs, NKG2D, DNAM-1, and CD16) as well as up-regulation of inhibitory receptors (killer-cell immunoglobulin-like receptors, KIRs, NKG2A). Alterations of NK cells lead to an impaired recognition of tumor cells as well as a decreased ability to interact with immune cells. Alternatively, cancer cells downregulate expression of ligands for NK cell-activating receptors and up-regulate expression of the ligands for inhibitory receptors. A better knowledge of the extent and the mechanisms of these defects will allow developing pharmacological strategies to restore NK cell ability to recognize and lyse tumor cells. Combining conventional chemotherapy and immune modulation is a promising approach likely to improve clinical outcome in diverse neoplastic malignancies. Here, we overview experimental approaches as well as strategies already available in the clinics that restore NK cell functionality. Yet successful cancer therapies based on the manipulation of NK cell already have shown efficacy in the context of hematologic malignancies. Additionally, the ability of cytotoxic agents to increase susceptibility of tumors to NK cell lysis has been studied and may require improvement to maximize this effect. More recently, new strategies were developed to specifically restore NK cell phenotype or to stimulate NK cell functions. Overall, pharmacological immune modulation trends to be integrated in therapeutic strategies and should improve anti-tumor effects of conventional cancer therapy.

Keywords: cancer, immune escape, NK cell, NCR, NKG2D, KIR, immunotherapy

INTRODUCTION

Natural killer (NK) cells are key components of the innate immunity and substantially contribute to anti-tumor immune responses (1–3). The role of NK cells in immune surveillance is linked to many aspects of the NK cell biology. First, NK cells directly recognize and lyse cancer cells. Besides this direct effect, NK cells are also able to initiate anti-tumor immune responses via the secretion of various cytokines such as IFN-γ and TNF-α (1, 4).

Triggering of effector functions of NK cells is the result of a balance between activating and inhibitory signals provided by a large set of activating or inhibitory receptors. The most commonly described activating receptors involved in anti-tumor immunity are NKG2D, DNAM-1, and the natural cytotoxic receptors (NCR), NKp30, NKp44, and NKp46. Hence, NCR are NK-activating receptors of primary importance in immune surveillance and response in the context of cancer (5–7). NKp30, NKp46 are expressed by all NK cells, whereas NKp44 is only expressed by activated NK cells (8–11). The acquisition of NCR during NK cell maturation correlates with the acquisition of cytolytic activity against tumor target cells (12). NKG2D is an activating receptor also expressed by, but not restricted to, all NK cells. Ligands for NKG2D include proteins related to non-classical HLA-I such as MICA, MICB, or the structurally related ULBP1–6 (13, 14). Inhibitory receptors belong to the killer-cell immunoglobulin-like receptors (KIRs) or to the C-type lectin CD94/NKG2A heterodimer (15). These receptors recognize HLA-I and the non-classical HLA-E and inhibit NK cell activation.

The fundamental role of NK cells in oncology has been widely demonstrated in both hematologic and solid neoplasms. The relevance of this concept is illustrated by many examples in clinical practice, such as the success of hematopoietic stem cell

transplantation in hematologic malignancies (16–19), poor NK cell functions associated with increased incidence of cancer (20), the importance of NK cells for the response to chemotherapy and radiotherapy (21, 22), or the use of parameters related to NK cell functions as prognostic biomarkers (23–25). Thus, NK cells can be used as prognostic biomarkers, as well as therapeutic targets or therapeutic agents.

However, although NK cells can kill target cells spontaneously without prior stimulation, a delicate balance between inhibitory and activating signals tightly regulates their activation (1, 26). In the context of cancer, this balance is often deregulated through various mechanisms (27). First of all, cancer cells are able to induce a down-regulation of activating receptors (notably NCR and NKG2D,) as well as an up-regulation of the NK cell inhibitory receptors (23, 24, 28, 29). Then, tumor cells usually poorly express ligands for activating receptors, and/or overexpress ligands for inhibitory receptors (30–32). Finally, the release of various factors such as cytokines or reactive oxygen species (ROS) within the tumor microenvironment impairs the crosstalk between NK cells and dendritic cells (DCs), enhancing the phenomenon of tumor escape (33–35).

Many efforts have been developed in the past few years to restore NK cell functionality in cancer patients. In this review, we focus on NK cells as a cornerstone to restore or improve anti-tumor immunity. We overview different pharmacological strategies aiming at counteracting the effect of tumor cells on NK cell functionality (**Figure 1**). Taking into account the crucial importance of NK cells for maintenance of a prolonged response to treatment, therapeutic strategies improving or restoring NK cell functions in combination with standard treatment regimens are expected to broadly impact patients' clinical outcome.

INDUCING NATURAL CYTOTOXIC RECEPTORS EXPRESSION

Natural cytotoxic receptors expression is classically downregulated during cancer progression, regardless of the type of cancer (23, 24, 28, 29). The mechanisms involved in NCR down-regulation still need to be further defined. Restoring NCR expression may render NK cells more efficient against tumor cells. So far, clinical strategies aiming at restoring NCR expression remain to be proposed. However, taking into account the strong prognostic value of NCR expression, therapeutic strategies aiming at inducing their expression is expected to improve clinical outcome. Therefore, targeting events interfering with the expression of these receptors is certainly a relevant therapeutic option (23, 25). Among possible mechanisms, Transforming Growth Factor beta 1 (TGF-β1) downregulates NKp30 and NKG2D expression on NK cells, leading to a decreased ability of NK cells to kill target cells (23, 36–38). The release of TGF-β1 is done either by the tumor cell or by regulatory T cells (Tregs). Other tumor-released soluble factors are involved in NCR down-regulation, such as Activin-A, indoleamine dioxygenase (IDO), or prostaglandin E2 (PGE2) (34, 39, 40). Similarly to other activating receptors defect, the down modulation of NCR is somehow dependent on the pressure exerted by tumor cells, which reflects a pathway for tumor evasion. Hence, in acute myeloid leukemia (AML) patients, the low NCR expression acquired during leukemia development is restored in patients achieving complete remission (23). Some recently published data suggest that NCR down-regulation is consecutive to NK activation in the tumor, leading to an exhaustion of the NK cells and a subsequent down-regulation of the NCRs (41).

CYTOKINES

Amongst the efficient ways to improve NCR expression on NK cells, the use of cytokines, mainly IL-2, IL-15, and IL-21, may be promising. NK cell differentiation is cytokine-dependent (29). High baseline levels of circulating IL-2 constitute an independent prognostic factor for head and neck cancer patients (42).

IL-2

IL-2 is FDA-approved for cancer indications, which is not the case for IL-15 and IL-21. Most clinical trials using cytokines alone or in combination with chemotherapy or radiotherapy are set with IL-2. Conclusions of clinical trials report modest anti-tumor activity when used in monotherapy. Among its diverse immunostimulatory potentials, IL-2 is able to induce expression of NKG2D and NKp46 on NK cells (43, 44). However, following IL-2 stimulation, the NK cytolytic functions do not seem to reach normal cytolytic activity when compared to healthy volunteers (44). Moreover, IL-2 fails to induce NK cell proliferation compared to healthy volunteers, and increases the rate of apoptotic NK cells (44). Some authors evidenced the critical role of IL-2 for the development and peripheral expansion of regulatory T cells (45), which is not the case for IL-15 and IL-21. Noteworthy, the use of IL-2, especially at high doses, might be limited to *ex vivo* expansion of NK cells for problems of *in vivo* toxicity (46).

IL-15

IL-15 plays a major role in the proliferation, differentiation, survival, and functions of T and NK cells (29, 47). Exposure of NK cells to low doses of IL-15 significantly improved NKp30, NKp46, NKG2D, and NKG2C surface expression. Accordingly, this increase of receptor expression was correlated with an increase of natural cytotoxicity against autologous AML blasts (29, 48). In addition, in hematologic malignancies, low levels of circulating IL-15 after bone marrow transplantation were predictive of risk of relapse (49). In line, NK cell recovery in stem cell transplantation is strongly correlated with plasmatic concentrations of IL-15 (48).

IL-15 serum concentration increases dramatically following administration of cytotoxic agents (29, 49). For some authors, this elevation of serum IL-15 could be related to the depletion of lymphoid populations that normally consume circulating IL-15 or to inflammation induced by chemotherapy (48). *In vivo*, injections of the IL-15/IL-15Rα heterodimer result in significant expansion of γδ, $CD8^+$ T, and NK cells (47). Recently, this cytokine has become available for use in early phase clinical trials as an alternative to IL-2 (29, 47). IL-15 is currently assessed as a therapy for various solid tumors including refractory metastatic melanoma, metastatic renal cell cancer. IL-15 is also assessed as an adjuvant of chemotherapy and vaccines strategies or prior to stem cell therapy and NK cells infusion.

IL-21

IL-21 shares significant structural homology with IL-2 and IL-15 (50). In phase I trials, this cytokine shows a favorable safety

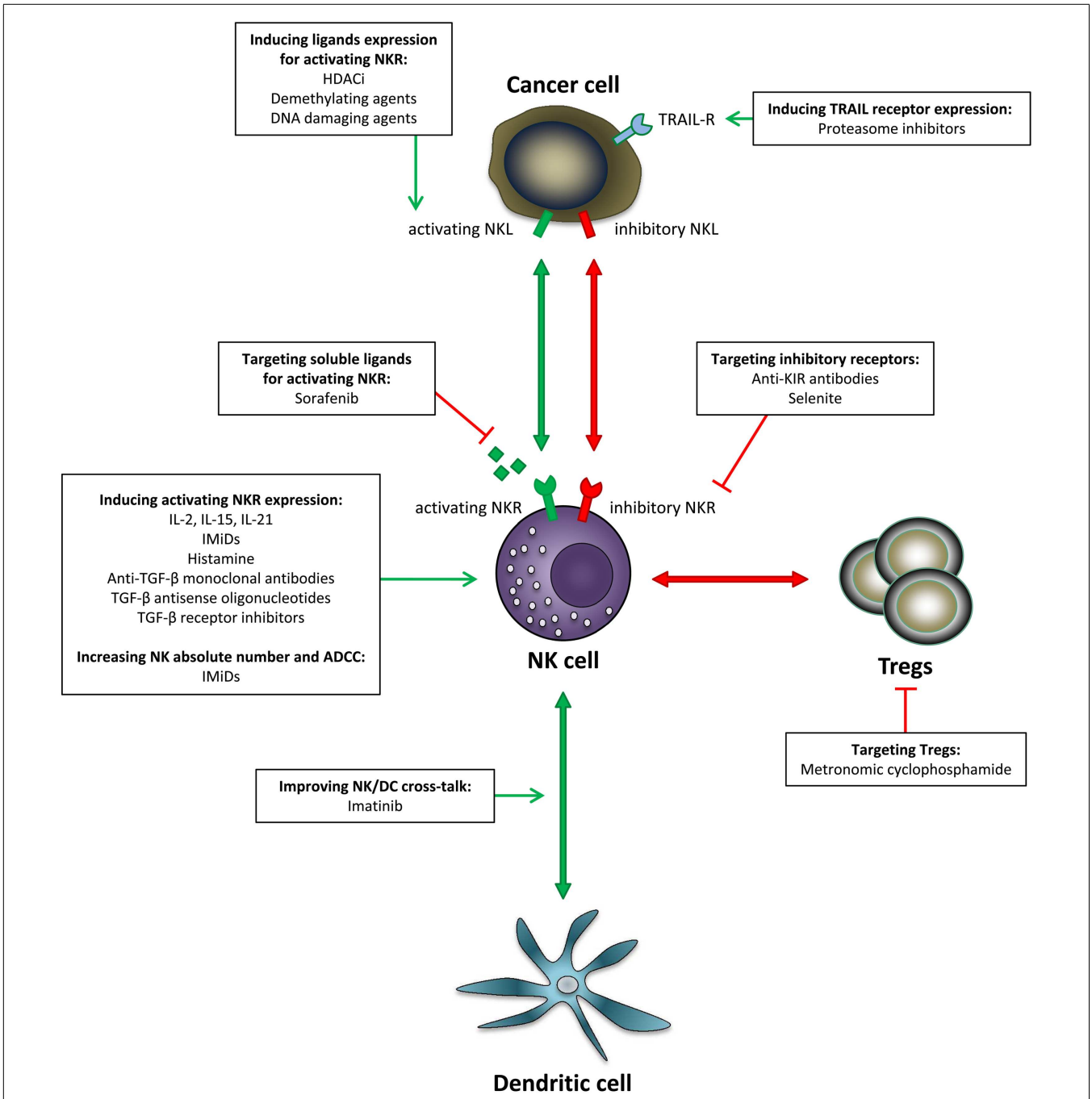

FIGURE 1 | Pharmacological strategies aiming at improving NK anti-tumor functions. Various options have been developed to restore NK cell functionality in cancer: induction of NK triggering receptors, induction of NK ligands expression on the target cells, blockade of inhibitory signals, as well as stimulation of NK/DC crosstalk. In addition, increasing NK number and improving ADCC can enhance this effect. NKL, natural killer ligand; NKR, natural killer receptor.

profile and signs of clinical activity (51). Although some reports demonstrated a deleterious effect of IL-21 by reducing activating receptor expression (NKG2D, NKp44), its main effect is to enhance NK cell functions. Hence, IL-21 is capable of inducing NK cell maturation and NKp46 and NKp30 expression (12, 52, 53). *Ex vivo*, IL-21 stimulates the production of IFN-γ and cytotoxic properties of NK cells (53). Several clinical trials reported the effect of IL-21 therapy on immune system after administration in patients with metastatic melanoma and renal cell carcinoma (51). Although NK and T-cell numbers were temporarily decreased during administration of IL-21, the cells had higher expression of CXCR3, HMMR, IFN-γ, perforin, and granzymes at the mRNA level. Evidence of NK cell activation was further confirmed by enhanced ability of NK cells from patients to lyse K562 target cells

(51). These results were confirmed in a phase II trial for metastatic melanoma (54).

IMMUNOMODULATORY DRUGS

Immunomodulatory drugs (IMiDs) present another therapeutic option to increase activating receptors expression. Two molecules are currently developed in oncology: lenalidomide, FDA-approved in hematologic malignancies, and pomalidomide. These drugs present anti-angiogenic and anti-proliferative activity, and their effect on the immune system, particularly on NK cells, is probably part of their mechanism of action. For instance, immunomonitoring of patients treated with immunomodulatory drugs, IMiDs have been associated with an increased expression of NKp44 and NKp46, in multiple myeloma (MM), myelodysplastic syndrome but also in solid tumors (55, 56). Interestingly, this effect of lenalidomide may not be a direct effect on NK cells because this effect was not observed *in vitro* on purified NK cells (57). In this study, IMiDs-treated NK cells displayed a lower NKp46 expression, although this had no functional consequences on cytolytic functions of NK cells.

HISTAMINE

Blocking phenomenon responsible for NCR down-regulation is another potential strategy to induce indirect NCR expression. Thus, ROS, PGE2, and IDO, which are present in the tumor microenvironment, appear to be relevant targets (33–35). Romero et al. demonstrated that histamine was able to prevent NKp46 and NKG2D down-regulation mediated by mononuclear and polymorphonuclear phagocytes ROS production (35). Moreover, histamine maintains the cytolytic activity of NK cells toward leukemic cells despite the presence of phagocytes. A phase III clinical trial assessed the efficacy of post-consolidation immunotherapy with IL-2 and histamine dihydrochloride for patients with AML in complete remission. This treatment was shown to significantly improve leukemia-free survival, with mild to moderate side effects (33).

INDUCING NKG2D EXPRESSION

NKG2D down-regulation on circulating NK cells in cancer patients compared to healthy volunteers was described in various cancer types, including breast cancer, glioma, melanoma, and lung cancer (58–62).

CYTOKINES

Few pharmacological agents are able to directly increase the expression of NK-activating receptors. Until now, the only described possibility to directly induce NKG2D expression on NK cells is the use of immunostimulatory cytokines. *Ex vivo*, IL-15 was shown to be able to induce a dramatic increase of NKG2D expression (63, 64). Although the use of IL-15 is still restricted to phase I and II clinical trials, conventional chemotherapies are able to induce a huge increase of the circulating IL-15 (29).

TGF-β PATHWAY

A second strategy allowing NKG2D restoration in the cancer context is indirect up-regulation by blocking the agents responsible for NKG2D down-regulation. For instance, stroma-derived factors in the tumor microenvironment, in particular TGF-β, display an immunosuppressive activity on most anti-tumor immune effectors, and an indirect immunosuppressive effect via the inhibition of MICA transcription (38, 65). Besides immune suppression, stroma-derived factors also present direct effects on the tumor cell since TGF-β promotes tumorigenesis and epithelial–mesenchymal transition (66). *In vitro*, TGF-β inhibits the expression of NKp30 and NKG2D (37) and blood concentration of TGF-β1 was shown to inversely correlate with NKG2D expression at the surface of NK cells of cancer patients and has been linked with impaired NK cytotoxicity (58, 60). TGF-β antagonizes the IL-15-induced proliferation and gene expression associated with NK cell activation, inhibiting the expression of NK cell activation receptor molecules (67). Moreover, *ex vivo* addition of neutralizing anti-TGF-β monoclonal antibodies completely restores surface NKG2D expression at the surface of NK cells and partially restores NKp30 expression (60, 67). In addition, blocking TGF-β completely restores IFN-γ production by tumor-associated NK cells (67).

Some approaches aiming at decreasing circulating TGF-β in patients are currently under investigation (68). These early stage clinical trials currently assess several approaches, mainly the use of anti-TGF-β monoclonal antibodies and antisense oligonucleotides. For example, fresolimumab (GC-1008), a fully humanized pan-neutralizing antibody directed against all the three isoforms of TGF-β, has been assessed in renal cell carcinoma and in metastatic melanoma (68, 69). In this phase I/II trial, fresolimumab was safe and well-tolerated with no dose-limiting toxicities and displayed encouraging results.

The impact of TGF-β blockade on immune parameters was recently assessed in patients with malignant pleural mesothelioma treated with fresolimumab (70). Fresolimumab had no effect in the expression of NK, $CD4^+$, or $CD8^+$ T-cell-activating and inhibitory markers, other than a decrease in the expression of 2B4 and DNAM-1 on NK cells, although TGF-β serum concentrations were markedly decreased. The authors conclude that acute changes in serum TGF-β concentration are not associated with the set of biomarker changes that were predicted based on animal models. No effect was detected on the expression of NKG2D nor NKp30, and the effect on DNAM-1 expression, although significant, was minor (70).

Another possibility to decrease TGF-β in the tumor milieu is the use of antisense oligonucleotides. Some of these compounds are currently in clinical evaluation. Belagenpumatucel-L, a therapeutic vaccine comprised of four TGF-β2 antisense gene-modified allogeneic NSCLC cell lines was assessed in grade III/IV NSCLC patients. In a phase II study, positive clinical outcomes were correlated with immune response to the vaccine and induction of immune enhancement of tumor antigen, but the effect on NK cells was not assessed (65). This compound is still currently investigated in non-small cell lung carcinoma in phases II and III trials.

Alternatively, SD-208, a TGF-β receptor I kinase inhibitors, restores the lytic activity of polyclonal NK cells against glioma cells in the presence of recombinant TGF-β or of TGF-β-containing glioma cell supernatant (71). This molecule is able to restore NKG2D expression on NK cells, whose expression was altered *in vitro* by cancer cell lines supernatants or direct inhibition with recombinant TGF-β (72).

To conclude, NKG2D expression has never been shown to present a prognostic value unlike NKG2D ligands expression, thus suggesting that the best strategy to target the NKG2D/NKG2D ligand system might be to induce ligands expression rather than the receptor itself.

INDUCING LIGANDS EXPRESSION FOR NK-ACTIVATING RECEPTORS

The main ligands for NKG2D are the MHC class I chain-related molecules MICA and MICB and the ULBP1–4. These ligands have been extensively studied in various malignancies. Ligands of DNAM-1 are CD112 (Nectin-2) and CD155 (Poliovirus receptor, PVR). Ligands of NCRs have been elusive for many years and although pathogen-related ligands have been suggested (hemagglutinins, heparate sulfates), only ligands for NKp30 have been identified. B7-H6, an Ig molecule from the family of B7 molecules has been identified as NKp30 ligand (73). B7-H6 is expressed by several cell lines and by primary tumors (74). Mechanisms of induction of B7-H6 expression have been described in non-transformed cells with TLR agonists as well as the pro-inflammatory cytokines TNFα and IL-1β (75). In primary tumors, recent experimental data suggest that B7-H6 expression is regulated by HDACs, in particular HDAC3 (74). In addition, BAG6/BAT3, a nuclear protein localized at the plasma membrane or on exosomes of tumor cells, has also been assigned as an NKp30 ligand (76). The importance of ligands expression for tumor cell recognition by NK cells is a key factor for anti-tumor immune response, as illustrated by the strong prognostic value of MICA/MICB, RAET1G, and ULBP2 expression in colorectal cancer and breast cancer (30–32). Tumor cells poorly express ligands for NK-activating receptors, and tumor ligands expression is inversely correlated with clinical stage (77).

HISTONE DEACETYLASE INHIBITORS

Histone deacetylase inhibitors were successfully introduced as anti-cancer agents for their ability to block gene transcription and promote cell differentiation. These molecules induce cell cycle arrest and induce apoptosis of tumor cells, with minimal effects on normal tissue (78). Unexpectedly, their effect on anti-tumor immunity is part of their mechanism of action.

The main impact of these molecules on immunity is mediated through up-regulation of tumor antigens, in particular NKG2D ligands (79). HDACi-mediated immune modulation is also linked to the ability of these molecules to enhance immune recognition and lysis of the tumor cells by T cells and NK cells (79). To date, two molecules, romidepsin and vorinostat, have received approval from the FDA for the treatment of cutaneous T-cell lymphoma. *In vitro*, romidepsin, vorinostat, and sodium valproate were shown to increase MICA/B and ULBPs expression on various cancer cell lines and primary tumor cells, and render the target cells more sensitive to NK cell lysis (80–84). Depending on the authors, this mechanism was found to be GSK3- or ERK-dependent (81, 83).

Induction of MICA and MICB expression was associated with a shedding of the soluble forms of these NKG2D ligands, sMICA and sMICB (82). This raises the question of the potential counterbalancing of the clinical benefits in this particular case, since increase of the serum concentrations of sMICA and sMICB are responsible for NKG2D endocytosis and degradation, and represents a mode of T-cell silencing and immune escape (62, 82). Thus, Poggi et al. monitored NKG2D ligands shedding following treatment of AML patients treated with valproic acid. In this study, MICA, ULBP2, and ULBP3 expression on blasts was significantly increased after treatment with valproic acid. No ligand shedding was detected despite a strong up-regulation of the ligands on leukemic cells. Consequently, leukemic cells from patients treated with valproic acid, become able to trigger lytic granule exocytosis by autologous $CD8^+$ T and NK cells (85).

However, some studies evidenced that HDACi down-regulate ligands for other NK cells-activating receptors, such as B7-H6, a ligand for NKp30, and impair tumor cell recognition by NK cells. These results were obtained with first and second generation HDACi (vorinostat, trichostatin A, valproic acid, and apicidin) on various cancer cell lines (74). Moreover, treatment of human NK cells with trichostatin A, valproic acid, or sodium butyrate affects the functional response of human NK cells, evidenced by a strong inhibition of IFN-γ secretion and a decreased ability to lyse target cells (86). Furthermore, the authors evidenced a down-regulation of activating receptors NKG2D and NCRs on resting and cytokine-stimulated NK cells.

Another study assessed the effect of vorinostat and valproic acid on NK cells. At therapeutic concentration, these drugs induced the down-regulation of NKp30 and NKp46, and inhibited IL-2 activation of NK cells, thus suppressing their cytolytic activity toward leukemic cell lines. This effect seems to be mediated by the inhibition of NFκB. In addition, the authors showed that vorinostat was toxic to NK cells in the range of therapeutic concentrations (87).

DEMETHYLATING AGENTS

The hypomethylating drugs decitabine and azacytidine are epigenetic drugs that are currently used in treatment of hematological malignancies (88). Besides their direct effect on the tumor cell, these drugs probably act through their impact on innate immunity. *In vitro*, both drugs induce ULBP1 and MICB on cell lines and primary tumor cells when incubated with either decitabine or 5-azacytidine (89, 90). This effect was related to promoter DNA methylation and DNA damage and correlates with enhanced NK cytotoxicity (90, 91).

However, DNA methylation is an important regulator of KIR expression by NK cells, potentially impacting on NK cell functions (92, 93). Hence, 5-azacytidine induces an increase in the percentage of KIR^+ NK cells upon treatment with clinically relevant concentrations of 5-azacytidine, which correlated with an impaired granzyme B and perforin release, IFN-γ production, and decreased cytotoxicity (91, 94). However, this effect seems to be restricted to 5-azacytidine, since decitabine increases NK cell cytotoxicity and enhances IFN-γ production, in a dose-dependent manner (91). These results were confirmed in recent studies in different settings. Recently, Cerdeira et al. tested the effect of 5-azacytidine in hypoxic conditions with addition of TGF-β. Although the authors confirmed the impact of this drug on KIR expression, however, the cytotoxicity of NK cells cultured in these specific conditions was not affected (92).

For some authors, the results obtained *in vitro* in such settings are debatable. Indeed, since 5-azacytidine and decitabine are nucleoside analogs, these molecules require DNA replication to be incorporated into the DNA strand. *In vitro* studies using resting NK cells are therefore more likely to reflect the direct mRNA effect of such drugs than the effect of hypomethylation (88). Thus, Kopp et al. studied the effect of decitabine on proliferating NK cells. The authors show that decitabine negatively affects NK cell viability and proliferation in a dose-dependent manner. Simultaneous increase in KIR and NKp44 expression and decrease in NKG2D expression was evidenced. However, the impact on NK functionality in terms of toxicity was biphasic, with decreased toxicity at low doses and increased toxicity at high doses. Since the target cells used in these experiments lack class I HLA, this effect is independent of KIR up-regulation. Whether this increased cytotoxicity is maintained in the presence of HLA-positive targets remains to be determinate (88).

To conclude, further investigation is required to determine whether epigenetic drugs adversely affect NK cell survival, proliferation, or functions when administrated to patients.

DNA-DAMAGING AGENTS

Some conventional chemotherapeutic agents can induce immunogenic cell death, e.g., tumor cell apoptosis and stress signals that lead to the surface expression of ligands for NKG2D and DNAM-1 (95, 96). This DNA damage pathway can be activated by several mechanisms, during the course of chemotherapy with DNA-damaging agents such as doxorubicin, mitoxantrone, cisplatin, and oxaliplatin (8, 95–99). This particular mode of cell death displays damage-associated molecular patterns, e.g., exposure of calreticulin endoplasmic reticulum proteins at the surface of the pre-apoptotic cell, as well as secretion of ATP (100).

The oncogenic stress induced by these DNA-damaging agents stimulates various aspects of anti-cancer immunity, including activation of NK cells via ULBP1, MICA/B, and PVR expression at the surface of the cancer cell in an ATM (ataxia telangiectasia, mutated), ATR (ATM- and Rad3-related) protein kinases, and/or P53-dependent manner (8, 96–99). Other agents are able to induce stress conditions, leading to the expression of ligands for NKG2D and DNAM-1, such as IMiDs and proteasome inhibitors (22). These results await clinical confirmation with immunomonitoring studies of patients undergoing DNA-damaging agent therapy.

TARGETING SOLUBLE LIGANDS FOR ACTIVATING RECEPTORS

The expression of NKR ligands at the surface of cancer cells appears to be a good prognostic factor. However, the shedding of soluble ligands in the circulation strongly impairs NK cell functions and has been linked with tumorigenesis and tumor progression (101) and high serum concentration of ULBP2 presents a strong prognostic value in breast cancer, colorectal cancer, and melanoma (30–32). Noteworthy, the discovery of B7-H6 and BAG6, ligands for NKp30, included the detection of soluble forms, which may compete for cell–cell interaction with membrane-bound ligands, although only soluble/exosome-bound BAG6 has been detected in a cancer situation (75, 102).

The prototypical example of ligand shedding is the release of soluble MICA/MICB (sMICA/sMICB), typically by A disintegrin and metalloproteases (ADAMs) (103, 104). These proteases are overexpressed in malignant tissues compared to normal tissues (105, 106). As a consequence, serum concentrations of soluble ligands for NKG2D are elevated in various malignant conditions (103). The ligation of these soluble ligands induces internalization of NKG2D and its subsequent degradation, leading to an overall down-regulation of the receptor at the surface of NK cells. In various cancers, high levels of circulating ligands for NK-activating receptors correlated with a poor prognosis. Direct pharmacologic inhibition of these metalloproteases is still in preclinical evaluation.

SORAFENIB

Sorafenib is a multi-target tyrosine kinase inhibitor targeting RAS/RAF/MAPK as well as VEGFR and PDGFR signaling pathways, implicated in cell proliferation and angiogenesis. Sorafenib is indicated in renal cell carcinoma, hepatocellular carcinoma, thyroid cancer and melanoma. *In vitro*, this molecule presents interesting off-target effects on ADAM9 expression as evidenced by a recent study on the human hepatocellular carcinoma cell line HepG2. In this study, sorafenib was able to strongly decrease ADAM9 expression at the proteic and transcriptional level, which correlated with a decrease of sMICA concentration in the culture supernatant and enhanced sensitivity to NK cell lysis. In addition, ADAM9 inhibition increases the expression of membrane-bound MICA on the tumor cell, enhancing the NK sensitivity of hepatocellular carcinoma cells (105).

Controversial data were published about effects of sorafenib on NK cells. NK cell function is inhibited by sorafenib as a consequence of impaired phosphorylation of PI3K and ERK, which directly control NK cell reactivity (107). Immunomonitoring of patients with renal cell carcinoma and melanoma treated with sorafenib failed to evidence modification of pERK1/2 expression in peripheral-blood NK cells after short-term or long-term administration (108). In addition, sorafenib may also positively (Th1) or negatively (DCs) impact other aspects of anti-tumor immunity (61, 109, 110). Whether this action is positive or negative remains to be determinated, as well as the overall "immune benefit" of such antagonistic effects on anti-tumor immunity, besides their direct pro-apoptotic effect on the tumor cell.

TARGETING INHIBITORY RECEPTORS

Although activating NK receptors are crucial, triggering of NK cell effector functions is prevented by the expression of the inhibitory receptors KIR and NKG2A. Although in some examples of solid cancer, KIR and NKG2A expression is altered, generally expression is maintained and tumor cells may maintain sufficient amounts of HLA molecules to ensure inhibition of NK cells and evade killing. Moreover, some tumors display decreased expression of TCR-dependent HLA molecules while maintaining a normal expression of KIR-dependent HLA molecules (111). High HLA-E expression has been observed in several solid cancers (112, 113) and leukemias (114). Consequently, as 20–70% of NK cells express NKG2A, HLA-E expression by tumor cells impairs the anti-tumor activity of a predominant proportion of NK cells.

ANTI-KIR MONOCLONAL ANTIBODIES

Among the strategies to improve the recognition of tumor cells by NK cells, blocking the inhibitory interactions is appealing. The most advanced therapeutic compound as for today is the anti-KIR monoclonal antibody, IPH2101. This fully humanized antibody blocks the interaction of the major KIR expressed by NK cells with their cognate ligands, i.e., HLA-C. This reagent has been tested in early phase clinical trials and was shown to be well-tolerated in patients suffering from AML (115). In some instance, NK cells from treated patients expressed the activation marker CD69 and IFN-γ or MIP-1β was detected in the sera of patients. Another clinical trial in patients with MM has also shown that IPH2101 is safe and also enhances *ex vivo* NK cell cytotoxicity against MM cells (116). IPH2101 (and its replacement IPH2102) is therefore a novel immune-therapeutic agent that may improve anti-tumor activity of patients. More trials are programed and already necessary but yet this reagent has reached the promises for clinical use against cancer cells.

SELENITE

As mentioned above, control of NK cell activation is either achieved by KIR/HLA interactions but also NKG2A/HLA-E interaction. In healthy individuals, at steady state, the two systems compensate for each other to ensure a total control of NK cell reactivity. Regarding NKG2A-mediated inhibition of NK cells by HLA-E expressing tumor cells, very few data are available. Interestingly, an FDA-approved reagent, selenium, may be a promising tool. Supplementation with selenium has been associated with reduced risk of solid cancer (117). The mechanism of action of selenium is not entirely known, but it induces apoptosis of tumor cells by generating an oxidative stress, which may be more effective on tumor cells compared to healthy cells (118, 119). Alternatively, selenium blocks the synthesis of HLA-E and consequently increases cytotoxicity mediated by NKG2A-positive NK cells (120). This effect, combined to the direct toxicity on tumor cells may result in reduced disease progression and improved survival. Sodium selenite is currently under investigation in several clinical trials for the treatment of different cancers.

Altogether, targeting inhibitory NK receptors reflects a novel orientation taken for innovative therapeutic approaches, as it represents another way to counteract the immune escape via ligands for inhibitory receptors. Of note, this strategy relies on the expression of activating ligands by leukemic cells. Hence, removing of inhibition will allow NK cells killing their targets provided that they express the ligands for activating NK receptors.

ALTERNATIVE PATHWAYS TO IMPROVE NK ACTIVITY

INCREASING NK CELL LYSIS CAPACITY WITH IMiDs

Immunomodulatory drugs are capable to enhance monoclonal antibodies anti-tumor activity. First *in vitro*, Wu et al. have shown an enhancement of NK cell-mediated tumor cell ADCC by lenalidomide for a variety of rituximab-treated NHL (non-Hodgkin lymphoma), cetuximab-coated CRC (colorectal cancer), and trastuzumab-coating breast cancer cell lines (121, 122). Another team highlighted the enhancement of ADCC by lenalidomide *in vitro*. They have shown an increase of Raji cell apoptosis mediated by PBMC combination with rituximab by lenalidomide (123). In the first case, the effect was observed on purified NK cell but Wu et al. have explained that this mechanism is dependent on the presence of antibody and either interleukin-2 or interleukin-12. In the second case, Zhu et al. have observed this effect on PBMC. Finally, the researches of Hayashi et al. have shown that IMiDs-enhanced NK cell ADCC by triggering IL-2 production from T cells (124). All these works suggest that *in vitro* IMiDs-positive effect on NK cell ADCC could be dependent on IL-2.

In animal models, lenalidomide or pomalidomide in combination with rituximab improves severe combined immunodeficient (SCID) lymphoma-bearing mouse survival compared to rituximab in monotherapy (125). Three years later, the same team explained this enhancement of anti-tumor activity by an expanding, activating, and trafficking of NK cells into the tumor bed, which facilitate a more efficient ADCC. The IMiDs effect on NK cells in this model is also associated with DC activation and production of chemokines and pro-inflammatory cytokines (126).

In the same way, IMiDs are also capable to enhance natural cytotoxicity of NK cell against cancer cells. First, Davies et al. highlighted the potency of thalidomide, lenalidomide, and pomalidomide to increase PBMC cytotoxicity toward MM tumor cells (cell lines and patient cells) *in vitro*. They presented this effect as an NK-dependent effect (127). Then, Zhu et al. have shown the similar effect with lenalidomide and pomalidomide on K562 and PC-3 cell lines (i.e., enhanced PBMC-mediated tumor cell apoptosis). They have also shown that NK cells are essential in inducing cancer cell apoptosis (123). In the same manner as ADCC, Hayashi et al. have explained this IMiDs enhancement of NK cell cytotoxicity via induction of IL-2 production in T cells (124).

In line with *in vitro* studies, IMiDs also increased NK cell natural cytotoxicity in patients suffering from MDS or solid tumors (56). At last, IMiDs have an important property toward NK cell numbers. Hence, the number and the localization of NK cells in cancer patients is often correlated with prognosis (24, 25, 128–130).

Davies et al. observed that thalidomide treatment for MM patients resulted in an increase of absolute NK cell numbers (127). This observation was confirmed with lenalidomide in some metastatic malignant melanoma patients and other advanced cancers (131), and in children with solid tumors or MDS (56). This effect was also highlighted in lenalidomide and pomalidomide treated mice (lymphoma-bearing SCID mice) at the tumor site. Reddy et al. have shown in their study an increase of tumor central infiltration by NK cells in mice treated by lenalidomide or pomalidomide compared to DMSO-treated mice. They could explain that by the IMiDs effects on DCs stimulation and modification of the cytokine microenvironment (126).

INDUCING TRAIL RECEPTOR EXPRESSION ON TARGET CELLS

Proteasome inhibitors are a class of anti-cancer drugs that are used in first line of treatment of MM, and that are currently evaluated in hematologic and solid malignancies. These molecules disrupt proteasome activity, resulting in cell growth arrest, apoptosis, angiogenesis inhibition, and decreased binding of tumor cells to stromal cells (132). *In vitro*, bortezomib was shown

to sensitize tumor cell lines as well as primary tumor cells to perforin/granzyme-mediated NK-tumor cytotoxicity. This effect was found to be dependent on augmentation of tumor caspase-8 activity as well as on up-regulation of Fas and TNF related apoptosis-inducing ligand (TRAIL) receptor on tumor cells, thus inducing target apoptosis by NK cells through Fas/FasL and TRAIL/DR5 interactions (133–135). Other proteasome inhibitors such as the b-A15 share this property (136). In addition, proteasome inhibitors up-regulate ULBP1 and ULBP2 expression (137–139). This effect is accompanied by a down-regulation of HLA class I molecules (140).

In vivo, bortezomib sensitizes tumors to killing by NK cells. This anti-tumor effect is enhanced upon depletion of Tregs (134, 141). Based on these results, a non-randomized phase I study is currently ongoing in order to evaluate the safety and the anti-tumor effects of adoptively infused *ex vivo* expanded autologous NK cells against metastatic cancers or hematological malignancies sensitized to NK TRAIL cytotoxicity with bortezomib (134). However, bortezomib paradoxically renders tumor cells resistant to killing by tumor-specific T cells, thus potentially counterbalancing the benefits obtained through the sensitization to killing by NK cells (136, 142). In addition, *in vitro* assays evidenced that bortezomib presents pro-apoptotic effects on NK cells, and induces a down-regulation of NKp46 expression with subsequent decrease in NKp46-mediated activity (143). b-AP15, a new proteasome inhibitor, appears to overcome this deleterious effect on T cells: *in vitro* evaluation of this molecule was shown to sensitize tumor cell lines to both NK and T cell-mediated killing (136). However, at equipotent doses, this molecule seems to be more toxic to NK cells than bortezomib (144).

IMPROVING NK/DC CROSSTALK

The relevance of the NK/DC crosstalk has been demonstrated in various physiopathological settings and alterations of these interactions have been shown to contribute to tumor progression (145). Imatinib mesylate is a tyrosine kinase inhibitor that inhibits the tyrosine kinase encoded by the bcr-abl oncogene and tyrosine kinases encoded by the c-kit and the PDGFR oncogenes. Targeting these tyrosine kinases directly induces apoptosis of the cancer cell, which constitutes the main mechanism of action of imatinib. Besides this direct anti-proliferative effect, an "off-target" effect, inducing DC-mediated NK activation was described by Borg et al. (145). In this study, patients with GIST were assessed for NK cell functions during the course of treatment with imatinib. Anti-tumor response correlated with enhanced NK-mediated anti-tumor response, thus bringing out a new mechanism of action of this molecule. The authors then defined immunologic responder patients with increased RFS. In a more recent study conducted in GIST patients, the authors validated the concept, showing a correlation between clinical outcome and NK cell activation induced by therapy with imatinib (21). Immunomonitoring of NK cell functions included IFN-γ production and NKG2D expression. Although IFN-γ production was associated with clinical outcome, enhanced NKG2D-dependent lysis observed at 1 year of imatinib therapy did not impact survival (21). Interestingly, this DC-mediated NK activation seems to occur in lymph nodes where imatinib promotes the formation of immunologic synapses with resting or preactivated NK cells as a consequence of the blocking of KIT signaling in DCs (21, 97).

DEPLETING Tregs

Tregs inhibit antigen-specific immune response both in a cytokine-dependent and cell contact-dependent manner (146–148). Tregs alter both T cells and NK cells proliferation and activity through the down-regulation of NKG2D (147–149). Increased frequency of Treg cells and low T effector (Teff)–Treg ratios are associated with a poor clinical outcome and a lack of treatment response (147, 150–153). Impairment of Treg activity by either specific blockade or depletion can enhance immune response against tumor-associated antigens (147, 148). To date, drugs that specifically target Tregs are not available (153).

Although cyclophosphamide is immunosuppressive at high doses, this molecule displays particularly interesting immunostimulatory properties in metronomic scheduling (iterative administration of low doses) mainly by its ability to suppress $FOXP3^+$ regulatory T cells (95, 149, 154) and to induce TH2/TH1 to TH17 shifts in cytokine production, induction of TH17, and resetting of dendritic cell homeostasis (153, 155). In murine models, metronomic cyclophosphamide strongly induces NKp46 expression as well as perforin and granzymes (156). Importantly, immunomonitoring studies evidenced that low-dose cyclophosphamide regimen restores patients' T cells and NK cells functions as evidenced by killing assays (149, 157). Metronomic cyclophosphamide is currently tested in combination with anti-cancer vaccines, for its ability to suppress Tregs in order to facilitate vaccine-induced tumor rejection (153). Despite metronomic cyclophosphamide provides promising clinical results, some authors point the absence of randomization in these trials (158).

CONCLUDING REMARKS

Accumulating evidence based on immunomonitoring analyses highlights immune parameters as strong prognostic factors, both in hematopoietic and solid neoplasms. These conclusions provide a strong rationale for developing therapeutic strategies aiming at restoring key immune parameters. Among the major mechanisms used by tumor cells to escape immunity, the evasion from receptor–ligand-mediated anti-tumor activity by NK cells represents the most prevalent pathway. Hence, the recognition of tumor cells by NK cells via NCR or NKG2D-activating receptors is often impaired in various cancers and enhancing NK cell functions appears as one of the most promising approaches. One important question remains the ability of a cancer cell to overcome immune suppression upon exposure to immunostimulating drugs. Recent studies suggest that NK cells on tumor site exhibit a phenotype of exhaustion and terminal differentiation. Restoring NK functionality in this context could be of limited interest since these cells may hardly become highly anti-tumoral. This parameter should be considered to maximize the effects of such approaches.

To conclude, targeting immune evasion mechanisms, in association with conventional chemotherapy, may improve clinical outcome and is clinically feasible with limited side effects. To date, clinical application of this concept is mainly limited to drugs designed to target cancer cells, with off-target effects on the immune system. The problem of these strategies is that the

overall benefit on the different immune effectors is sometimes hard to predict, and can be deleterious on crucial immune effectors, although restoring other cells. New strategies aiming at specifically restored immune functions will be potentially more efficient, and are currently in preclinical and clinical development. Further development of these immune therapies urges to associate clinical trials with translational immunology and immunomonitoring. A better knowledge regarding immune evasion mechanisms will definitely provide the absolutely required bases for the next-generation immune cancer therapies.

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NK cell subset redistribution during the course of viral infections

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Natural killer (NK) cells are important effectors of innate immunity that play a critical role in the control of human viral infections. Indeed, given their capability to directly recognize virally infected cells without the need of specific antigen presentation, NK cells are on the first line of defense against these invading pathogens. By establishing cellular networks with a variety of cell types such as dendritic cells, NK cells can also amplify anti-viral adaptive immune responses. In turn, viruses evolved and developed several mechanisms to evade NK cell-mediated immune activity. It has been reported that certain viral diseases, including human immunodeficiency virus-1 as well as human cytomegalovirus infections, are associated with a pathologic redistribution of NK cell subsets in the peripheral blood. In particular, it has been observed the expansion of unconventional $CD56^{neg}$ NK cells, whose effector functions are significantly impaired as compared to that of conventional $CD56^{pos}$ NK cells. In this review, we address the impact of these two chronic viral infections on the functional and phenotypic perturbations of human NK cell compartment.

Keywords: viral infection, immune escape, immune activation, innate immune response, physiophysiological interaction

INTRODUCTION

In the absence of drugs that are able to eradicate and cure viral infections, the presence of efficient immune responses is key in the control and clearance of virally infected cells. Natural killer (NK) cells represent the first line of defense against viral infections, as it became clear since the first experimental evidence in the late 1980s reporting severe and recurrent herpes virus infections in a young patient with NK cell deficiency (1). The fact that NK cells do not need a prior antigen sensitization makes them ready to fight against pathogens starting from the early phases of innate immune responses through several effector functions controlled by a dynamic balance between inhibitory and activating NK cell receptors (NKRs) (2). Indeed, NK cells are able to lyse "non-self" cellular targets while sparing normal cells that express adequate levels of "self" major histocompatibility complex of class I (MHC-I) molecules. This cytolytic function is regulated by a heterogeneous family of inhibitory NKRs (iNKRs) that bind specifically to either classical or non-classical human leukocyte antigen (HLA) alleles (3). Diminution or absence of expression of HLA-I molecules on the surface of virally infected cells results in reduced engagement of iNKRs which, in turn, allow a large group of activating NKRs (aNKRs) to trigger cytotoxicity. The "on signal" exerted by aNKRs to trigger NK cell killing depends on the induced expression of putative ligands for activating receptors on virally infected target cells. The recognition of these specific ligands is required for the engagement of aNKR-mediated downstream pathways associated with the NK cell release of lytic granules (4–13).

Upon activation, NK cells also produce several chemokines such as CCL3/MIP1α, CCL4/MIP1β CCL5/RANTES, and cytokines such as interferon-γ (IFN-γ), tumor necrosis factor (TNF), and granulocyte/macrophage colony-stimulating factor (GM-CSF). These soluble factors play not only an important regulatory role in hematopoiesis and cellular activation, but are also involved in the suppression of human immunodeficiency virus-1 (HIV-1) replication through non-cytolytic mechanisms (2, 10, 14–17). NK cells are also endowed with the ability of either priming or taking part to cellular networks of interactions. In fact, it has been shown that NK cells are engaged in an active and bi-directional cross talk with autologous dendritic cells (DCs) through a process that requires both NK cell–DC cellular interactions and secretion of specific cytokines (18–25). Furthermore, monocytes/macrophages and neutrophils have been shown to regulate the recruitment and the activation of NK cells, which, in turn, can eliminate overstimulated macrophages (26–29). It has been also reported that human neutrophils are able to establish a network with both NK cells and 6-sulfo LacNAc+ DCs (slanDC). This "*mènage à trois*" involves direct reciprocal interactions as well as positive amplification loops mediated by cell-derived cytokines with the aim of inducing IFN-γ production by NK cells (30). The final outcome of

these synergic NK cell interactions is the coordination and optimization of both innate and adaptive immunity in response to inflammatory stimuli such as viral infections at tissue sites (31).

Under physiological conditions, the distribution of the surface markers CD56 and CD16 (FcγRIII) defines two subsets of $CD14^{neg}/CD3^{neg}/CD19^{neg}$ NK lymphocytes: the $CD56^{bright}/CD16^{neg\text{-}dim}$ ($CD56^{bright}$) population that accounts for ~10% of blood NK cells and the $CD56^{dim}/CD16^{bright}$ ($CD56^{dim}$) cells that comprise for ~90% of circulating NK cells (17, 32). $CD56^{bright}$ NK cells exert only marginal cytotoxic capacity and yet produce high amounts of cytokines like IFN-γ, TNF, and GM-CSF. Their degree of proliferation in response to activation stimuli is much higher as compared to that of $CD56^{dim}$ NK cells. Given the pleiotropic roles of the cytokines on multiple immune and non-immune populations, $CD56^{bright}$ NK cells have been generally referred to as regulatory NK cells. Conversely, $CD56^{dim}$ NK cells were originally identified as the main subset endowed with cytotoxic capacity, although subsequent works indicated that they can also produce relatively high amounts of pro-inflammatory cytokines following the engagement of aNKRs (32–36). The different functional outcomes of $CD56^{bright}$ and $CD56^{dim}$ NK cells are associated with different repertoires of NKRs and with distinct homing capacities that are determined at the level of chemokine receptor expression on the cell surface. Indeed, $CD56^{dim}$ NK cells preferentially migrate to inflamed peripheral tissues on the basis of their increased expression of CXCR1, CX3CR1, and ChemR23, while the $CD56^{bright}$ subset expressing CCR7 preferentially homes to secondary lymphoid organs (37–39). Remarkably, recent data indicate that, in several pathological conditions including viral infections, $CD56^{dim}$ NK cells may also express CCR7 *de novo* and migrate toward lymph nodes (40–42).

It is well known that viruses remarkably affect NK cell homeostasis, phenotype, and functions, thus highlighting the key roles played by NK lymphocytes in the physiopathology of chronic and inflammatory viral disorders. This review provides an updated summary of the virally induced changes of NK cell phenotype and functions and of their implications in NK cell physiology and physiopathology.

NK CELL RESPONSES TO HIV-1

HIGH FREQUENCIES OF $CD56^{neg}$ NK CELL SUBSET IN HIV-1 INFECTION

Although the NK cell population is mainly composed by the two $CD56^{bright}$ and $CD56^{dim}$ subsets, low frequencies of a $CD14^{neg}/CD3^{neg}/CD19^{neg}/CD56^{neg}/CD16^{bright}$ ($CD56^{neg}$) population are also detected in healthy donors (16, 43). This unusual and rare population has been substantially ignored until mid 1990s, when it has been described that the decrement of absolute numbers of circulating NK cells during the course of HIV-1 infection is associated with expansion of an unconventional subset of $CD56^{neg}$ NK lymphocytes (44). This report opened a new research topic in the field of NK cell biology and many groups, including ours, highlighted the great importance of $CD56^{neg}$ NK cell in the physiopathology of HIV-1 infection. It then became evident that NK cells are remarkably affected by the deleterious effect of ongoing HIV-1 replication. Although NK cells are not productively infected by HIV-1, high and chronic levels of viremia significantly impair NK cell-mediated host immune responses, thus leading to a defective control of viral spreading and, subsequently, to disease progression. This is due, at least in part, to the defective capacities of NK cells from viremic HIV-1-infected patients to eliminate autologous HIV-1-infected $CD4^{pos}$ T cells. Moreover, NK cells from the same individuals displayed impaired killing of cell targets either tumor-transformed or infected by opportunistic pathogens as well as weaker production of anti-viral cytokines/chemokines and defective interactions with autologous DCs (10, 17). In turn, dysfunctions in NK-DC crosstalk impair the maturation of DCs that, instead of priming an effective adaptive immune response by presenting HIV-1 antigens to T cells, contribute to disseminate the infection in secondary lymphoid organs (23). These NK cell aberrancies are a direct consequence of the HIV-1-driven expansion of the highly anergic $CD56^{neg}$ NK cell subset. In patients with chronic or late stage HIV-1 infection and high viral loads, decreased frequencies of $CD56^{dim}/CD16^{pos}$ NK cell populations are counterbalanced by increased percentages of these dysfunctional $CD56^{neg}$ cells expressing an aberrant repertoire of inhibitory and aNKRs. This experimental evidence clarified that, rather than an absolute decrement of total circulating NK cells (44), HIV-1 viremia is associated with a significant and pathological redistribution of NK cell subsets associated with impaired anti-viral responses (12, 16, 23, 45–53). The sequential deregulation of NK cell subset has been reported to start from the early phases of HIV-1 infection due to the presence of surface markers highly sensitive to viral replication (33, 53). In particular, it has been reported that the c-lectin-type molecule Siglec-7 (also known as p75/AIRM1), an inhibitory receptor constitutively expressed on all NK cells, is the first marker to be down-regulated during the early phases of HIV-1 infection before the loss of CD56. Siglec-7 down-modulation is preserved throughout the course of the infection and depends on the level of viral replication. Indeed, the small cohort of individuals that do not progress toward AIDS (i.e., the long-term non-progressors) and who naturally display low or undetectable HIV-1 viremia keep a normal distribution of NK cell subsets as identified by the expression of Siglec-7 and CD56. Since all these NK cell phenotypic and functional abnormalities are reversible following a successfully suppression of viral replication, the pathological redistribution of NK cell subsets can also be used to monitor the effectiveness of antiretroviral therapy (ART) (17).

Finally, we recently reported that the NK cell modulation of Siglec-7 in HIV-1 infection is directly involved in HIV-1 pathogenesis (54). In fact, chronic high levels of viral replication lead to a decreased surface expression of Siglec-7 on NK cells counterbalanced by increased levels of soluble Siglec-7 detected in the plasma of viremic HIV-1-infected patients. This soluble form of Siglec-7 is able to directly bind the glycoprotein 120 expressed on HIV-1 envelope and facilitates the infection of $Siglec\text{-}7^{neg}/CD4^{pos}$ T cells. In contrast, high levels of HIV-1 viremia do not alter the constitutive expression of Siglec-7 in monocytes and macrophages, whose susceptibility to HIV-1 infection is enhanced by the direct interaction between the virus and this lectin-type receptor (33, 54). These data suggest that, similar to other members of Siglec family (55–57), both membrane-bound and soluble Siglec-7 greatly increase the susceptibility of $CD4^{pos}$ cell targets expressing CCR5 or CXCR4 to be infected by HIV-I (54).

ORIGIN OF THE CD56^{neg} NK CELL SUBSET

Natural killer cells resulted not to be directly infected by HIV-1 (45) and, therefore, it is unlikely that the expansion of CD56^{neg} NK cell is HIV-1 specific. Indeed, although the origin of CD56^{neg} cells is still being debated, it later became clear that high frequencies of this pathological subset are associated with the presence of chronic and systemic inflammation, which is a hallmark of chronic HIV-1 infection (10, 12). Several studies then investigated other human disorders characterized by high levels of systemic immune activation and reported similar increased percentages of circulating CD56^{neg} NK cells. Among these diseases, there are hepatitis C virus (HCV) (58, 59), human cytomegalovirus (HCMV) (60), hantavirus (61), treponema pallidum (62) infections, post-transplant lymphoproliferative malignancies driven by Epstein–Barr virus (EBV), (63) and autoimmune disorders such as myasthenia gravis (64) and dermatomyositis (65). Expansion of CD56^{neg} NK cells has been described both in HCV as well as in HCV–HIV co-infected patients (59). However, the increase of this aberrant subset is much more contained in mono-infection by HCV compared to HCV–HIV co-infection. Therefore, additional studies are required to clarify whether the accumulation of CD56^{neg} NK cells is a hallmark of chronic HCV infection. Finally, a significant proportion of CD56^{neg} cells have also been found in umbilical cord blood and in healthy infants and are characterized by impaired anti-viral activities (66–69).

The fact that high frequencies of CD56^{neg} NK cells are found in so many different either pathological or physiological conditions underline that their ontogenesis relies on mechanism(s) associated with activation of the immune system and not with a specific viral infection or inflammatory disorder. Moreover, in all the above-mentioned disorders as well as in umbilical cord blood, CD56^{neg} cells share the same phenotypic and functional features: (i) low expression of natural cytotoxity receptors (NCRs) and Siglec-7; (ii) reduced cytolytic potential; (iii) decreased production of anti-viral cytokines and chemokines. In regard to their ontogeny, it has been first postulated that this "anergic" CD56^{neg} cells could arise from a failure of NK cell development and/or from inadequate cell stimulation. This theory was mainly supported by experimental evidence showing that the incubation *in vitro* of CD56^{neg} cells with IL-2, IL-12, and IL-15 induces cell proliferation and restores the classical distribution of CD56 and repertoire of NKRs (44, 59, 67). We have to point out though that therapies with anti-viral drugs (in case of HIV-1 and HCV infections) or with immunosuppressants (for myasthenia gravis and dermatomyositis) restored physiological NK cell phenotype and functions (16, 59, 64, 65).

Unfortunately, there are no reports showing that an *in vitro* setting could reproduce the expansion of CD56^{neg} cells and this hampered our capacity to disclose the mechanistic insights highlighting this phenomenon. Our current knowledge of NK cell ontogenesis states that CD16^{neg} immature NK (iNK) cells expressing low levels of CD56 and NCRs precede CD56^{bright} NK cells in development (43, 70). Although sharing these phenotypic features with iNKT, CD56^{neg} NK cells also express many NK cell-specific receptors that are not found on iNK cells, including KIRs, CD94/NKG2A, NKG2D and CD16 (10, 43). iNK cells produce high amounts of GM-CSF but not other inflammatory cytokines following phorbol myristate acetate (PMA)/ionomycin stimulation, and they are not even able to kill target cells nor to produce cytokines (70). Moreover, iNK cells develop into CD56^{bright} NK cells after stimulation with IL-15, thus suggesting they are precursors of these cells *in vivo* (70). On the contrary, CD56^{neg} NK cells retain inflammatory cytokine production and killing capacity, albeit at impaired level compared to conventional CD56^{pos} NK cells (10, 17). Finally, CD56^{neg} cells but not iNK cells have been recently shown to express CD57 (71), a marker of terminal cell differentiation (72). Taken together, these experimental findings strongly suggest that CD56^{neg} NK cells do not derive from iNK cells.

An alternative hypothesis proposed that CD56^{neg} NK cells are mature lymphocytes that recently engaged target cells. Under normal circumstances, NK cells are capable of killing multiple target cells, thus resulting in a reduced, but never complete, loss of perforin and granzyme B (73). In HIV-1-infected patients, decreased granzyme B and perforin expression and increased surface expression of CD107a in the absence of *ex vivo* stimulation suggests that CD56^{neg} NK cells engage target cells *in vivo*. Authors also argued that this hypothesis is further supported by the increased expression of CD95 on CD56^{neg} cell subset, thus indicating a more pronounced activation compared to their CD56^{dim} cell counterpart (71). However, the down-regulation of CD56 as a consequence of recent activation is still awaiting confirmation by additional studies. Since the transcriptome of CD56^{neg} NK cells has been found to be more similar to myeloid cells than to traditional CD56^{dim} NK cells (74), CD7, a surface protein expressed on thymocytes and mature T cells, has been proposed as an additional informative marker for their identification. In this regard, CD56^{neg} NK cells have been reported to be a mixed population of CD7^{pos} true NK cells and CD7^{neg} myeloid cells present at a low frequency in healthy donors and expanded in HIV-1 viremic subjects (71).

NK CELL RESPONSES TO HUMAN CYTOMEGALOVIRUS

Similar to what it has been observed in humans (1), mice infected with murine cytomegalovirus (MCMV) and depleted of NK cells were unable to control infection and displayed disseminated MCMV in the lungs and in the liver (75). The anti-viral NK cell responses are particularly relevant when viruses exploit mechanisms of immune evasion, leading to the down-regulation of MHC-I molecules and thus escape from the CD8^{pos} T cell-mediated cytolytic activity. Multiple HCMV-derived proteins have been demonstrated to interfere with the transport or the expression of MHC-I on the cell surface (76). On the other hand, HCMV-infected cells up-regulate ligands for the aNKR NKG2D, including MICA, MICB, and UL-16 binding proteins (ULBPs), thereby facilitating NK cell activation (77–79). On average, 50–80% of the human population in Western countries is HCMV-infected, but the virus does not harm the health of immune-competent individuals, unless in specific situations such as maternal HCMV reactivation or primary infection during pregnancy. Instead, immune-compromised individuals, such as those infected with HIV-1 or those who received bone marrow transplantation (BMT), are particularly susceptible to CMV reactivation (80). Following BMT, NK cells recover faster than CD8^{pos} T cells (2–3 weeks vs. 4–6 weeks, depending on the type of transplantation) and possibly mediate a first line of protection against viral dissemination (81). Different groups have recently shown that patients experiencing

HCMV reactivation following BMT display highly mature KIR^{pos}, NKG2A^{neg}, NKG2C^{pos}, and CD57^{pos} NK cells that are not found in uninfected recipients (82–84). A similar NK cell phenotype is observed following acute HCMV infection in healthy individuals (72). Interestingly, modification of the NK cell surface phenotype did not change with resolution of the infection (83), thus suggesting a stable imprinting in the NK cell maturation stage. Similar to what it has been observed in HCV and HIV-1 infections (33, 43), HCMV-reactivations in patients undergoing umbilical cord blood transplantation induce the expansion of the CD56^{neg}/CD16^{pos}/Siglec-7^{neg} NK cell subset (60). The expansion of anergic CD56^{neg} NK cells following HCMV reactivation likely occurs when T cell immunity is impaired and supports the hypothesis that HCMV has a role in immune-senescence (85, 86).

Multiple components of the immune system are thus engaged to protect the host from reactivating CMV replication. The sole NK cell response is likely not sufficient in this regard and must act in concert with recovering CMV-specific CD8^{pos} T cells with the support of CMV-specific CD4^{pos} T cells. This is well demonstrated in individuals infected with HIV, where the loss of antigen-specific CD4^{pos} T cells causes increased susceptibility to opportunistic infections, including CMV reactivation (87). These data altogether suggest that NK cells could be exploited together with CD8^{pos} T cells in the therapeutic treatment of CMV reactivation.

HCMV AND MEMORY NK CELLS

Studies in mice led to the demonstration that a population of "memory" NK cells develop following acute infection with MCMV. In particular, it has been reported that NK cells expressing the activating Ly49H receptor undergo a clonal-like expansion upon recognition of the MCMV-encoded m157 antigen and generate long-lived memory NK cells (88). Similarly in humans, it has been recently demonstrated that NKG2C^{pos} NK cells remarkably increase in frequency following HCMV infection or reactivation and can persist for years (60, 72, 89–92). Such expanded NKG2C^{pos} NK cells may exert an efficient anti-viral activity by producing higher amounts of cytokines, in particular IFN-γ, hence suggesting that "memory-like" NK cell responses may occur in humans as well. However, NKG2C cannot be considered as a univocal marker of "memory-like" NK cells. Indeed, recent reports suggest that also other NKRs that are preferentially found in terminally differentiated NK cells, including activating KIRs or CD57, are up-regulated following HCMV reactivation. In this context, a number of recent studies suggest that the presence of activating KIRs correlates with protection against viral infections (84, 93).

Despite "memory-like" NK cells mostly display a CD56^{dim}/CD16^{pos} phenotype (60, 72), they have been shown to share some phenotypic traits with CD56^{neg}/CD16^{pos} NK cells as well. These similar traits between "memory-like" and CD56^{neg}/CD16^{pos} NK cells are not supported by functional features, as the former are expected to display increased functional capacity while the latter are well known to be impaired in a number of effector functions. Further studies are needed to confirm if the KIR^{pos}/NKG2A^{neg}/NKG2C^{pos} CD57^{pos} NK cells represent the human memory NK cell counterpart and to test whether they share any of the CD56^{neg}/CD16^{pos} NK cell properties. Importantly, it would be interesting to determine whether continuous stimulation of NK cells by persistent, but undetectable viral replication, in infected individuals plays a role in maintaining these mature NK cell phenotypes.

CONCLUDING REMARKS

Viruses employ several strategies to escape from NK cell-mediated clearance of virally infected cells or secretion of anti-viral cytokines. In particular, it became clear that viruses are able to affect the functional status and the homeostasis of NK cells through the modulation/engagement of several surface receptors and through the expansion of unconventional NK cell subsets. Although many aspects of NK cell physiology have been disclosed by taking lessons from the physiopathology of viral infections, several fundamental questions still remain to be answered. First of all, the origin and the expansion of the CD56^{neg} NK cells subset that represents the largest fraction of total NK cells in late stages of HIV-1 infection and that highly contributes to the lack of viral control and to disease progression. Disclosing the mechanisms underlying the high frequencies of this highly anergic NK cell population is key to better understand not only NK cell development but also to make it possible the manipulation of these cells *in vitro* in order to improve their anti-viral potential and provide a better control of viral replication and spreading.

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Baseline and dynamic expression of activating NK cell receptors in the control of chronic viral infections: the paradigm of HIV-1 and HCV

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Natural killer (NK) cell function is regulated by a balance between the triggering of activating and inhibitory receptors expressed on their surface. A relevant effort has been focused so far on the study of KIR carriage/expression setting the basis for NK cell education and self-tolerance. Focus on the evolution and regulation of activating NK receptors has lagged behind so far. Our understanding of activating receptor expression and regulation has recently improved by evidences derived from *in vitro* and *in vivo* studies. Virus infection – either acute or chronic – determines preferential expansion of NK cells with specific phenotype, activating receptors, and with recall-like functional activity. Studies on patients with viral infection (HIV and HCV) and specific diverging clinical courses confirm that inter-individual differences may exist in baseline expression of natural cytotoxicity receptors (NKp46 and NKp30). The findings that patients with divergent clinical courses have different kinetics of activating receptor density expression upon NK cell activation *in vitro* provide an additional, time-dependent, functional parameter. Kinetic changes in receptor expression thus represent an additional parameter to basal receptor density expression. Different expression and inducibilities of activating receptors on NK cells contribute to the high diversity of NK cell populations and may help our understanding of the inter-individual differences in innate responses that underlie divergent disease courses.

Keywords: natural cytotoxicity receptors, NKp46, NKp30, HIV, HCV, regulation

INTRODUCTION

Natural killer (NK) cells represent a cellular component of the innate immune system. They circulate in peripheral blood and peripheral tissues, and may be more abundantly recovered in secondary lymphoid organs and in some non-lymphoid organs (e.g., the liver). They are characterized by considerable cytotoxic activity, which is due to the constitutive expression of perforin and granzyme, which may be promptly released upon cell triggering (1, 2). In addition to this unique feature, which contributes to the high efficiency with which NK cells are suited to kill virally infected or tumor cells, their function also includes the production of cytokines, such as IFN-γ, tumor necrosis factor (TNFα) and G-CSF, and the early release of chemokines (MIP-1a/b, RANTES) (3). NK cell function is finely regulated by the interplay of a wide array of activating and inhibitory receptors expressed on their surface (1). The accurate regulation of this signaling system guarantees that under baseline conditions their exceptional ability to rapidly kill is harnessed by inhibitory receptors mostly (but not exclusively, e.g., Siglec7 and IRP60) specific for "self" MHC class I molecules (2), which turn NK cells "off" and normally prevent NK-mediated lysis of HLA class I^+ autologous cells.

Natural killer cell activating stimuli are manifold and act through triggering of different groups of receptors expressed on their surface (4). Activation of NK cells is determined by triggering the major activating receptors, which include NKp46, NKp30, NKp44 (i.e., natural cytotoxicity receptors, NCRs) NKG2D, and FcγR (CD16) as well as other receptors and co-receptors including, NKG2C (a lectin-type triggering receptor which dimerizes with CD94), 2B4 (CD244), NKp80, DNAM-1, and NTB-A (5). Stimuli delivered through other groups of receptors may also determine NK cell activation including toll-like receptors (TLRs) as TLR2, TLR3, TLR7/8, TLR9, and interleukin receptors (IL-2, IL-12, IL-15, IL-18) and combinations thereof (e.g., IL-2 + IL-15, IL-2 + IL-12, and IL-12 + IL-18) (5–7).

The NK cell receptors repertoire is germ line-encoded and does not undergo somatic recombination. This provides the basis for inclusion of NK cells among innate host defenses as an effective and apparently basic unsophisticated innate defense system which

does not need specific recognition of foreign antigens (e.g., from pathogens or tumors).

INVOLVEMENT OF NK CELLS IN THE TUNING AND CONTROL OF IMMUNE RESPONSES

The original view of NK cells as a purely "primitive," muscular, short-lived, rapid responder cell type has undergone considerable revision and has been considerably updated over recent years. NK cells are presently known to represent long-lived innate cells, whose functional spectrum extends beyond classical search-and-destroy patrolling activity and/or early recruitment of immune responses. NK cell function indeed also includes regulation of other innate and adaptive functions through their direct or indirect reciprocal interaction (crosstalk) with macrophages, polymorphonuclear cells (6, 7), fibroblasts (8), DC (9–11), and T cells.

Natural killer cells interact with and respond to either pathogen-infected or tumor-associated macrophages, and their response is modulated by macrophage functional polarization. The functional interaction of NK cells with pro-inflammatory (M1) or anti-inflammatory (M2) macrophages relies on DNAM-1, 2B4, and NKp46 receptor signaling, in addition to membrane-bound macrophage-derived IL-18 (12–14). Considering macrophages infected with specific pathogens, different sets of activating receptor–ligand interactions drive NK cell activation in a more pathogen-dependent pattern (6, 11, 15). A decreased NK cell response has been described during their interaction with M2 cells compared to M1 macrophages, or in the tumor environment where NK cell activating receptors are down-regulated (13, 14). In view of the reported recognition of mycobacteria-infected macrophages by NK cells via NKp46 (15), decreased signaling via NKp46 in patients with overt secondary pulmonary TB (16) could represent for example a possible mechanism participating in the so far poorly understood mechanism of exit from latent TB (17).

The reciprocal interaction of NK cells and DC is often referred to as "crosstalk" and involves multiple receptors and cytokines. NK cells and DC show anatomical and functional co-localization in T cell areas of lymphnodes (18) and in inflamed tissues (19). Human DC activate NK cells, via direct interaction and involvement of NKp30 (10, 20) and DNAM-1 (9). NK cells determine lysis of immature HLA class $I^{-/low}$ DC, while mature DC are protected from NK-mediated lysis by high density expression of HLA class I molecules that interact with inhibitory receptors (e.g., KIR) expressed on activated NK cells (11). In turn, NK cells induce DC maturation via TNFα and IFNγ production (10). This DC–NK cell interaction provides a mechanism to edit DC responses and their repertoire by selecting optimally mature DCs (21) and inducing DC to express high amounts of membrane-associated IL-15 (22) with impact on downstream adaptive responses (23). These observations are further supported by the demonstration, *in vitro* with bacterial infection (24) and *in vivo* during HIV infection that the presence of reduced NCR expression (25) and NK cell subset alterations (26) leads to reduced killing of immature DC (20).

Involvement of NK cells in the shaping of adaptive responses extends beyond their crosstalk with DC. In fact, NK cells also directly interact with T cells, favoring antigen-specific $CD8^+$ T cell responses (22, 23, 27). The reciprocity of this circuit has been elegantly shown in the macaque model where Ag-specific $CD4^+$ T-central memory lymphocytes support NK cell activation and function in SIV-controller donors (28).

Therefore, a relevant part of the tuning activity of NK cells on the function and control of other cells is based on direct cell-to-cell interactions and involves activating NK cell receptors. Consequently, different activating NK cell receptor molecule densities may have an impact on their crosstalk with other cells of the immune system. The purpose of this review is to provide a reading frame to differences in static and dynamic NCR expression in subjects displaying clinical divergence upon infection with different viruses.

INNATE OR NOT INNATE, THIS IS THE QUESTION

Until recently, the prevailing expert view attributed to NK cells a limited degree of variability in response to pathogens, and basically assumed stereotyped responses. This concept practically ruled out the possibility of ranges of variability of NK cell responses either against different pathogens within the same subject or to the same pathogen within different patients. This view has been steadily upgraded in recent years. It has been shown for example in mice that infection with viruses and other pathogens determines the expansion of specific NK cell subsets (29–31) which maintain for prolonged periods of time the ability to produce increased amounts of TNFα and IFNγ. This observation is reminiscent of memory T cell function thus suggesting a possible memory-like feature of NK cells. Subsequent observations in human beings showed that also human CMV infection leads to expansion of a subset of $NKG2C^+$ NK cells (32, 33) with memory-like properties. Increased proportions of $NKG2C^+$ NK cells persist (34) after acute infection into latency, and may be observed also after bone marrow transplantation (34, 35). Additional evidences of transient NK cell expansions in human beings are provided by infection with chikungunya and hantavirus (36, 37), and may persist up to 60–90 days. Also in these instances the expanded cells are exclusively $NKG2C^+$, and their triggering results in increased and rapid reactivity with production of IFNγ upon re-challenge. These observations are clearly different from T cell memory, which is conventionally defined by (life)long-lasting antigen-specific recall ability, increased memory T cell receptor (TCR) density, antigen-specific TCRs, and specific markers identifying memory cells (CD45RO vs. CD45RA) (31, 38). In mice, $LY49H^+$-MCMVm157 antigen-specificity and increased protection to Mouse CMV (MCMV) challenge support a resemblance with memory T cell protection (31, 39). In human beings, on the other hand, the expansions are more time-limited with the possible exception of HCMV infection and latency. These human NK cell subset expansions are stereotyped and monomorphic since only $NKG2C^+$ cell expansions are reported, irrespective of the invading pathogen (either HCMV, or hantavirus or chikungunya).

Altogether, the description of pathogen-induced recall NK cell reactivity, even if not fitting with a long-lasting heterogeneous T cell memory (40, 41), has the merit of further expanding our understanding of NK cell function advancing our view beyond the original "first-line of defense" towards a wider horizon of multifaceted NK cell function.

An interesting contribution to the notion of a polymorphism in NK cell responses has been recently provided by mass cytometry study of NK cell receptor carriage (42) showing that up to 30,000 different phenotypic NK cell populations may be harbored in one individual. In addition, environmental factors appear to heavily influence activating receptor carriage, while inhibitory receptor diversity seems to be largely genetically determined (42). In line with this observation, NK cell responses induced by virus(es) are accompanied, not only by expansion of a subset of peripheral NK cells which may be NKG2C$^+$ (33), but also by changes in triggering receptor expression (e.g., NCRs) (34).

NCR EXPRESSION AND HCV INFECTION

Natural killer cell triggering receptors recognize specific ligands induced by either cell transformation or infection [e.g., B7-H6 (41, 43)]. Although cellular ligands for NCRs are still not yet well characterized (e.g., NKp46-L, NKp44-L, and other NKp30-ligands), a range of pathogens including influenza virus, parainfluenza virus, West Nile virus (WNV), dengue virus, and mycobacteria have been shown to interact with NCRs, either directly or after their infection of target cells (44). Accordingly, NK cell first-line defenses against pathogens and regulation of immune responses may be more intertwined than expected at a first look. Indeed, the same receptors that are involved in recognition of infected cells may be also involved in direct pathogen detection and in crosstalk with other cells of the immune system (e.g., monocytes and dendritic cells). Hence, individual differences in baseline NCR expression may underlie and affect divergent host responses to the pathogen.

Natural killer cell triggering is proportional to both the actual number/density of a given triggering receptor expressed on NK cells and to the density of the respective ligand(s) expressed on target cells. At any given level of KIR/HLA interaction and of NK-ligand expression, changes in activating receptor expression density determines proportional changes in NK cell cytotoxic activity(45, 46).

Accordingly, wide inter-individual variations in triggering receptor expression could contribute, at least in part, to the different clinical courses (e.g., from mild disease to life-threatening clinical course) that are observed in patients infected by the same pathogen. When for example considering NKp46 and influenza hemagglutinin (HA), NKp46 (and NKG2D) is necessary for the activation of the human response to influenza infection (47, 48). In mice, induced deletion of the human NKp46 homolog (NCR1) determines lethal influenza infection (49). In addition, NK cell function is impaired in aging mice infected with influenza virus, with reduced production of IFN-γ also upon stimulation with anti-NKp46 mAbs, in line with the suggestion of reduced receptor expression (49, 50).

The possibility that different levels of NCR expression may correlate to different clinical courses emerges also from studies in patients with HCV infection. A relevant association exists between outcome of acute HCV infection and germline carriage of KIR genes and HLA C supertypes (51, 52). Also activating NK cell receptor expression has been shown to associate with different disease courses during HCV infection. In the acute phase of HCV infection, an increase in CD56^{bright} NK cells is observed, and is accompanied by a reciprocal reduction in CD56^{dim} cells (53). In patients who spontaneously clear the virus (HCV), the increase in CD56^{bright} NK cells is transient, with subsequent decline within 1–3 months. This change is however permanent in those who fail to clear HCV and proceed to chronic infection with virus replication (53). Interestingly, NKp30 expression is increased in NK cells from multiply HCV exposed-uninfected intravenous drug users. In these patients, enhanced IL-2-induced cytolytic activity against the NK-sensitive cell line K562 has been also reported (54). The same mechanism of NKp30-associated protection applies to the control of *in vitro* hepatocyte infection (54). In a recent and different setting, increased NKp46, NKp44, and NKG2A expression was detected in NK cells from HCV exposed healthcare workers (HCW), who did not develop disease (55). Interestingly, in this series, HCV-specific T cell responses to non-structural gene products were detected in the absence of B cell responses and of HCV-specific antibody production. This observation is reminiscent of a phenomenon occurring in HIV-uninfected children born to HIV-seropositive mothers. These children lose maternal antibodies are uninfected and seronegative, but show HIV-specific cytotoxic activity by CD8$^+$ T cells (56, 57). An analogy is evident in results obtained 20-years apart in different models of human infection and raises the possibility that inducibility of NCRs on NK cells in some patients may be associated with discordant T and B cell responses and with protection from infection.

Differences in NCR expression are detected also when exposed patients become acutely infected and display HCV viremia. In this case, lower frequencies of NKp46- and NKp30-expressing NK cells are observed compared to healthy donors, and this phenotype correlates with HCV clearance (58). Thus, NCR (NKp46 and NKp30) expression on peripheral NK cells is different when acutely infected patients (low NCRs) are compared to exposed-uninfected patients (high NCRs). The observed difference may be for example due to inherent, preexisting baseline differences that become evident following patient selection in different series. Alternatively, in patients who resolve acute infection, the lower expression of NKp46- and NKp30 molecules on NK cells could be due to increased margination of NK cells to the site of active HCV replication (i.e., liver) (59). A further possible explanation for these differences could be represented by different inductions of NCR expression in different patient groups upon challenge with the virus (HCV). In such a scenario, early responders would rapidly upregulate NCR expression, avoid establishment of infection, and evolve to the clinical state of exposed-uninfected subjects.

During chronic HCV infection, imbalances in peripheral NK cells were originally described, with a deficient ability to activate DCs due to the interaction of NKG2A with HLA-E expressed on hepatocytes (60). This interaction is associated to IL-10 production (60, 61) and to reduced ability of IFNγ production upon IL-12 stimulation or with other stimuli (62). With regard to NCR expression, either increases or decreases in NCR expression are observed (61, 63) and are related to their ability to respond to IFN-α-containing treatment regimens. Indeed, patients who clear the infection upon dual treatment with pegylated-IFN-α + ribavirin (PegIFNα–ribavirin) have lower baseline (pre-treatment) expression of NKp30 and of CD85j, compared to those who subsequently fail the treatment (null responders, partial responders) (64). The decreased expression of NKp30 in these patients is not reflecting

a defect in NK cell function, but rather an individual difference in the regulation of receptor expression. This is confirmed by the successful induction *in vitro* of NKp30 expression on purified NK cells cultured in the presence of IFN-α, with correspondingly increased receptor-mediated function. These observations are in line with evidences deriving from the study of intrahepatic interferon-stimulated gene (ISG) expression (65, 66). In the liver, ISG is already upregulated before treatment in patients who will not respond to IFN-α and ribavirin dual treatment. Patients who will clear virus upon treatment, on the contrary, have baseline lower ISG expression which may be induced during treatment (66). Thus, inherent individual regulation of crucial gene expression is present during chronic HCV infection and extends from ISG in hepatocytes to NCRs.

Therefore, differences in baseline NCR expression and in NK cell phenotype can be accurately detected (42) and are associated with diverging clinical courses in subjects exposed to HCV. Lower NCR expression, albeit inducible, represents an advantage and appears to be inherently regulated in a subset of chronically infected patients. In this context, the increased NCR expression observed on NK cells in exposed-uninfected patients may be the result of repeated HCV challenge in patients with lower baseline NCR expression.

NCR EXPRESSION AND DIVERGING CLINICAL COURSES IN HIV INFECTION

During HIV infection, the virus targets NK cell-mediated responses with similar or possibly higher intensity compared to other arms of innate or adaptive immunity. NK cells are part of the early response that controls acute viremia during primary HIV infection. Similar to HCV exposed-uninfected patients (54, 55), increased NK cell activity has been detected also in HIV exposed-uninfected patients (67). Activating receptor expression was not addressed in this work, and materials for monitoring (anti-NCR mAbs) were not yet available at the time. Although the mechanisms leading to increased NK cell function in those HIV exposed-uninfected remain still not defined, increases in the expression of triggering receptors on NK cells could have been possible, similar to what has been observed after exposure to HCV (54, 55). If so, this would fit in a broad concept of an advantage against infection in subjects whose NK cells achieve rapid dynamic increases in NCR expression after virus challenge.

Once HIV viremia is established, NK cell derangement can be detected in infected patients soon thereafter (68–70) and includes imbalances in activating and inhibitory receptor expression, altered circulation of NK cell subsets, and impairment of NK cell function (25, 26, 71, 72). Viremic HIV-patients have dramatic decreases in activating receptor expression (NKp46 and NKp30) on NK cells *in vivo* (25), up to one-third of circulating NK cells display activation markers (HLA-DR and CD69) (72) and an apparently poorly functional subset of $CD56^{-}CD16^{+}$ appears, which displays expression of low levels of NCRs (26, 72). The impairment of NK cell function has a relevant impact on DC editing. In addition to the reduced activating receptor expression on NK cells and appearance of poorly functional $CD56^{-}CD16^{+}$ NK cells in peripheral blood, HIV infection reduces the expression on $CD4^{+}$ T and other target cells of ligands of activating NK cell receptors which are important in triggering NK cell cytotoxicity and cytokine production (e.g., PVR, NKp46-L, and NKp30-L) (73–76). Thus, while HIV adopts multiple strategies to evade NK cell surveillance, conserved (or restoration of) activating receptor function may represent a fundamental barrier to virus spread.

From a clinical standpoint, during the first years of the epidemic, it became soon clear that consistent clinical variability could be observed among patients even in the absence of successful treatment. A benign disease course in otherwise untreated patients was identified through the observation of HIV-infected patients with long-term non-progressing disease (i.e., high $CD4^{+}$ T cells $>450/\mu l$ and low level viremia, LTNP) and of patients with high $CD4^{+}$ T-lymphocytes and undetectable viremia (elite controller, EC) (77, 78). In addition, upon progressive disease with decreasing $CD4^{+}$ cell counts, diverging courses are observed. At any given $CD4^{-}$ count, opportunistic infections or cancers may appear in some – but not all – patients (79–81) and AIDS-defining disease including Kaposi Sarcoma, non-Hodgkin Lymphoma, or Tuberculosis may occur before $CD4^{+}$ counts fall below 200–350/μl (82–85). On the other hand, in the pre-highly active antiretroviral treatment (HAART) era, some untreated patients could reach very low $CD4^{+}$ T cell counts without progressing to AIDS and showed surprisingly preserved NK cell numbers and function (86). Following HAART, NK cells fail to fully recover IFN-γ production and phenotype (87, 88) and surprisingly maintain high levels of activation, as defined by HLA-DR expression (46, 89). This extensive list of examples shows the profound impact of personal clinical divergence in everyday HIV clinical practice.

Divergent clinical courses during HIV infection cannot be fully accounted for by $CD4^{+}$ cell numbers alone. Data from NK cell function and receptor expression may be used in this context to help understand existing differences. When antiretroviral treatment is interrupted in chronically infected patients, viral replication invariably resumes even after thorough and extensive treatment (90). Trials of $CD4^{+}$-guided treatment interruptions (CD4GTI) in patients with high $CD4^{+}$ T cell showed that the rate of $CD4^{+}$-cell loss after discontinuation of antiretroviral treatment is dishomogeneous with ample divergence and inter-individual differences (91, 92). Factors associated with different rates of $CD4^{+}$ T cell loss upon CD4GTI include not only proviral DNA changes (93), increased proportion of $CD4^{+}CD127^{+}$ cells (94, 95), but also a different baseline NK cell expression and function (96). At baseline (i.e., before treatment interruption), patients with long treatment interruptions (i.e., without the need of resuming HAART) due to persistently high $CD4^{+}$ cell counts had lower proportions of $CD56^{bright}CD16^{\pm}$ NK cells, and also expressed lower levels of NKp30 and NKp46 activating receptors on NK cells (96). Interestingly, this observation is in agreement with the findings from chronically HCV-infected patients, where those who will clear the virus after PegIFNα–ribavirin treatment have low – and inducible – NCR (NKp30) expression on their NK cells at baseline (64).

In addition, inherent differences in NK cell receptor expression are observed also in HIV-patients with low $CD4^{+}$ cell counts ($<220/\mu l$) but divergent clinical course. Those who have AIDS-defining opportunistic infections (i.e., PCP and neurotoxoplasmosis) have lower NKp46 expression and low

DNAM-1/NKG2D/NCR:ligand ratios compared to patients who reach similarly low $CD4^+$ cell counts but do not develop AIDS (46). These results therefore indicate that similar to HCV patients, differences in NK cell regulation underlie divergent clinical courses also in HIV-patients, irrespective of their $CD4^+$ T cell count, of HAART or virus replication. Importantly, this view is not limited to chronic infections. Support to the general view of inherent specific NK cell signatures underlying divergent disease course is provided also from analyses in cancer patients with recurrent disease, either gastrointestinal stromal tumor (GIST) (97) or breast cancer (98).

INDUCIBLE NCR EXPRESSION AND CLINICAL DIVERGENT DISEASE COURSES

Although analysis of NCR expression on peripheral NK cells may reflect baseline regulations in NK cell subsets, it represents only a "frozen" view and does not provide insights on their inducibility or "dynamic" regulation. In this regard, *in vitro* study of NCR expression in purified NK cells recently revealed that different induction kinetics over time may be detected in HIV-patients (99). Indeed, when patients with non-progressive disease course (EC and LTNP) were compared to HAART-treated aviremic patients, relevant differences were detected. In EC/LTNP patients, purified NK cells display increased NKp46 expression 2 days after *in vitro* activation with IL-2, with subsequent return to baseline expression 4 days thereafter. Also NKp30 expression is induced in EC/LTNP upon *in vitro* activation with progressive increased densities until 4 days after activation. On the contrary, HAART-treated progressor patients with undetectable HIV-RNA do not show any induction of NKp46 or NKp30 (99). The conserved induction of NCRs in non-progressor patients provides the basis for an intact NK cell function, conserved crosstalk with DCs and downstream specific $CD8^+$ CTL responses (20, 100, 101). Interestingly, also in HCV-infected patients inducibility of NKp30 is associated with a different (improved) disease course compared to patients lacking this regulation (64). An additional observation of considerable interest in this context was the lack of inducibility of NKp44 in EC/LTNP patients compared to progressor patients (99). This apparent "fault" with failure to rapidly upregulate NKp44 molecule expression upon activation, might actually be highly protective once HIV infection has established. Indeed, HIV_{gp41} S3 peptide shedding in infected patients (102) induces expression of NKp44-ligands in uninfected $CD4^+$ T cells. The apparent "faulty" induction of NKp44 thus would avoid innocent bystander killing of $CD4^+$ NKp44-L^+ cells by activated NK cells *in vivo*. Support to this hypothesis is directly provided by the demonstration that HIV_{gp41} vaccination prevents from shedding of the S3 peptide and from NKp44-L expression thus protecting $CD4^+$ T_{CM}-lymphocytes in SHIV-infected non-human primates (103).

A limitation to interpretation of NCR expression/induction is represented by the lack of molecular and genetic proof so far in the regulation of NCR responses and inducibility, with only

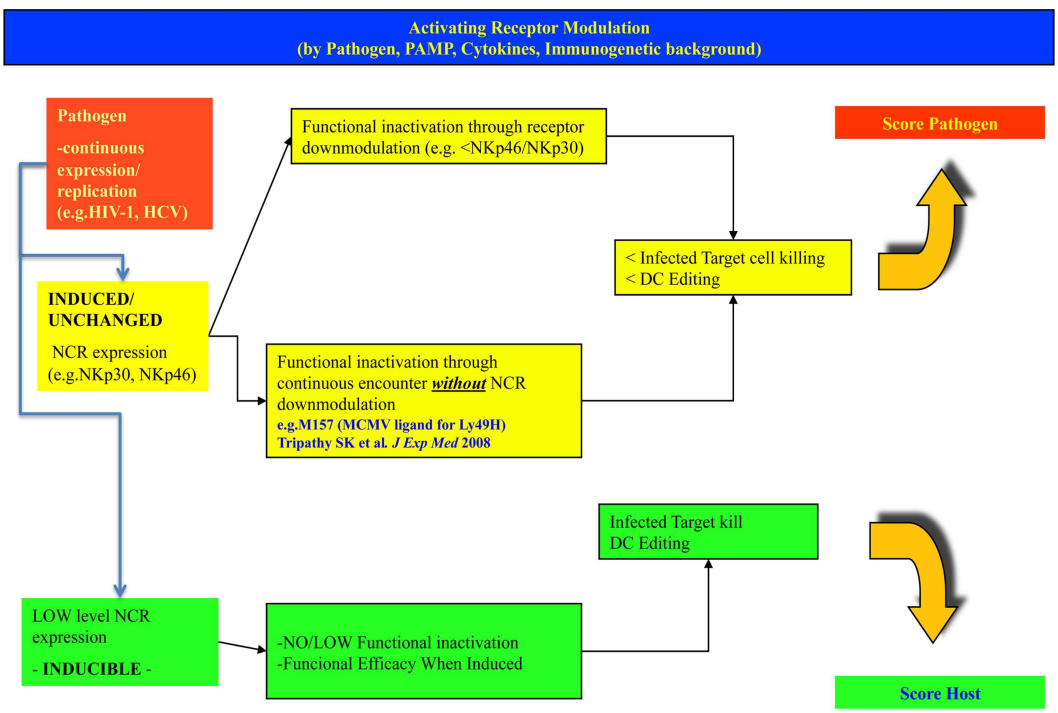

FIGURE 1 | Different levels and modulation of natural cytotoxicity receptors on NK cells may lead to different outcomes in the fight against invading intracellular pathogens. The figure indicates possible alternate patterns of NK cell NCR expression/induction upon infection with a pathogen. In the upper row, the case for high basal expression is shown, leading to receptor inactivation and progressive infection. In the lower row, the case of a low/inducible NCR expression is shown, with inducibility upon strong challenge with successful control of the pathogen. The hypothesis considers a spectrum of intermediate conditions (not shown here).

limited information being available (104). Also, there is poor understanding of the mechanism(s) underlying "protection" from adverse disease in the presence of low-inducible activating receptor expression. It has been suggested that low-inducible NCR expression may provide advantage in the case of overwhelming stimulation of NK cells due to a relative preservation from their excessive activation with detrimental activation of other innate and adaptive immune mechanism(s) (44). In this regard, it has been shown that homo-oligomerization of NKp30 in the plasma membrane of NK cells is favored by IL-2-dependent up-regulation of NKp30 expression and improves recognition and lysis of target cells by NK cells (105). Interestingly, independent studies showed that viruses may address NKp30 receptors inactivating their function, as shown for NKp30 signaling by vaccinia virus HA (106). Thus, low expression of NKp30 with maintained inducibility, as in the case of HCV and HIV (64, 96, 99), could represent an advantageous inherent individual trait. Subjects carrying this characteristic would evade NK cell-targeting by virus(es) due to lower exposure of a given NCR (e.g., NKp30) to hyperactivation and inactivation (107). At the same time, they would still be able to upregulate molecule density upon activation (e.g., IL-2 and IFNα) thus reaching critical receptor homo-oligomerization with binding to its ligand (e.g., infected cells and DCs).

Evidences supporting memory-like NK cell responses and those showing inducibility of NCR receptor expression on NK cells are derived from clearly distinct settings and should be considered unrelated unless differently proven. Indeed, changes in cytokine production and in activating receptor expression have been reported during memory-like NK cell responses following CMV, hantavirus, or chikungunya virus infection (34, 36, 37), while NKp30 inducibility has been observed in some HCV or HIV infected patients (16, 96, 99). In addition, memory-like NK cell responses have been so far attributed only to NKG2C$^+$ NK cells, while NCR inducible NK cell responses are largely NKG2C unrelated, and show a considerably shorter kinetic (2–4 vs. 30–60 days).

The concept of host antiviral NK cell-associated defenses may however be expanded to encompass both rapid inducibility of NK cell responses after virus infection and memory-like NK cell responses within the frame of NK cell diversity modulated by environmental factors (42). Diverging NCR inducibility, similar to memory-like NKG2C expansion both contribute to host protection and may represent different perspectives of a multifaceted ability of NK cells to adapt to tackle different invading pathogens.

CONCLUSION

Manifold inter-individual differences in the regulation of innate defenses have been described until very recently. The immunogenotype of several components of innate immunity strongly influences the risk to contract infections and also the outcome of their treatment. Genome-wide association studies showed that polymorphisms of innate components are associated to individual variable response to treatment and to disease progression. Nucleotide polymorphisms of TLRs [TLR-4 (108, 109), TLR-1 and 6 (110)] and of pentraxin-3 (111) are associated to increased risk of invasive aspergillosis or mycosis in bone marrow transplant

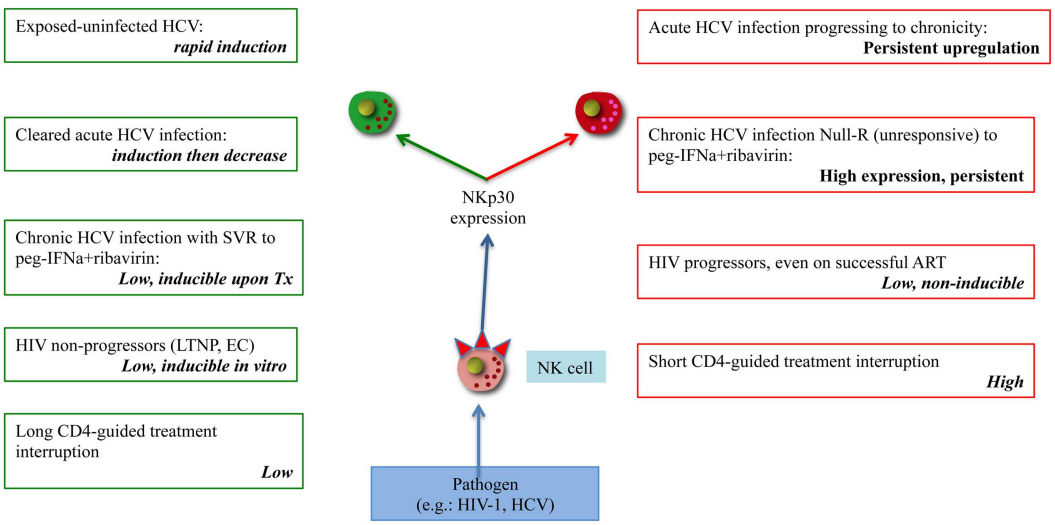

FIGURE 2 | Organization of evidences supporting clinical divergence in HIV-1 or HCV-infected patients according to NKp30 expression/regulation on peripheral NK cells. The left column lists evidences showing favourable disease course in the presence of receptor inducibility. The left column lists evidences for unfavourable disease course in the presence of high basal expression without inducibility or no inducibility regardless of expression.

patients (110, 111). Similarly, TLR3 polymorphisms have been associated to pneumonia development in children with influenza virus infection (112). In addition, IL-28B single nucleotide polymorphisms are associated to prognosis of treatment in HCV infection using IFN-α-containing regimens (113–116).

The presently discussed modulation of NCR expression (NKp30, NKp46, and NKp44) has been described also in chimpanzees (117), and thus appears to represent an innate mechanism of protection against chronic infections that is conserved in evolution and that provides inherent individual diversity in chronically infected (HIV and HCV) patients where it contributes to explain clinical divergence (**Figures 1** and **2**).

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Human decidual NK cells: unique and tightly regulated effector functions in healthy and pathogen-infected pregnancies

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NK cells present in the peripheral blood (PB) respond rapidly to pathogens or pathogen-infected cells by various means including cytotoxicity and release of cytokines and chemokines. In addition they modulate adaptive immunity via the interaction with dendritic cells. Decidual NK cells (dNK) are poorly cytotoxic in healthy pregnancy, both in humans and rodents, when compared to their PB counterparts. We will discuss recent findings that may contribute to answer the following questions: (i) Do dNK possess functional killing machinery in normal healthy pregnancy? (ii) If so, what are the regulatory mechanisms that negatively control this effector function? (iii) Have dNK from early pregnant uterus the intrinsic ability to kill pathogen-infected autologous maternal uterine cells and/or produce soluble factors that stimulate the anti-pathogen adaptive immune response? (iv) Do dNK undergo a receptor repertoire profile shift when they are in contact with pathogen-infected uterine cells? (v) Which pathogen-mediated signal(s) and molecular interactions subvert the inhibition of dNK cytolytic activity?

Keywords: decidual NK cell, pregnancy, cytotoxicity, cytokine, angiogenic factors, cytomegalovirus, pathogens

INTRODUCTION

During the last decade, an increasing body of data based on genetic and functional studies have provided compelling evidence that during early healthy pregnancy, decidual NK (dNK) cells, the dominant lymphocyte population accumulated in the *decidua basalis* where the trophoblast cells infiltrate, exhibit unique functional and phenotypic characteristics (1–5). More than 95% of dNK are $CD56^{bright}$ $CD16^{neg}$ $CD160^{neg}$ (6). As opposed to dNK, the major peripheral blood (PB)-NK cell subset is $CD56^{dim}$ $CD16^{+}$ $CD160^{+}$, a specific phenotype associated with cytotoxic activity (6, 7). dNK cells express the two-domain killer immunoglobulin-like receptor (KIR) at higher frequency than PB-NK (8). This KIR2D repertoire is biased toward recognizing HLA-C, the dominant KIR ligand expressed by fetal extravillous trophoblast (8). PB-NK mostly contribute to the innate immunity against pathogen-infected or tumor cells by their cytolytic activity as well as release of pro-inflammatory cytokines and chemokines (9). In healthy pregnant uterus, dNK are poorly cytotoxic and have specialized pregnancy-specific functions, including a capacity to produce angiogenic molecules and to control trophoblast invasion (10–15). Until very recently (16), little information existed on the dNK capability of limiting local decidual pathogen infection.

DECIDUAL NK CELLS PRODUCE SOLUBLE FACTORS THAT INFLUENCE PLACENTAL DEVELOPMENT IN NORMAL PREGNANCY

Human dNK, in contrast with PB-NK, control two crucial functions in early healthy pregnancy. Firstly, dNK promote vascular growth in the decidua through the production of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) (9, 13, 17–19) as well as of angiopoietin 1, angiopoietin 2, and TGF-β1 (15, 20). The release of these proangiogenic factors by dNK cells depends on the engagement of both NKp30 and NKp44 activating receptors by their specific ligands present on stromal decidual cells and extravillous trophoblast (17). Engagement of another NK cell receptor, KIR2DL4, by soluble HLA-G specific ligand was shown to induce the production of proangiogenic cytokines (21). Since KIR2DL4 is expressed by dNK either at the cell surface (22) or intracellularly (21), and soluble HLA-G is secreted by trophoblast (23), this receptor-ligand couple is likely to contribute to the remodeling of the maternal vasculature in early pregnancy. The same authors have demonstrated that the KIR2DL4-induced senescence secretome of PB-NK can promote vascular remodeling and angiogenesis (24). Analysis of the dNK cell transcriptome revealed a strong similar senescence signature (24). Secondly, dNK release interleukin-8 (IL-8) and interferon-inducible protein-10 (IP-10) chemokines that favor the migration of the extravillous cytotrophoblast into the *decidua basalis* by interacting with the CXCR3 and CXCR1 receptors expressed by the trophoblast cells (17). Migration of extravillous trophoblast cells results in the invasion of spiral arteries thus contributing to the uterine vascular remodeling crucial for the placental development and outcome of pregnancy (25). Such production of chemoattractants by dNK is due to the engagement of NKp30 and NKp44 by their specific ligands expressed by trophoblast and stromal decidual cells (17). Similar interactions between dNK and trophoblast

were observed in pregnant mouse, leading to mesometrial spiral artery remodeling (26–28). Activation of the KIR2DS1 dNK receptor by HLA-C2 was shown to produce the GM-CSF soluble factor, which enhanced migration of trophoblast (29). Other reports from our laboratory indicated that a specific engagement of NKp30 activating receptor on dNK induced the release of several soluble inflammatory and growth factors, including IFN-γ, TNF-α, MIP1-α, MIP1-β, and GM-CSF (22, 30). During viral infection, these molecules are important to recruit and activate eosinophils, macrophages, and dendritic cells (31).

DO DECIDUAL NK CELLS POSSESS FUNCTIONAL LYTIC MACHINERY IN NORMAL HEALTHY PREGNANCY?

Human PB-NK are important innate immune effector cells that rapidly respond to and destroy malignant or virally infected cells (32). PB-NK kill infected cells via the polarized release of cytotoxic granules and the formation of immune synapse between the target cell and the PB-NK. The balance between activating and inhibitory NK cell receptor engagement upon recognition of specific cellular ligands determines the degree of NK cell activation or inactivation. In contrast to PB-NK, it has been demonstrated that both human dNK and mouse uterine NK cells displayed a poor ability to kill various classical NK cell targets (12, 28, 33). dNK cells fail to polarize their microtubules organizing centers and perforin-containing granules to the synapse when they are in contact with HLA class I negative target cells (33). Although dNK cells fail to fully exert their lytic properties in normal pregnancy, they have lytic granules containing perforin, granzyme, and granulysin (34), indicating that they are potentially capable of cytolytic activity. Using redirected cell lysis assay, it has been shown that specific monoclonal antibody engagement of NKp46 activating receptor expressed on freshly isolated dNK induced intracellular calcium mobilization, granule exocytosis, and efficient P815 target cell lysis (22). It is of note that uterine NK cells in pregnant mice express the natural cytotoxicity receptor 1 (NCR1), an ortholog of human NKp46 also present in other species (35). This evolutionary conservation suggests that NKp46 could be involved in pathogen recognition (36). Experiments performed in mice homozygous for NCR1 loss revealed the role of NCR1 in the clearance of influenza virus infection (37). It has been demonstrated that granulysin release from mouse uterine NK cells in spontaneous abortion induced apoptosis of extravillous cytotrophoblast (28). All of these data strongly suggested that both human and mouse NK cells have the capability to exert a cytotoxic effector function in the pregnant uterus.

WHAT ARE THE REGULATORY MECHANISMS THAT NEGATIVELY CONTROL THE CYTOTOXIC FUNCTION OF DECIDUAL NK CELLS IN NORMAL PREGNANCY?

The importance of the balance between activating and inhibitory signals mediated by the specific engagement of activating and inhibitory NK cell receptors has been underlined by a number of reports (38). In addition to the inhibitory KIRs, three other dNK receptors play an inhibitory function, namely 2B4, CD94/NKG2A, and LILRB1/ILT2. Whereas 2B4 was shown to be expressed on all dNK (22, 39), both CD94/NKG2A and LILRB1/ILT2 are only present on a subset of dNK (8, 12, 22, 33). Specific interaction between inhibitory dNK receptors and either MHC class Ia (HLA-C) or MHC class Ib molecules (HLA-E, HLA-G) in human play an important role (40). We found that the co-engagement of CD94/NKG2A inhibitory receptor triggered a drastic inhibition of the cytolytic function of freshly isolated dNK from early *decidua basalis* (22). These results suggest that *in situ* the CD94/NKG2A receptor interaction with its HLA-E specific ligand expressed by trophoblast and other decidual cells is a dominant negative regulatory mechanism that likely prevents unwanted cytotoxicity toward non-infected fetal-derived trophoblast cells. Another inhibitory mechanism depends on the engagement of the LILRB1/ILT2 dNK receptor by HLA-G which blocks dNK cytotoxicity (41, 42). It was also demonstrated that VEGF-C released by dNK in early pregnancy induced up-regulation of TAP-1 in extravillous cytotrophoblast protecting them from cytolysis (13). Expression of the inhibitory form of 2B4 receptor on dNK also contributes to negatively regulate lytic function of dNK (39). Several regulatory mechanisms thus contribute to block the functional cytolytic machinery of dNK in normal pregnancy.

HAVE DECIDUAL NK CELLS FROM EARLY PREGNANT UTERUS THE INTRINSIC ABILITY TO KILL PATHOGEN-INFECTED AUTOLOGOUS DECIDUAL CELLS AND PRODUCE SOLUBLE FACTORS THAT STIMULATE THE ANTI-PATHOGEN ADAPTIVE IMMUNE RESPONSE?

A variety of pathogens, including human cytomegalovirus (hCMV) (10), human immunodeficiency virus 1 (HIV-1) (43), hepatitis C virus (44), toxoplasma (45), *Plasmodium falciparum* (46), bacteria in chorioamnionitis (10, 47) can infect the *decidua basalis* and potentially spread to the fetus through anchoring villi which are in contact with maternal blood of the intervillous space (23, 48). We will mainly focus on data dealing with hCMV which frequently infects decidua and has been detected in decidual stromal, macrophages, and endothelial cells (49). Areas of decidual hCMV infection were shown not to contain T cell accumulation, suggesting that, in addition to the humoral immunity (10), dNK could be another effector cell of the local anti-viral innate immunity preventing virus spread to the fetus. This hypothesis was recently validated by the observation that on biopsies of placental samples from hCMV termination, dNK have been identified in the vicinity of hCMV positive cells (16).

DECIDUAL NK CELLS FULLY EXERT THEIR KILLING EFFECTOR FUNCTION WHEN IN CONTACT WITH hCMV-INFECTED AUTOLOGOUS DECIDUAL FIBROBLASTS

A recent report from our lab has demonstrated that dNK play a role in the control of decidual cell hCMV infection (16). When exposed to hCMV-infected decidual autologous fibroblasts, a cell type abundantly present in decidual tissue, dNK acquires major functional and phenotypic changes (16). As opposed to dNK cells in contact to uninfected autologous decidual fibroblast, dNK cells engage immune synapse with hCMV-infected autologous stromal cells, polarize their lytic machinery toward infected target cells and efficiently kill them (16). Thus, the co-culture of dNK from early gestation with infected autologous decidual cells clearly enhances their cytotoxic ability.

DECIDUAL NK CELLS MODULATE THEIR CYTOKINE AND CHEMOKINES PRODUCTION WHEN IN CONTACT WITH hCMV- OR HIV-1-INFECTED DECIDUAL FIBROBLASTS

Co-culture of dNK from early gestation with hCMV-infected autologous fibroblast induced a significant increased secretion of VEGF-A, sICAM, CXCL-1, IL-6, Granzyme-B, and MCP-1 by comparison with dNK in contact with uninfected similar target cells (16). IL-6 is a multifunctional cytokine that includes directing T cells differentiation in adaptive immunity (50). Although the first trimester pregnancy decidua is permissive to HIV-1 infection *in vitro*, the frequency of early *in utero* mother-to-child transmission of HIV-1 is low, suggesting efficient inhibitory regulatory mechanisms (51). dNK secrete cytokines and chemokines known to inhibit HIV-1 infection like CCL-3 and CCL4 or CXCL-12 (51). The killing of HIV-1-infected decidual cells by dNK could be mediated by specific engagement of NK cell receptors but also by induction of apoptosis mediated by the Fas/FasL pathway (51) as dNK were shown to produce FasL (52).

DO DECIDUAL NK CELLS UNDERGO A RECEPTOR REPERTOIRE PROFILE SHIFT WHEN THEY ARE IN CONTACT WITH PATHOGEN-INFECTED UTERINE CELLS?

hCMV infection modulates dNK cell receptor repertoire ability to kill virally infected decidual cells. Such a killing requires interaction between dNK cell activating receptors and specific ligands expressed at the cell surface of autologous decidual infected cells. Co-culture of dNK for 48 h with hCMV-infected decidual fibroblasts and without addition of IL-2 or IL-15, significantly increased the number of $CD56^{dim}$ dNK, associated with appearance of CD16 (16). This dNK phenotype is consistent with acquisition of a cytotoxic profile (16). A recent study similarly reported a significant increase of the $CD56^{dim}$ $CD16^{+}$ dNK subpopulation as early as 12 h after *in vitro* infection with *Toxoplasma* when compared to uninfected dNK (53). Furthermore, hCMV infection induced increased levels of expression of NKp44 and NKG2C receptors (16). Another study found that hCMV infection promoted a persistent expansion of PB-NK cells that express the CD94/NKG2C activating receptor (54). It is of note that the CD94-NKG2 system is highly conserved in both rodents and primates (55), highlighting the critical activating function of this receptor/ligand couple. No change of NKp30 expression was noticed on dNK cells after contact with hCMV-infected fibroblast (16). When comparison was made between dNK and PB-NK, no significant change was observed in the expression of PB-NK cell repertoire after contact with hCMV-infected cells (16).

Disappearance of the $NKp46^{high}$ dNK subset and decrease of KIR2DL1, KIR2DL4, and LILRB1/ILT2 expression were also observed on dNK in contact with hCMV-infected decidual fibroblasts (16). Thus, exposure to hCMV-infected cells modulates dNK receptor repertoire. An up-regulation of the NKG2D activating

Table 1 | Decidual NK receptor-ligands interactions, signals and cytotoxic effector functions.

dNK co-culture with	dNK CD56 marker	dNK receptors	Decidual fibroblast ligands (L)	dNK cytotoxic signals	dNK cytotoxic effector function
Uninfected decidual fibroblasts	$CD56^{bright}$ ~95%	CD94/NKG2A[b]	HLA-E	Negative	NO
	$CD56^{dim}$ ≤5%	KIR2DL1[b]	HLA-C	Negative	
		ILT2[b]	HLA-G[d]	Negative	
		2B4[a]		Negative	
		NKG2D[c]	NKG2D-L: high level	Negative	
		CD94/NKG2C	NKG2C-L	?	
		KIR2DL4[b]	HLA-G[d]	?	
		NKp30[a]	NKp30-L: low level	No signal	
		NKp44[a]	NKp44-L: high level	No signal	
		NKp46[c]	NKp46-L: low level	No signal	
		$CD16^{neg}$			
hCMV-infected decidual fibroblasts	$CD56^{bright}$ ~48%	CD94/NKG2A: down	HLA-E: down	?	YES
	$CD56^{dim}$ ~40%	KIR2DL1: down	HLA-C	?	
		ILT2: down	HLA-G[d]	?	
		NKG2D[c]	NKG2D-L: down	Positive	
		CD94/NKG2C: up	HLA-E: down	Positive	
		KIR2DL4: down	HLA-G[d]	?	
		NKp30[a]	NKp30-L: up	No signal	
		NKp46: down	NKp46-L	No signal	
		NKp44: up	NKp44-L: down	Negative	
		CD16[b]			

[a] *20–40% positive cells.*

[b] *50–80% positive cells.*

[c] *100% positive cells (flow cytometry).*

[d] *HLA-G is expressed on extravillous cytotrophoblast present among decidual adherent cells.*

receptor expression was reported in *Toxoplasma*-infected dNK which correlated with enhanced cytolytic activity against human extravillous cytotrophoblast target cells (53). Based on these data, infection of decidua by other pathogens is likely to trigger a change in the repertoire profile of dNK receptors.

WHICH PATHOGEN-MEDIATED SIGNAL(S) AND MOLECULAR INTERACTIONS SUBVERT THE INHIBITION OF DECIDUAL NK CELL CYTOLYTIC ACTIVITY?

Using Fc-chimeric proteins, we found that hCMV infection of decidual fibroblast modulated the expression of several NKR ligands: NKp30L and NKG2DL were significantly increased whereas NKp44L and NKp46L decreased (16). Blocking the engagement of NKp44 with its specific ligand resulted in an increase killing of infected autologous fibroblast by dNK cells, suggesting the presence of an inhibitory form of NKp44. In contrast, blocking NKG2D ligation induced a decrease of dNK cytotoxicity. These observations suggested a crucial role of NKG2D-mediated cytotoxic function of dNK against hCMV-infected fibroblasts. Infection of decidual fibroblast resulted in a diminishment of HLA-E expression on their cell surface. Blockade of HLA-E ligation with its specific dNK cell receptor induced a decrease of dNK killing, suggesting that HLA-E on hCMV-infected autologous fibroblasts did bind the CD94/NKG2C activating receptor. In contrast, neither NKp46 nor NKp30 seemed to be implicated in dNK cell lytic response. dNK cell subset that expresses the LILRB1/ILT2 receptor could be involved in the control of HIV-1 infection as it has been demonstrated in the PB-NK counterpart (56).

All of these data, including those dealing with hCMV infection and summarized in **Table 1**, showed that pathogen-mediated signals (i.e., modulation of expression of dNK receptor-ligands systems) and molecular interactions (engagement of dNK receptors with specific pathogen-mediated ligands) contribute to subvert the inhibition of dNK cell cytotoxic activity observed in normal pregnancy.

CONCLUSION

Instead of being detrimental, dNK cells have positive effects in healthy human pregnancy as they promote placental development through the crucial release of angiogenic factors and attraction of extravillous trophoblast. After contact with hCMV-infected autologous decidual fibroblasts, dNK acquire cytotoxic capacity by subverting their killing inhibitory regulatory mechanisms occurring in normal pregnancy. Effector functions of dNK in pathogen-infected pregnancy require engagement of shifted repertoire of activating and/or inhibiting dNK cell receptors by specific ligands up- or down-regulated in pathogen-infected decidual cells, including HLA-E and NKG2DL. These results, if confirmed by further studies dealing with different viruses or pathogens, strongly suggest that, *in situ*, dNK are likely to contribute to local pathogen innate immune control and clearance during the initial phase of decidua infection.

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Beyond NK cells: the expanding universe of innate lymphoid cells

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For a long time, natural killer (NK) cells were thought to be the only innate immune lymphoid population capable of responding to invading pathogens under the influence of changing environmental cues. In the last few years, an increasing amount of evidence has shown that a number of different innate lymphoid cell (ILC) populations found at mucosal sites rapidly respond to locally produced cytokines in order to establish or maintain homeostasis. These ILC populations closely mirror the phenotype of adaptive T helper subsets in their repertoire of secreted soluble factors. Early in the immune response, ILCs are responsible for setting the stage to mount an adaptive T cell response that is appropriate for the incoming insult. Here, we review the diversity of ILC subsets and discuss similarities and differences between ILCs and NK cells in function and key transcriptional factors required for their development.

Keywords: innate lymphoid cells, IL-22, NKp44, cytokines, mucosal tissues

INTRODUCTION

The adaptive immune system has evolved antigen receptor diversity to cope with a large variety of pathogens and non-self antigens. It is well-established that, depending on the nature of the invader and the signals coming from the surrounding environment, the adaptive immune system mounts a specific effector T helper (Th) response that serves to control the trespasser and re-establish homeostasis. Functionally, Th1 eliminate intracellular pathogens, Th2 are critical in promoting the eradication of helminthes, and Th17 control fungal and bacterial infections (1).

In the last few years, it has become evident that the innate immune system displays a similar strategy in order to ensure a first line of defense against intruders via innate lymphoid cells (ILCs). Broadly, ILCs are defined by their lymphoid lineage and their lack of RAG-mediated recombined antigen receptors. Mirroring the Th subsets, ILC populations diverge from one another in their reliance on unique transcription factors and production of signature cytokines. In general, ILCs provide primary border patrol at mucosal surfaces sensing changes in the microenvironment and rapidly responding to insults by producing specific cytokines that limit the damage caused by the attacking pathogen or favor its clearance and elimination.

Until ILCs came into the spotlight, conventional NK cells (cNKs) (i.e., blood circulating $CD56^+$ cells in human and splenic, lymph node, and bone marrow $NKp46^+NK1.1^+$ cells in mouse) were the only innate immune cells known to respond to cytokines produced by antigen presenting cells (APCs), such as dendritic cells (DCs) and macrophages (2–4).

Natural killer (NK) cells can rapidly release IFN-γ in response to IL-12 and IL-18 and/or type 1 IFN, which are produced when viruses and bacteria infect or come into contact with APCs. Because of their capacity to immediately respond to cytokines, cNK cells can be viewed as the prototype of all ILC subsets. However, they have additional properties, such as cytotoxic ability, that set them apart from other ILCs and allow them to eliminate virally infected or tumor-transformed cells.

Here, we will review the lineage relationship between ILC subsets and cNKs based on key transcription factors that regulate their respective development as well as the current understanding their functional roles.

ILC HETEROGENEITY AND FUNCTIONAL SPECIALIZATION

Innate lymphoid cells, like Th cells, are heterogeneous and currently grouped in three major subsets: ILC1, ILC2, and ILC3 (5).

GROUP 1 ILCs

ILC1s produce the Th1 signature cytokine IFN-γ. They include cNK [reviewed in detail in Ref. (6, 7)] and several additional, recently described, subsets of IFN-γ-producing cells (8–10) (**Table 1**).

A brief overview of cNK cells

Conventional NK cells have been known and actively studied for almost four decades (11, 12). cNKs were first described as a cell subset capable of rapidly eliminating tumor-transformed or allogeneic cells without the need for prior sensitization and in the absence of RAG-recombined antigen receptor recognition (13, 14). Traditionally, cNK cells are thought to be critical in conferring protection from viral infections, such as CMV (15), and in the immunosurveillance of tumor-transformed cells (16, 17).

In humans, cNKs include the $CD56^{bright}CD16^-$ and the $CD56^{dim}CD16^+$ subsets present in peripheral blood. $CD56^{bright}$ NK cells are specialized in IFN-γ secretion in response to DCs/macrophages-derived cytokines, such as IL-12 and IL-18 (18) or T cell-derived cytokines, such as IL-2 (19), a functional feature

Table 1 | Emerging subsets of ILC1 and tissue-resident NKs.

	CD127	IL-15 dependence	IL-7 dependence	TBX21 dependence	Eomes dependence	E4BP4 dependence	Human counterpart
siLP ILC1	+++	Yes	No	Yes	No	Partial	Most likely $CD127^+CD56^-CD94^-$ mucosal cells
Intraepithelial ILC1	–	Partial	No	Yes	TBD	Yes	$CD56^+NKp44^+$ $CD103^+CD160^+$ cells in human tonsil and intestine
"Converted" or "plastic" ILC3	+++	No, but IL-15 can promote conversion	Yes	Yes	No	TBD	Mostly generated *in vitro*
cNKs (splenic, bone marrow, lymph nodes)	–	Yes	No	Yes	Yes	Yes	Most likely $CD56^{dim}CD16^+$ blood NKs
Thymic NK cells	+++	Yes	Yes	Yes	Yes	Yes	Most likely $CD56^{bright}CD16^-$ blood NKs
Liver-resident $CD49a^+DX5^-$ NKs/ILC1	–	Yes	TBD	Yes	No	No	TBD
Skin-resident $CD49a^+DX5^-$ NKs	–	Yes	TBD	Yes	TBD	No	TBD
Uterus-resident $CD49a^+DX5^-$ NKs	–	Yes	TBD	No	TBD	No	TBD
Salivary gland NKs	–	Yes	TBD	TBD	TBD	No	TBD

TBD, to be determined.

that place them close to other ILC1 subsets. $CD56^{dim}CD16^+$ NKs are specialized in cytotoxicity, since they can readily release lytic granules containing perforin and granzyme upon contact with sensitive targets. However, it has been shown that also $CD56^{dim}$ NK cells can produce IFN-γ, although with a more rapid kinetic and in a less sustained fashion than $CD56^{bright}$ NKs (20). In addition, it has been suggested that $CD56^{bright}$ can differentiate into $CD56^{dim}$ NKs upon activation (21, 22). $CD56^{bright}$ cells produce additional monokines such as GM-CSF, TNF-α, IL-13, and IL-10, suggesting that they may exert an immunoregulatory function in specific circumstances (23). In addition, $CD56^{bright}$ NKs express CCR7, CXCR3, and CD62L and they are thought to traffic to secondary lymphoid organs via high endothelial venules (HEVs) (24).

In mouse, cNK include mature ($CD11b^{high}CD27^{low}$) and immature ($CD27^{high}CD11b^{low}$) circulating splenic and bone marrow NKs (25), $CD127^+$ (the IL-7 receptor α chain) IL-7-dependent thymic-derived NKs (26), and different subsets of tissue-resident NKs (12, 27) whose nature, function, and relationship to other emerging subsets of ILC1 we are just beginning to understand. Tissue NKs include an abundant population of salivary gland NKs, which are poorly cytotoxic and poor producers of cytokines (28, 29), liver-resident $CD49a^+$ (VLA1) $DX5^-$ NK cells, and skin- and uterus-resident NK cells (30). Thymic NKs for their ability to produce IFN-γ, TNF-α, and GM-CSF in response to IL-12 are thought to represent the murine counterpart of human $CD56^{bright}$ NK cells. One unifying feature of all NK cell subsets, including thymic NKs (31), is their dependence on IL-15 and IL-15Rα for development, survival, and maintenance.

The major breakthrough in understanding the biology and function of cNK cells was the discovery of germ line-encoded cell surface receptors of the C-type lectin or of the immunoglobulin superfamily that deliver either inhibitory or activating signal to NK cells (3, 32–35). These receptors, some of which are clonally distributed, finely tune NK cytolytic ability and the capacity to release cytokines through recognition of MHC class I molecules or other counter-receptors specifically expressed by target cells upon infection, tumor transformation, or stress-related signals (36). The appreciation of the complex array of signaling receptors expressed by cNKs has challenged the idea that NK cells simply exert "natural" cytotoxicity, and has suggested that in addition to "missing self" (37), which leads to release of inhibition mediated by inhibitory receptors that recognize self MHC class I, NK cells have to integrate a complex network of clues, which depend on contact with the target cells and/or the surrounding microenvironment. Accordingly, recent work has suggested that engagement of cytokine receptors can influence NK cell recognition mediated by activating receptors, such as NKG2D (38). Importantly, expression of inhibitory and activating receptors on NK cells controls their education and "licensing" (39), as well as

their expansion during responses to pathogens. This expansion generates a long lived-memory-type NK cell progeny that is more effective in clearing infections upon a second challenge (6, 40).

Thus cNK cells possess some features adaptive immune cells that maximize their capacity to eliminate intruders while avoiding self-reactivity (7).

Emerging ILC1 subsets

One first subset of ILC1 is present in human mucosal tissues and expresses CD127 and the C-type lectin CD161, but does not express other markers of the NK lineage such as CD94, CD56, NKp44 (NCR2), or NKp46 (NCR1); although it rapidly responds to IL-12 and IL-18 by producing IFN-γ (8). These cells also do not express c-kit (the receptor for stem cell factor, SCF), which marks many other ILC subsets.

The second subset was identified in human tonsillar tissue and is characterized by the expression of several NK-related markers such as CD56, NKp46, and NKp44 (9). However, this subset also expresses markers of tissue-resident memory CD8 T cells such as CD103, CD49a, and CD101. These ILC1s are also present in mouse small intestine, have an intraepithelial location, and are distinguished by the expression of CD160, a receptor of the immunoglobulin superfamily that binds to the TNF receptor superfamily member HVEM. HVEM–CD160 interactions promote epithelial integrity by inducing the alternative NF-kB signaling pathway and Stat3 phosphorylation in epithelial cells (41). Intraepithelial resident ILC1s are distinguished by cNKs because they do not respond to IL-12 and IL-18. Alternatively, they secrete large amounts of IFN-γ upon stimulation with IL-15. Notably, intraepithelial ILC1s are only partially dependent on IL-15/IL-15Rα signaling for their development, a feature that set them apart from cNKs. Interestingly, these ILC1s seem to promote tissue damage in a mouse model of colitis induced by CD40 ligation in immune-deficient mice. Although the function of the newly described human ILC1 subsets has not been investigated in detail *in vivo*, they may play important pathogenic roles in human inflammatory bowel diseases (IBDs) as both $CD56^{-}CD127^{+}$ and $NKp44^{+}CD103^{+}$ ILC1s are increased in patients with Crohn's disease, as compared to control individuals (8, 9). For instance, earlier studies have suggested that IL-15 and retinoic acid are highly pathogenic factors in celiac disease, since they induce production of IL-12 by intestinal DCs (42). In light of the fact that one ILC1 subset is responsive to IL-12, while the other promptly reacts to IL-15, it would be interesting to explore the respective role of these ILC1 subsets in human celiac disease or mouse models of celiac disease.

A putative murine equivalent of the $CD56^{-}CD127^{+}$ human ILC1s has been recently described in small intestine lamina propria (siLP) (10). These cells are $CD127^{+}$, but also express markers of the NK lineage, such as NKp46 and NK1.1. Interestingly, siLP ILC1s, as cNKs, depend on IL-15, but not IL-7, for their development. *In vivo* these cells are major producers of IFN-γ and TNF-α in response to oral infection with *Toxoplasma gondii (T. gondii)* and promote clearance of this pathogen by recruiting inflammatory monocytes through the CCR1/CCL3 axis (43). Therefore, in these settings, siLP ILC1s play a major protective role. Most likely, clearance of *T. gondii* requires a strong Th1 environment, while in Crohn's disease a robust Th1 signature sustains autoimmune damage and tissue destruction, explaining why ILC1s may play protective or pathogenic roles in different scenarios and in different diseases.

Another subset of ILC1s is represented by the so-called "converted" or "plastic" ILC3s. These cells can be generated *in vitro* from ILC3s in response to cytokines such as IL-15, IL-2, IL-12, and IL-23 that induce IFN-γ production (8, 44). They can also be induced *in vivo* by transfer of $ROR\gamma t^{+}$ ILC3s and visualized by fate-mapping experiments in siLP (10, 45, 46).

GROUP 2 ILCs

ILC2s produce the Th2 signature cytokines IL-5 and IL-13 (47–50). They also produce amphiregulin and IL-9, and notably, IL-9/IL-9 receptor signaling is required for their survival (51). ILC2s are found in various tissues including adipose tissue-associated lymphoid structures, gut, lung (52) and, as recently described, the skin (53–55). They promote expulsion of parasites (56, 57) and maintain lung homeostasis (58) or drive airway hyper-reactivity during viral infections, such as influenza (59). They also contribute to the pathogenesis of atopic dermatitis (53–55). In visceral adipose tissue, ILC2s maintain metabolic homeostasis by recruiting eosinophils, which sustain macrophage alternative activation (60, 61). ILC2s rapidly respond to the alarmin IL-33, to the IL-17 family member IL-25 and to TSLP in skin (62). They are characterized by the expression of CD127, c-kit, Sca1, and ST2 (the receptor for IL-33). Human ILC2s express CD161 and high levels of the prostaglandin D2 receptor CRTH2 (63). Notably, ILC2s are highly enriched in nasal polyps of patients with chronic rhinosinusitis (63), suggesting that they might play a fundamental role in human Th2-mediated diseases such as asthma and atopic dermatitis. A subset of cells named multipotent progenitor type 2 (MPP^{type2}), originally classified within ILC2s because of their ability to expand in response to IL-25, have been recently shown to contain progenitors giving rise to myeloid cells, such as macrophages and eosinophils (50). Because of the distinct transcriptional profile and functional potential that distinguishes MPP^{type2} from other ILC2s, and ILCs in general, these cells are no longer classified as Group 2 ILCs.

GROUP 3 ILCs

ILC3s produce the Th17 signature cytokines IL-17 and/or IL-22 (64–69). ILC3s include $Lineage^{-}$ (Lin)$ROR\gamma t^{+}$ $CD4^{+}$ Lti-like cells originally described in the 1990s (70), Lin^{-} $ROR\gamma t^{+}$ $CD4^{-}$ Lti-like cells, NCR^{+} ILC3s originally named NK-22 (65), and colonic $Sca1^{+}$ $Thy1^{high}$ ILCs (71). ILC3s rapidly respond to IL-23, a member of the IL-12 family. They also express the IL-1 receptor and respond to IL-1β (44, 72). ILC3s are mainly found in mucosal tissues, such as small and large intestine, Peyer's patches (PP), and gut-associated lymphoid tissue (GALT). Small numbers of ILC3s are present in spleen (73) and lung (74). By producing IL-22, ILC3s protect intestinal epithelium *in vivo* from attaching and effacing bacteria, such as *Citrobacter rodentium*, a mouse model of human enteropathogenic *E. coli* (75). IL-22 acts selectively on stromal and epithelial cells by inducing STAT3 phosphorylation, leading to multiple downstream events including the rapid production of the antimicrobial peptides alpha and beta defensins, as well as the promotion of epithelial cell survival and proliferation

(75, 76). ILC3s and ILC3-derived IL-22 are critical in containing dissemination of commensal bacteria in immune-deficient animals. In the absence of ILC3s, host-derived bacteria of the *Alcaligenes* species disseminate to peripheral organs and induce systemic inflammation (77). IL-22 also acts on intestinal epithelial stem cells, and radio-resistant IL-22-producing ILC3s from the recipient are key to limit the severity of intestinal damage during graft versus host disease (GVHD) (78).

While in the short term IL-22-mediated survival and proliferation of epithelial cells may favor tissue healing and repair, prolonged IL-22 signaling, and sustained epithelial proliferation may drive tumor formation (79). Accordingly, recent evidence has linked colonic ILC3s to colon cancer in a genetically prone bacterial-driven model of colon cancer (80). ILC3s have also been involved in other human disease, such as Crohn's disease (81) and psoriasis (82).

NCR^- ILC3s have been shown to negatively regulate adaptive CD4 T cell responses to commensals. This process does not depend on IL-17 or IL-22 but requires MHC class II expression on ILC3s and, by mechanisms yet to be completely elucidated, restricts CD4 T cell proliferation to commensal antigens (83).

Interestingly, in human tonsil, ILC3s produce GM-CSF, BAFF, and LIF in addition to IL-22 and express high levels of CD40L and RANKL (44). GM-CSF promotes accumulation of granulocyte-monocyte progenitors (GMPs) and mediates immunopathology in a mouse model of T cell-mediated colitis (84). Surprisingly, in steady state conditions, ILC3-derived GM-CSF is essential to induce development of $CD103^+CD11b^+$ DCs that instruct differentiation of $FoxP3^+$ Treg cells in an IL-10, TGF-β, and retinoic acid-dependent fashion. ILC3s produce GM-CSF selectively upon stimulation with IL-1β, which is released by macrophages activated by microbial products (85). Selective loss of GM-CSF in ILC3s results in impaired oral tolerance to dietary antigens.

CD40L expression and BAFF production by ILC3s enhance antibody secretion by marginal zone B (MZB) cells (86). Interestingly, ILC3s activate marginal reticular cells (MRCs) by providing TNF-α and lymphotoxin (LT). They also receive survival signals from MRCs, including IL-7. The interaction between MZB cells and ILC3s also involves expression of DLL1 by ILC3s, a Notch ligand that may activate Notch2 on MZB cells (86). Furthermore, in these settings, GM-CSF produced by ILC3s promotes APRIL secretion by neutrophils, boosting their B helper phenotype and further promoting IgM, IgG, and IgA production by MZB cells. $ROR\gamma t^+$ LTi-like ILC3s induce T cell-independent IgA production in isolated lymphoid follicles (ILFs). The simultaneous stimulation of stromal cells by LTi-like cells, via LT/LTβR, and by bacteria, via TLRs, induces recruitment of DCs and B cells promoting formation of ILFs and boosting T cell-independent IgA production in ILFs (87). Type 3 ILCs have also been shown to control T cell-dependent IgA production through release of soluble LTα3, which induces T cell homing to the gut (88). Ultimately, reduction in IgA levels due to absence or dysfunction of ILC3s in small intestine will induce changes in the microbial communities that may cause immunopathology. Collectively, these studies indicate that ILC3s may provide help to B cell responses at mucosal barriers by multiple mechanisms that may cooperate in providing optimal protection from environmental insults.

The relevance of LIF production and RANKL expression by ILC3s has yet to be understood. However, RANKL has been shown to be essential for the differentiation of the specialized microfold cells (M cells) that overlay the dome region of PP (89). Therefore, RANKL expression by ILC3s could be relevant in the context of PP function and biology and in the transport of particulate antigen from the lumen to APCs located in the sub-epithelial dome area of PP.

Although a transient burst of IL-22 from ILC3s seems to play protective effects in many scenarios and in particular during bacterial infections, a recent study has defied this view. IL-22 induces robust expression of antimicrobial proteins, such as lipocalin and calprotectin. These proteins are known to sequester metal ions including iron, zinc, and manganese. By subtracting these ions to commensal bacteria, IL-22 favors expansion of pathogenic bacteria, such as *Salmonella typhimurium* that are resistant to ion starvation (90). Therefore, in some circumstances, ILC3s may tip the balance in favor of pathogens, rather than protect from their attack.

In addition to sensing cytokines released in the surrounding microenvironment, ILC3s are also sensitive to nutrients. Recent work has shown that vitamin A deficiency results in decreased numbers of ILC3s in the intestine, which increases susceptibility to bacterial infections. However, the same deficiency induces expansion of ILC2s, which protect from nematode infections (91). Moreover, vitamin A intake by pregnant mothers controls the pool of LTi-like $CD4^+$ ILC3s in embryos, the size of lymph nodes and PP, and the efficacy of immune responses to viral infections (92). These findings again emphasize the idea that ILCs are exquisitely sensitive to environmental cues and continuously adapt to rapidly changing settings, such as the ones present at mucosal surfaces.

THE NETWORK OF MASTER REGULATORS OF ILC DEVELOPMENT

Until recently, the general consensus was that the very same transcription factors that drive Th1, Th2, and Th17 commitment and differentiation also regulate ILC1, ILC2, and ILC3 development, respectively. Moreover, it seemed that cNKs and the other ILC groups significantly differed in their developmental requirement for transcription factors.

This concept has been recently challenged by a number of studies suggesting that developmental requirements for NK cell subsets and ILCs are more complex than originally anticipated (**Figure 1**). Here, we will highlight the most recent progresses in the field.

All ILC groups, including cNKs, depend on Id2 (47, 93). Id2 is a transcriptional repressor that, by blocking members of the E2 family of transcription factors, suppresses the B cell fate potential in a progenitor cells that develop downstream of the common lymphoid precursor (CLP). A recent study that took advantage of a reporter mouse expressing GFP under the control of the Id2 promoter to track Id2-expressing cells has shown that Id2 is not expressed in CLPs. Id2 is, however, expressed at very high level in all ILCs, including cNKs (94). In NK cells, Id2 is not required for the NK cell lineage specification, since development of $CD122^+NK1.1^-$ NK cell progenitors is not impaired in Id2-deficient mice. Nevertheless, Id2 expression in NK cells is required for acquisition of a fully mature phenotype. The few NK cells that

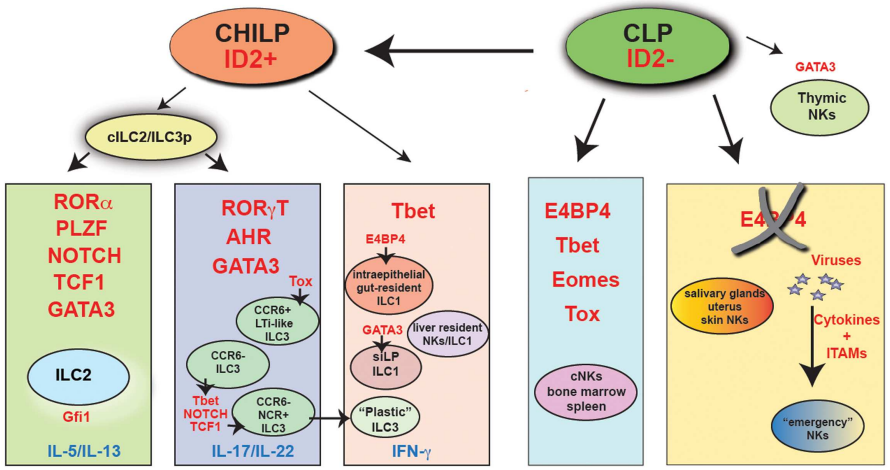

FIGURE 1 | The major transcriptional pathways that control ILC and NK cell development. Indicated in red are the key transcription factors that have been shown being absolutely required *in vivo* for the development of each cell subset. "Emergency" NK cells are NK cells that develop in the absence of E4BP4, following viral infection. It is presently unclear whether these cells are Tbet-dependent. CHILP, common helper innate lymphoid precursor; CLP, common lymphoid progenitor; cILC2/ILC3p, common ILC2/ILC3 progenitor.

develop in the absence of Id2 have a phenotype that closely resemble thymic NK cells, express high levels of CD127 and produce large amounts of IFN-γ (95). In committed B cells, the transcription factor EBF1 mediates Id2 suppression (96). According to the idea that Id2 is globally required for development of all ILCs, EBF1-deficient pro-B cells acquire the capacity to differentiate into T cells and into functionally competent ILC2s and ILC3s (97). Importantly, the generation of an Id2-reporter mouse has allowed the identification of a common ILC progenitor, or CHILP (common "helper-like" ILC lineages progenitor) (10) that can generate $CD127^+NKp46^+$ siLP ILC1, ILC2, and ILC3 *in vivo* and *in vitro*. This Lin^-Id2^+ progenitor also expresses CD127 and the integrin α4β7 but does not express CD25 (which is present on differentiated ILC2) or CD122, a marker of NK cell precursors. Intriguingly, this precursor is only slightly affected by IL-7 deficiency and can generate liver-resident $CD49a^+Eomes^-$ NK cells, but not cNKs (10). Further confirming the existence of a common ILC progenitor, an other recent study performing fate-mapping experiments shows that ILCs, including intraepithelial ILC1, ILC2, and NCR^+ ILC3, derive from a common committed precursor present in fetal liver and adult bone marrow that transiently expresses the transcription factor PLZF (encoded by *Zbtb16*) (98). This transcription factor was known to be required for NKT development, but it is dispensable for cNK cells and $CD4^+$ LTi-like cells (99). Reconstitution experiments with *Zbtb16*-deficient or -sufficient bone marrow cells in lethally irradiated mice indicated that *Zbtb16* is absolutely required for ILC2s development only. On the contrary, it seems to be dispensable for NCR^+ ILC3s reconstitution, despite its high expression in this subset. As *Zbtb16* is controlled by Id2 (100), the PLZF-expressing common ILC precursor may be downstream of the $Id2^+CD127^+CD25^-α4β7^+$ CHILP.

Most likely downstream of the CHILP, all ILC3 subsets require the Th17 master regulator RORγt (101, 102). In the absence of RORγt, Lti, and Lti-like cells fail to develop and RORγt-deficient mice do not possess lymph nodes, PP, cryptopaches (CP) nor ILFs (103, 104), which derive from CP under signals coming from the microbiota through the nod-like receptor (NLR) NOD1 (105). ILC3s also require aryl hydrocarbon receptor (AHR) (106–108), which drives IL-22 secretion in Th17 cells (109, 110). In the absence of AHR, both Lti-like ILC3s and NCR^+ ILC3s are severely diminished. In addition, small intestine CP and ILFs are absent, while colonic patches develop normally (111). Some studies have suggested that exogenously provided AHR agonists, such as tryptophan derivatives added to the diet, play a role in expanding and/or maintaining ILC3s (106, 112). Recent work has also shown that AHR agonists derived from commensal bacteria, such as lactobacilli, increase IL-22 production by ILC3s in the context of a tryptophan rich diet and this, in turn, confers resistance to infection by the opportunistic fungus *Candida albicans* (113). It is also possible that endogenous AHR ligands, such as kynurenine, produced by the enzyme tryptophan dioxygenase (TDO), may play a critical role in ILC3 biology (114).

Unexpectedly, AHR, in ILC3 cells, restrains adaptive Th17 responses. In AHR-deficient mice that lack ILC3s, Th17 cells in siLP undergo an uncontrolled expansion that leads to tissue damage. This Th17 proliferation is due to a dysregulation in the intestinal microbial niche that allows excessive growth of *segmented filamentous bacteria* (SFB), commensals known to drive Th17 differentiation (115).

In addition to RORγt and AHR, ILC3s also depend on Notch signaling for their development (111, 116). In RBP-$J_k^{-/-}$ mice that lack all Notch signaling, NCR^+ ILC3s are selectively reduced. Notch also promotes $ROR\gamma t^+$ ILC differentiation from adult bone marrow precursors (117). Recent studies have also shown that NCR^+ ILC3s, which develop post-natally and reach maximal expansion 4–6 weeks after birth in mouse small intestine LP (118),

express Tbet (encoded by *Tbx21*) and require Tbet for development (46, 116, 119, 120). Induced by IL-23 and cues from the microbiota, Tbet is essential to instruct IFN-γ production and protection from pathogens such as *S. typhimurium* (46). Tbet expression seems to induce Notch, as Tbet haplo-insufficiency results in reduced Notch expression (116). However, it is conceivable that AHR and Tbet may cooperate in inducing Notch and final maturation of NCR^+ ILC3s.

The requirement for Tbet in NCR^+ ILC3s is somewhat surprising since human tonsil ILC3s do not express Tbet *ex vivo* (44). In fact, in steady state conditions, Tbet expression is limited to ILC1s and cNKs, while RORγt is selectively expressed by ILC3 (9).

Human tonsil ILC3s also express high levels of CCR6 and can easily be sorted at high purity based on NKp44 and CCR6 expression (44, 121), while mouse NCR^+ ILC3s are CCR6 negative (46). Whether the discrepancies between the mouse and human system are linked to tissue specific differences (intestine versus tonsil) still remains to be determined. It is conceivable that Tbet may be needed at later stages of ILC3s differentiation, which may be poorly represented in the tonsil. In agreement with this hypothesis, tonsil ILC3s are characterized by an intrinsic plasticity and, when stimulated with cytokines such as IL-2 and IL-15 *in vitro*, can generate IFN-γ producing pro-inflammatory cells that acquire some features of cNK (8, 44). Specific engagement of cell surface receptors expressed by ILC3s by cognate ligands present in some tissues but not in others may also be an issue. Indeed, a recent study has suggested that stimulation of ILC3s via the DAP12-associated activating receptor NKp44 induces production of TNF-α and convert them to a more inflammatory-prone cell type (122). Experiments in mice have suggested that ILC3 conversion to an IFN-γ/TNF-α secreting pro-inflammatory cell type can occur *in vivo* (45). Moreover, recent fate-mapping experiments, aimed to track cells that expressed RORγt at any stage of their life cycle, show that "plastic" or "converted" ILC3s are present within the $ROR\gamma t^-NKp46^+NK1.1^+$ population in siLP and cluster with siLP ILC1s and cNK (10).

Finally, STAT3 expression in ILC3s is required for robust IL-22 production and protection by intestinal infection with *C. rodentium* (123).

Group 2 ILCs necessitate RORα (124), Gfi1 (125), and Gata-3 for their development (94, 125–127). Gfi1 specifically controls the response to IL-33 and TSLP and the production of IL-5, but not IL-13, in ILC2s (125). The requirement for Gata-3 by ILC2s closely resembles the need for Gata-3 in Th2 differentiation. However, Gata-3 is also necessary for thymic NK cell development (26). Moreover, a recent study has indicated that Gata-3 is required for the generation of ILC3s (128), before the identification of the CHILP (10). Transfer of Gata-3-deficient fetal liver precursor cells in mice lacking ILCs prevents reconstitution of all $ROR\gamma t^+$ ILC3s. This finding has led to the hypothesis that a common precursor might exist between ILC2s and ILC3s (128). In agreement with this view, another transcription factor TCF-1 (encoded by Tcf7) is required for ILC2s and NCR^+ ILC3s (129, 130). TCF-1 in ILC2s acts downstream of Notch and forced expression of TCF-1 can bypass Notch requirement for ILC2 generation. TCF-1 also induces Gata-3, which is responsible for upregulation of *Il-17rb* (the receptor for IL-25) and *Il2ra* (CD25) in developing ILC2s. However, in the absence of Gata-3, TCF-1 directly controls the expression of *Il7r* (CD127) (130). In NCR^+ ILC3s, TCF-1 acts downstream of Tbet and Notch to promote ILC3 development (129).

Gata-3 is also required to induce differentiation and/or maintenance of siLP ILC1s (10), as deletion of Gata-3 in $NKp46^+$ cells results in a dramatic reduction of this ILC subset, while does not affect ILC3s or cNKs. In addition, early deletion of Gata-3 in hematopoietic cells abolishes the development of all $CD127^+$ ILCs (131).

While it is not clear whether TCF-1 is also required for siLP ILC1s, the data available suggest that some transcription factors may be required at multiple stages of ILC differentiation and may be turned on and off at different transitional steps with a hierarchy that is still poorly defined.

Intraepithelial ILC1s and cNKs cells share some requirements in transcription factors for their differentiation. To begin, both cell populations require Tbet for development (9, 132). In addition, Tbet is necessary for the development of liver-resident NK cells that express Trail, CD49a, lack DX5, and Eomes (133) and have features of tissue-resident memory cells based on parabiosis experiments (30, 134). Although these cells were originally thought to represent an NK immature cell subset (135), more recent data suggest that they represent a distinct lineage of NK cells that share part of their transcriptional program with NKT cells (133). While it is not clear whether intraepithelial ILC1s derive from the CHILP, liver-resident NK cells could represent a true subset of ILC1s related to the siLP ILC1s, since they appear to differentiate from the CHILP (10).

Intraepithelial ILC1s and cNK cells also require E4BP4 (encoded by *Nfil3*) to develop (9, 136, 137). E4BP4 in NK cell development was previously thought to act directly downstream of IL-15R, which is required for NK cell maintenance (138, 139). However, more recent data suggest that viral infections can rescue NK cell development independently of E4BP4 in a process that requires inflammatory cytokines such as type 1 interferon and IL-12 along with ITAM signaling (140). E4BP4-independent NK cells are functional and persist in the periphery in an IL-15 dependent fashion. Ablation of E4BP4 after the earlier steps of NK cell commitment completely bypasses the requirement for E4BP4 in NK cell development (140). In addition, E4BP4 is less stringently required for NK cell development outside of the bone marrow. Liver-resident $Trail^+$, $DX5^-$ NK cells develop in the absence of E4BP4, especially when T cell competition is eliminated (30, 141, 142). Salivary gland NKs also do not require E4BP4 (29). The role for E4BP4 in thymic NK cell development is still controversial. Thymic NK cells seem to develop normally *in vitro* from E4BP4-deficient double negative (DN)1 and DN2 thymocytes (141). However, a second study indicates that thymic NKs *in vivo* develop along an E4BP4-dependent pathway (142). According to this report, E4BP4 is necessary to enforce Eomes expression in cNKs as well as in thymic NKs. Further confirming that E4BP4 may be necessary to drive Eomes expression, another report demonstrates that E4BP4 is strictly required for the transition from CLP to NK cell progenitor and regulates Eomes and

Id2 expression in NK cells (143). Challenging the previous concept that E4BP4 acts downstream of the IL-15R (136), this study shows that E4BP4 operates upstream of IL-15 signaling and in the absence of E4BP4 CD122$^+$ NK precursors fail to develop. E4BP4 expression is detected earlier that Tbet, Eomes, or Id2 expression and chromatin immunoprecipitation experiments demonstrate that E4BP4 promotes transcription of Eomes and Id2 by binding directly to the regulatory regions of their genes (143). These data are, however, in conflict with the study by Seillet et al., which shows that Id2 expression is normal in the absence of E4BP4 (142). Further studies will be necessary to elucidate the exact hierarchy in which transcription factors need to be expressed in order to grant successful development and maturation of NK cells and ILCs.

Finally, cNK cells share with CD4$^+$ LTi-like cells the requirement for the HGM-box transcription factor superfamily member TOX. TOX-deficient animals lack NK cells and do not develop lymph nodes nor PP (144). Although it has been hypothesized that TOX controls induction or maintenance of Id2 in precursor cells, the exact mechanisms by which TOX regulates development of cNKs and LTi-like cells and whether it is necessary for other ILC subsets is unclear.

CONCLUDING REMARKS

Studies over the past few years have indicated that adaptive immunity has adopted modules already established in the innate branch of the immune system to counteract pathogens and to maintain homeostasis at mucosal surfaces, which are constantly exposed to environmental insults and non-self-microbial communities. The identification of innate subsets that mimic Th1, Th2, and Th17/Th22 Thelper cells has greatly advanced our understanding of how immune responses are orchestrated and shaped. Future challenges in the field will be to understand whether additional subsets of ILCs are present that prevent or control autoimmune processes, i.e., whether an innate functional equivalent of regulatory T cells is in place. In addition, it will be important to delineate whether dedicated subsets of APCs or stromal cells are located in mucosal tissues that can preferentially activate a particular ILC group, or whether this activation process depends on specific signals provided by the intruder. Elucidating whether diverse inflammatory milieus can elicit different responses and immunological outcomes from the same ILC group will be also important. In the long term, a better understanding of the very first events that take place early on during the initiation of an immune response may be key in designing better strategies for vaccine development and therapeutic intervention.

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Recognition strategies of group 3 innate lymphoid cells

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During the early phase of an inflammatory response, innate cells can use different strategies to sense environmental danger. These include the direct interaction of specific activating receptors with pathogen-encoded/danger molecules or the engagement of cytokine receptors by pro-inflammatory mediators produced by antigen presenting cells in the course of the infection. These general recognition strategies, which have been extensively described for innate myeloid cells, are shared by innate lymphoid cells (ILC), such as Natural Killer (NK) cells. The family of ILC has recently expanded with the discovery of group 2 (ILC2) and group 3 ILC (ILC3), which play an important role in the defense against extracellular pathogens. Although ILC3 and NK cells share some phenotypic characteristics, the recognition strategies employed by the various ILC3 subsets have been only partially characterized. In this review, we will describe and comparatively discuss how ILC3 sense environmental cues and how the triggering of different receptors may regulate their functional behavior during an immune response.

Keywords: innate lymphoid cells, ILC3, RORγt, NKp44, NCR, AHR

INTRODUCTION

Innate lymphoid cells (ILC) represent a family of innate effectors lacking recombination activating gene (RAG)-dependent rearranged antigen receptors and myeloid and dendritic cell (DC) markers. ILC are developmentally related as they all depend on the common cytokine receptor γ-chain as well as on the transcriptional repressor inhibitor of DNA binding 2 (ID2) for their development. ILC comprise of different effector populations characterized by distinct patterns of cytokine production and lineage-specific master transcription factors, tailored to their exclusive role in host defense. Thus, they closely resemble the heterogeneity of $CD4^+$ T helper (T_H) cell subsets. ILC can be grouped into three functionally distinct groups: group 1 ILC, among which Natural Killer (NK) cells are the main population, group 2 ILC (ILC2), and group 3 ILC (ILC3). ILC react promptly during infections or inflammatory responses and play an important role in tissue homeostasis, as well as in immune reactions against infectious microorganisms and transformed cells (1). While the recognition strategies employed by NK cells have been extensively investigated, the analysis of the receptors mediating the activation of ILC3 and ILC2 is still at the beginning. In this assay, we will review the current understanding of the signals able to activate and induce effector functions in ILC3 and discuss it in the context of NK cell recognition strategies.

GROUP 3 ILC

Group 3 ILC or ILC3 are characterized by the expression of the transcription factor RORγt, which is critical for their development and function. A population of ILC3 emerges already during embryogenesis and corresponds to the previously described lymphoid-tissue inducer (LTi) cells, which are strictly required for the prenatal development of lymph nodes (LN) and Peyer's patches (PP). In addition to RORγt, LTi cells express the IL-7 receptor (IL-7R or CD127), the stem cell factor (SCF) receptor c-kit (or CD117), IL-17, IL-22 and a number of tumor necrosis factor (TNF) family members, such as lymphotoxin (LT) $\alpha_1\beta_2$ (2–8). While LT is crucial for the formation of secondary lymphoid structures, the role of IL-17 and IL-22 during embryogenesis is not clear, as both IL-22- and IL-17-deficient mice display normal lymphoid structures (9). ILC3 expressing IL-17 and IL-22 have also been identified after birth and are located mainly at mucosal surfaces (7, 10, 11). Mouse ILC3 can be dissected according to CD4 and CCR6 (11, 12). $CCR6^+$ ILC3 share several features with LTi cells, being enriched in IL-17 and CD4 expression. Conversely, $CCR6^-$ ILC3 do not express IL-17 or CD4 but produce IL-22. Among $CCR6^-$ ILC3, a particular subset has been described, which is characterized by the expression of the NK cell activating receptor (actR) NKp46 and partial expression of NK1.1, producing not only IL-22, but also some IFN-γ (12–18). While all subsets depend for their development on RORγt, only $CCR6^-$ ILC3 require the transcription factor T-bet, which is important for the differentiation toward $NKp46^+$ ILC3 (12, 17, 18). Mouse ILC3 display a certain level of plasticity *in vitro* and *in vivo* (1, 19, 20). IL-7 and microflora have been reported to stabilize RORγt expression in small intestine (SI) lamina propria (LP) $NKp46^+$ and $NKp46^-$ ILC3, enabling them to maintain their phenotype of IL-22 producers. However, $NKp46^+$ ILC3 from spleen or colon tend to lose RORγt expression while acquiring a full NK cell phenotype, which renders them almost undistinguishable from conventional NK cells. However, by using RORγt fate mapping ($ROR\gamma t^{fm}$) to visualize $NKp46^+$ cells derived from $ROR\gamma t^+$ progenitors, it could be shown that a large fraction of colon LP and a minority of splenic $NKp46^+$ cells actually represent ILC3, which have lost RORγt expression, as indicated by their phenotype of $ROR\gamma t^{fm+}$ $ROR\gamma t^-$ cells (20).

Similar to their mouse counterpart, human ILC3 are characterized by the expression of RORγt, IL-7R, c-kit, $LT\alpha_1\beta_2$, and IL-22. Human fetal LTi, displaying LTi-like *in vitro* activity, express IL-17 and IL-22 but not CD4. After birth, human ILC3 can be mainly

found not only in the gut LP but also in tonsils, from where they have been mostly isolated and characterized. Tonsil ILC3 are homogenously CD127^{hi}, c-kit^{+}, and LT$\alpha_1\beta_2^{+}$ and can be further dissected according to the expression of NKp44 and CD56 (7, 16, 21, 22). Although in one of the first reports, human IL-22-producing RORγt^{+} ILC3 were identified as Lin^{-} CD56^{+} NKp44^{+} cells and termed NK-22 (16), tonsil and gut LP CD56^{-} NKp44^{+} and CD56^{+} NKp44^{-} cells are also enriched in *RORC* transcripts. They display a partially overlapping phenotype compared to their CD56^{+} NKp44^{+} counterpart, thus suggesting that a large fraction of tonsil lineage (Lin)$^{-}$ CD127^{hi} c-kit^{+} cells, which do not express the NK cell marker CD94 or the ILC2 marker CRTH2, might be bona fide ILC3 (21–23). It has been proposed that CD56^{-} ILC3 might represent the counterpart of CD4^{+} mouse LTi-like cells (7). However, some major differences between the mouse and human counterparts should be mentioned. In humans, ILC3 homogenously express CCR6, while lacking CD4 (7, 16). IL-17-producing ILC3 can be found among CD56^{-} NKp44^{-} ILC3 especially in fetal LN and gut LP of patients with Crohn's disease, but not in tonsils (21, 24). Importantly, IL-22 expression is strictly confined to human NKp44^{+} ILC3 subsets (especially CD56^{+}), which largely co-express NKp46 (16, 21, 22). Conversely, mouse IL-22 is preferentially expressed by NKp46^{-} ILC3 (25). Moreover, in contrast to the NKp46^{+} T-bet^{+} RORγt^{+} mouse ILC3 subset, T-bet and IFN-γ proteins are not produced *ex vivo* by tonsil-derived ILC3, although expression can be induced after *in vitro* culture (7, 16, 19, 22). In addition to the cytokines mentioned, human tonsil ILC3 have also been shown to express IL-26, GM-CSF, TNF, CCL20, LIF, IL-5, and IL-13. Intriguingly, human ILC3 produce consistent amounts of IL-2, whose *in vivo* function remains to be elucidated (7, 16, 19, 22, 26). Similarities and differences among human and mouse ILC3 subsets in comparison with splenic or blood NK cells are depicted in **Table 1**.

ILC3 FUNCTIONS

ROLE OF ILC3 FOR THE FORMATION OF SECONDARY LYMPHOID ORGANS AND POST-NATAL INTESTINAL LYMPHOID CLUSTERS

LTi cells play a pivotal role during prenatal organogenesis of LN and PP. The most important effector molecules for lymphoid organogenesis are the TNF superfamily members, in particular LT$\alpha_1\beta_2$ which, by triggering the LTβ-receptor (LTβR) on mesenchymal stem cells, induces the expression of adhesion molecules and chemokines, such as VCAM-1 and CXCL13. As a consequence, B cells, T cells, and DC can be recruited to form the LN (36). In addition, ILC3 are also required for post-natally developing intestinal lymphoid organs such as cryptopatches (CP) and isolated lymphoid follicles (ILF) (37). CP are lymphoid clusters located in the LP in between the gut crypts and comprise mainly of ILC3 surrounded by a wall of DC. When B cells are recruited to CP, ILF are formed, which represent important sites of T cell-independent IgA production. While the formation of CP is independent of the intestinal microbiota, formation of ILF requires signals from intestinal bacteria (27, 38–42).

MAINTENANCE OF EPITHELIAL BARRIER FUNCTION

ILC3 are critically involved in the maintenance of the barrier function, due to the production of cytokines instructing epithelial cell functions. Their ILC3 signature cytokine IL-22 is certainly a main mediator of the cross talk between epithelial cells, immune cells, and the commensal microflora. IL-22 belongs to the IL-10 family and binds the IL-22 receptor, a heterodimer comprising of IL-10R2 and IL-22R1, which is exclusively expressed on epithelial cells and signals mainly via STAT3. IL-22 induces the production of antimicrobial peptides and proteins, such as β-defensins, RegIIIβ and RegIIIγ, calgranulins S100A8, S100A9, and lipocalin-2 by epithelial cells (43–46). Furthermore, IL-22 induces epithelial cells to secrete elevated levels of mucus-associated molecules like Muc1, Muc3, Muc10, and Muc13, thus increasing the mucus production, whereby the translocation of commensal bacteria across the epithelial barrier during inflammation is reduced. *In vivo*, IL-22 induces the migration of epithelial cells and promotes wound healing during inflammation (47–50). Similar to its role in the intestine, IL-22 exerts tissue repairing functions also during liver inflammation (51–53). The ILC-mediated regulation of epithelial cell functions is strictly linked to their role in the containment of commensal and pathogenic microbes. Indeed, an intact ILC compartment is important for preventing peripheral dissemination and systemic inflammation of commensal bacteria such as *Alcaligenes species*, residing within PP and mesenteric LN of healthy humans and mice (54). Moreover, it was shown that IL-22 from ILC3 is important to contain the expansion of commensal-segmented filamentous bacteria (SFB), known to promote Th17 cells (55). On the other hand, microbiota can modulate production of IL-22 by ILC3 (25), although ILC3 development seems to be independent of gut flora or SFB (11, 56).

ILC3 also play an important role in the defense against pathogen infections, such as *Citrobacter rodentium*, a murine pathogen that models human enterohemorrhagic and enteropathogenic *Escherichia coli* infections. Protection against *C. rodentium* is mediated by IL-22, which is mainly produced by NKp46^{-} ILC3 in an IL-23-dependent manner (46, 57). Despite initial evidences for a role of NKp46^{+} ILC3 in the defense against *C. rodentium* infection (14, 16), these cells appear to be dispensable (12). Although T cells also importantly contribute to produce IL-22 after infection, early production of IL-22 is crucial for *C. rodentium* resistance, as *Il22*$^{-/-}$ mice rapidly succumb within the first 8–12 days after infection (46, 58).

IFN-γ produced by T-bet-dependent CCR6^{-} ILC3 has been shown to contribute to the response against *Salmonella typhimurium* infection in mouse (12). Other reports have also recently outlined the importance of IL-17A and IL-17F production by ILC3 for the protection against mucosal *Candida* infections (59).

Due to their ability to modulate epithelial cell functions as well as to respond against commensal bacteria and pathogens, ILC3 also participate in the complex regulation of inflammatory bowel disease (IBD), displaying rather a dual role. Indeed, several investigators have suggested a protective role for IL-22, likely produced by ILC3, in innate and adaptive IBD models (47, 60). On the other hand, expression of IL-17 and IFN-γ from ILC3 has been implied to drive inflammation in innate IBD models, such as anti-CD40 or *Helicobacter hepaticus*-induced colitis (20, 61).

Table 1 | Phenotype of LTi, ILC3 subsets, and NK cells in human and mouse.

	Mouse				Human			
	Fetal LTi	**NCR$^-$ ILC3**	**NCR$^+$ ILC3**	**Spleen NK cells**	**Fetal LTi**	**NCR$^-$ ILC3**	**NCR$^+$ ILC3**	**Blood NK cells**
SURFACE MARKERS								
CD127 (IL-7Rα)	+	+	+	−	+	+	+	lo/−
CD117 (c-Kit)	+	+	+	lo/−	+	+	+	lo/−
CD122 (IL-2Rβ)	ND	lo	lo	+	ND	lo	lo	+
NK1.1/CD161	−	−	lo/−	+	±	+	+	+/lo
CCR6	+	±	−	−	+	+	+	−
NKG2D	−	−	+	+	−	lo/−	lo/−	+
NKp30	NA	NA	NA	NA	±	±	+	+
NKp44	NA	NA	NA	NA	lo/−	−	+	+[a]
NKp46	−	−	+	+	−	±	+	+
CD56	NA	NA	NA	NA	lo/−	±	±	+
Perforin	−	−	−	+/lo	−	−	−	+/lo
CD16	ND	−	−	±	−	−	−	±
Ly49/KIR	−	−	−	±	−	−	−	±
CD94	ND	−	±	±	−	−	−	±
CD4	+	±	−	−	−	−	−	−
RANKL	+	+	+	lo/−	+	+	+	−
CYTOKINES								
TNF	+	+	+	+	+	+	+	+
IFN-γ	−	lo/−	lo/−	+	−	lo/−	lo/−	+
IL-17	+	±	−	−	+	lo/−	lo/−	−
IL-22	+	+	+	−	+	−	+	−
GM-CSF	ND	ND	ND	ND	ND	+	+	+
IL-2	ND	ND	ND	ND	ND	+	+	−
LTA/LTB	+	+	+	lo	+	+	+	lo
TRANSCRIPTION FACTORS								
T-bet	−	±	+	+	−	−	−	+
Eomes	−	−	−	+	−	−	−	+
RORγt	+	+	+	−	+	+	+	−
Gata-3	−	lo	lo	lo/−	lo/−	lo/−	lo/−	lo/−
AhR	+	+	+	−	+	+	+	−

NCR$^+$ refers to NKp46$^+$ ILC3 in mouse and NKp44$^+$ ILC3 in humans. + Indicates high level expression, − indicates no expression, ± indicates bimodal expression, and lo indicates low expression of the molecules according to published reports (1, 4, 6, 7, 11, 13, 14, 16–18, 21–23, 25, 27–35).

NA, not applicable as gene is not conserved in the respective species. ND, not described.

[a] *Expressed only on activated-NK cells.*

AhR, aryl hydrocarbon receptor; CCR6, C–C chemokine receptor type 6; GM-CSF, granulocyte-macrophage-colony stimulating factor; IFN-γ, interferon-γ; IL, interleukin; KIR, killer-cell immunoglobulin-like receptor; LT, lymphotoxin; LTi cell, lymphoid-tissue inducer cell; NK cell, natural killer cell; RANKL, receptor activator of NF-κB ligand; RORγt, retinoic acid receptor-related orphan receptor-γt.

REQUIREMENTS FOR ILC3 ACTIVATION

Although many reports have clarified the functions of ILC3 and their role in the defense against pathogens, their receptor repertoire and the main signaling pathways able to trigger effector functions in ILC3 have not been extensively investigated. It was shown that, similar to NK cells, ILC3 can be mainly activated by cytokines released by the epithelium or antigen presenting cells (APC). More recently, receptors mediating a direct sensing of the environment by ILC3 have been also described. Here, we will revise the main findings concerning the recognition strategies that enable ILC3 to mediate their effector functions in response to perturbation of the epithelial barrier.

CYTOKINE RECEPTORS

Both human and mouse ILC3 constitutively express the receptors for IL-7 and TSLP (IL-7R or CD127), IL-15/IL-2 (IL-2Rβγ), IL-23 (IL-23R), IL-1 (IL-1R), and SCF (c-kit). Among these cytokines, IL-7, TSLP, and SCF are required for ILC3 development and induce, together with IL-2/IL-15 and IL-1, proliferation of ILC3. Conversely, IL-23 and IL-1 play an important role in inducing ILC3 effector functions *in vitro* and *in vivo* (7, 8, 13, 16, 19, 62, 63). IL-23 is a heterodimer composed of subunits p19 and p40. The cellular sources are predominantly activated macrophages and DC (64). IL-23 was initially described as the main stimulus for induction of IL-22 expression in ILC3 (13, 16). Nevertheless, in

IL-23p19-deficient mice or in wild-type mice treated with neutralizing anti-IL-23R antibody, the production of IL-22 by ILC3 is unaffected at steady state, suggesting that constitutive production of IL-22 by ILC3 is IL-23-independent (25). However, IL-23 becomes an important stimulus for innate production of IL-22 and IL-17 during *C. rodentium* and *H. hepaticus* infection (46, 61). In humans, IL-23 promotes IL-22 expression *in vitro*, together with IL-1 (65). Moreover, it induces IL-17 production in $CD56^-$ ILC3 isolated from the gut LP of patients affected by Crohn's disease (24). Altogether, these data imply the IL-23 axis as an important pathway of ILC3 activation during inflammation.

IL-1β is a key pro-inflammatory cytokine, which can be produced by different cell types (66). IL-1β does not only induce ILC3 proliferation, especially in combination with IL-7 and IL-2/IL-15 (19), but also induces the accumulation and activation of ILC3 during the course of *H. hepaticus* infection (67). IL-1β synergizes with IL-23 or IL-7 in stimulating ILC3 to produce IL-22 (22, 23). The importance of IL-1β for cytokine expression by ILC3 was also demonstrated by the decrease in basal as well as in IL-23-induced production of IL-22 observed in the presence of anti-IL-1R1-blocking antibodies or in mice deficient for IL-1R adaptor molecule, MyD88 (56).

In addition to their role in development of ILC3, IL-7, TSLP, and SCF also likely contribute to ILC3 maintenance post-natally (68). IL-7 enhances $LT\alpha_1\beta_2$ expression (69, 70) and stabilizes *in vivo* the expression of RORγt on ILC3, thus preventing their conversion into IFN-γ-producing ILC3 (20). The main source of these cytokines is mainly non-hematopoietic cells such as fibroblasts, epithelial cells, and different types of stromal cells. Thus, it would be interesting to understand whether these cells get directly or indirectly instructed from ILC3 to produce IL-7, TSLP, and SCF.

ENVIRONMENTAL SENSORS

Apart from cytokine receptors, ILC3 are capable of directly recognizing environmental cues. Here, we will describe the main receptors that have been reported to enable ILC3 accomplishing this task.

Aryl hydrocarbon receptor

Both mouse and human ILC3 express the transcription factor aryl hydrocarbon receptor (AhR), belonging to the basic helix–loop–helix/Per–Arnt–Sim (bHLH/PAS) family of proteins. After engaging its ligand, AhR translocates from the cytoplasm to the nucleus where it pairs with AhR nuclear translocator (ARNT or HIF-1β) and then binds to xenobiotic response elements (XRE) present in the regulatory regions of AhR target genes (71, 72). Relevant target genes encode xenobiotic-metabolizing enzymes, including the cytochrome p450 superfamily members CYP1A1, CYP1A2, and CYP1B1. Several endogenous molecules, including metabolites of tryptophan and arachidonic acid have been shown to activate AhR. Among exogenous molecules functioning as AhR agonists, plant phytochemicals, such as polyphenols and glucosinolates as well as environmental toxins (dioxin) have been described. Moreover, bacterial metabolites have also been reported to display AhR agonist functions. Thus, AhR mainly acts as an environmental sensor (71, 73).

The number of post-natal ILC3 as well as the development of CP and ILF is drastically reduced in *Ahr*-deficient mice. Conversely, the number of fetal LTi and secondary organ development is not perturbed. The decrease in ILC3 observed in *Ahr*-deficient mice is not evident until the third week, suggesting that environmental stimuli may contribute to the differentiation, survival, and post-natal expansion of ILC3. Indeed, ILC3 from *Ahr*-deficient mice displayed lower expression of c-kit and IL-7R, as well as of the anti-apoptotic genes *Bcl2* and *Bcl2l1*. Moreover, IL-22 but not IL-17 expression by ILC3 in SI and colon was consistently reduced in *Ahr*-deficient mice (28–30). Despite the evidence of a central role of AhR in the maintenance and functions of ILC3, the endogenous and exogenous ligands driving this process still remain unclear. Kiss et al. have proposed a major role for AhR ligands derived from food components. Indeed, they could show that mice fed with phytochemical-free diets had a phenotype similar to *Ahr*-deficient mice (28). However, by using a different diet to feed mice, these data could not be confirmed by Lee et al. (29). Can ILC3 sense commensal bacteria via AhR? AhR ligands from bacterial metabolites can modulate ILC3 functions. It was recently shown that under conditions of unrestricted tryptophan availability, *Lactobacilli species* can produce an AhR ligand (indole-3-aldehyde) enhancing IL-22 expression in ILC3, which allows the survival of mixed microbial communities and provides colonization resistance to *Candida albicans* (74). In the future, it would be of great interest to understand whether ILC3 can employ AhR-dependent strategies to directly sense pathogens and produce effector cytokines.

Toll-like and other pattern recognition receptors

Pattern recognition receptors (PRR), such as Toll-like receptors (TLR), are mainly expressed on APC, such as macrophages or DC. Engagement of PRR by pathogen-derived ligands induces APC activation and production of pro-inflammatory cytokines (75). TLR2 and the C-type lectin receptor dectin-1 (or CLEC7A) bind β-glucans, i.e. structural cell wall polymers of fungi, and are implicated in the immune response to *Candida albicans* (76). Since both, the IL-17/IL-22 axis and ILC3 play a role in the defense against *Candida albicans* infection (59, 77), the role of PRR recognizing fungal components has been investigated. Injection of TLR2 and dectin-1 ligands can boost IL-17 and IL-22 production by mouse ILC3 as well as by γδ T cells (10, 78). However, while mouse IL-17-producing γδ T cells express both receptors, and directly respond to TLR2 stimulation, mouse ILC3 apparently lack TLR2 and are not directly activated by TLR2 ligands (26). By using quantitative RT-PCR, Crellin et al. observed broad expression of many TLR transcripts, including TLR1, 2, 5, 6, 7, and 9 by *ex vivo* isolated human ILC3 as well as by cloned ILC3 lines, although the degree of expression was still lower than on monocytes. Interestingly, only TLR2 agonists were able to induce cytokine production by human ILC3 in the presence of cytokines like IL-2, IL-15, and IL-23 (26). Thus, TLR2 engagement in ILC3 seems to act as a costimulus, rather than as a trigger on its own, as is the case when TLR agonists stimulate myeloid cells. This finding is in line with previous observations on other lymphocyte subsets, such as T cells and NK cells (79, 80). TLR2 triggering induces different responses depending on the cytokine employed as costimulation. TLR2 engagement

induces IL-5 and IL-13 in the presence of exogenous IL-2 or IL-15, but not of IL-23. In contrast, IL-22 can be induced by either combination of signaling pathways (26). In light of its role in fungi recognition, TLR2 engagement might contribute to human ILC3 activation during *Candida* infection. Moreover, polymorphisms of TLR2 are linked with the disease phenotype in IBD. However, it remains to be established whether TLR2 activation of ILC3 plays a role *in vivo* (81).

NK cell activating receptors

Both human and mouse ILC3 subsets express NK cell actR, which have been described to mediate NK cell cytotoxicity and production of cytokines, such as IFN-γ and TNF, upon recognition of cognate cellular and viral ligands. NK cell actR include NK group 2, member D (NKG2D), DNAX accessory molecule (DNAM)-1, 2B4, CD94/NKG2C, and the natural cytotoxicity receptors (NCR), namely NKp46 (also known as NCR1 or CD335), NKp44 (also known as NCR2 or CD336), and NKp30 (also known as NCR3 or CD337). Human activating killer Ig-like receptors (KIR) and NKp80 or mouse Ly49 and NK1.1 also function as actR in NK cells (82, 83).

As previously mentioned, a subset of T-bet-dependent mouse ILC3 expresses NKp46 and has also been named NCR$^+$ ILC3. NKp46$^+$ ILC3 isolated from the SI LP of B6 mice largely co-express NKG2D and 2B4, while only a part of these cells co-express NK1.1 (15, 16). As engagement of actR induces effector functions in NK cells, actR triggering was also explored as a potential activating stimulus in ILC3. Engagement of NKp46, NK1.1, or 2B4 did not succeed in inducing IFN-γ or IL-22 expression in SI LP NKp46$^+$ ILC3 (63). Conversely, NK1.1 but not NKp46 triggering was sufficient to drive IFN-γ and TNF, but not IL-22, expression in splenic NKp46$^+$ RORγt^{fm+} RORγt$^-$ ILC3 (22). A consistent fraction of human ILC3 derived from tonsils and gut LP expresses NKp44, NKp46, and NKp30, although to lower levels compared to NK cells. NKG2D or CD94/NKG2C is conversely not expressed by human ILC3. Thus, among NK cell actR, human ILC3 preferentially express NCR (7, 16, 21, 22, 65). In a recent study, we could show that among the different NK cell actR expressed by ILC3, namely NKp46, NKp30, and CD2, only engagement of NKp44 results in a strong cytokine response by ILC3 (22). Thus, the basic biology of NKp44 and its role on human ILC3 will be further discussed.

NKp44

NKp44 belongs to the NCR family, which represents type I membrane proteins of the immunoglobulin superfamily. NKp44 is not conserved between humans and mouse. In contrast to the other NCR, NKp44 is not expressed on resting human NK cells but it is up-regulated on their surface after IL-2 stimulation and upon engagement mediates the killing of susceptible tumor cell lines (84, 85). Conversely, NKp44 is detectable *ex vivo* on ILC3 and selectively marks the IL-22-producing subset in human tonsil and gut LP (16, 21, 22). NKp44 as well as NKp30 and NKp46 comprises of three domains: the extracellular ligand-binding domain, the transmembrane one, and a short cytosolic tail lacking intracellular signaling activity and therefore associating with ITAM-containing adaptor proteins (82, 83). Engagement of NCR results in recruitment and activation of zeta chain-associated protein kinase 70 (ZAP-70) and spleen tyrosine kinase (SYK), leading to the activation of several downstream signaling molecules, including phosphatidylinositol 3-kinase (PI3K) and phospholipase C (PLC)-γ1 or PLCγ2 (86). Unique among the NCR, NKp44 is coupled to a dimer of the ITAM-containing adaptor DNAX-activation protein (DAP)12 for downstream signal transduction and triggering of NKp44 in IL-2-activated NK cells leads to cytotoxicity of tumor target cells (84). Engagement of NKp44 in *ex vivo* isolated ILC3 is sufficient to induce cytokine production, demonstrating that ILC3 can directly sense the environment and be activated in the absence of pro-inflammatory cytokines (22). NKp44 triggering in *ex vivo* isolated ILC3 selectively induces the expression of TNF and IL-2 as well as a coordinated pro-inflammatory program, while cytokine stimulation (IL-23, IL-1, and IL-7) preferentially induces IL-22 and GM-CSF expression. Thus, ILC3 are able to switch between IL-22 or TNF production, depending on the triggering stimulus. However, combined engagement of NKp44 and cytokine receptors results in a strong synergistic effect both at transcriptome and protein level (22).

Which ligand is recognized by NKp44 expressed by NK cells or ILC3? Although several tumor cells and bacteria can bind NKp44–Ig fusion protein (87, 88), and NKp44 blocking via specific antibodies can decrease activated-NK cell-mediated cytolysis (84), as well as ILC3 cytokine production (22), the identity of NKp44 ligands triggering NK cells and ILC3 remains elusive. Discovering cellular ligands of the NCR still represents a great challenge, and only few cellular and viral ligands have been identified (89), such as the NKp30 ligands BAT3 (or BAG6) and B7-H6, which are expressed or released by tumor cells (90, 91). NKp44 has been described to bind to sialylated and sulfated cellular proteoglycans, hemagglutinin (HA) from influenza virus, and other viral HA-neuraminidase proteins, and HA engagement of NKp44 results in NK cell activation (92, 93). Surprisingly, influenza virus HA does not trigger NKp44-mediated cytokine expression in ILC3 (22). However, as sialic acid moieties attached to the stalk domain of NKp44 contribute directly to HA binding (93), glycosylation pattern of NKp44 expressed by NK cells or ILC3 should be analyzed. In addition, an inhibitory ligand of NKp44, namely proliferating cell nuclear antigen (PCNA), has also been reported (94). Very recently, a truncated form of the mixed lineage leukemia-5 (MLL5) protein has been described being an activating ligand of NKp44 and named NKp44L (95). Unlike MLL5, which is present in the nucleus and cytosol, NKp44L displays a unique C-terminal sequence that is required for its localization at the cell surface and its interaction with NKp44. NKp44L expression is present on the surface of several tumor cell lines, which are susceptible to NK cell-mediated lysis and their killing can be reduced using an anti-NKp44L antibody (95). As the nature of the NKp44 ligands eliciting inflammatory signatures in ILC3 remains elusive, it would be of great interest to test whether NKp44L could also trigger ILC3 cytokine production. In particular, it would be important to investigate whether, in addition to tumor cell lines, NKp44L would be also up-regulated on damaged or infected epithelial cells. NKp44 recognition of ligands expressed by microorganisms or intestinal epithelial cells could participate in fighting selected pathogens, restraining gut microflora, or even regulating epithelial

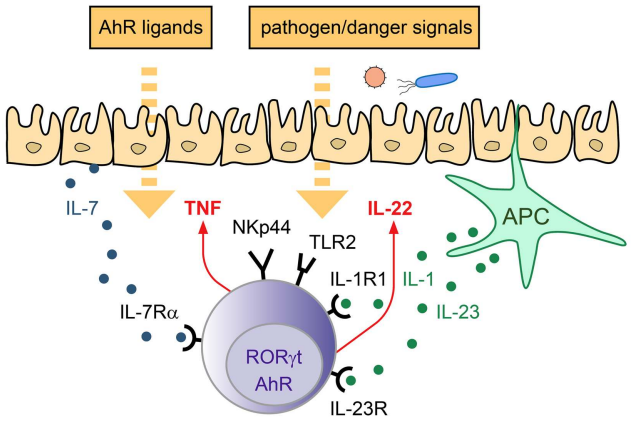

FIGURE 1 | Strategies of ILC3 to sense the environment. LP-resident ILC3 can sense the environment via their receptors for cytokines, actR, AhR, and PRR. Depending on the triggering stimulus production of pro- and anti-inflammatory cytokines can be induced, which mediate the response to pathogens and promote tissue homeostasis/repair.

cell homeostasis at steady state or during inflammation. In this context, it was recently reported that NKp44L is present on the surface of normal human articular chondrocytes (96). As ILC3 accumulate in the synovial fluid of patients affected by rheumatoid arthritis and can mediate proliferation of fibroblast-like synoviocytes in a TNF- and IL-22-dependent manner (97), the role of NKp44–NKp44L interactions in mediating this process should be investigated. Revealing the nature of NKp44 ligands relevant for ILC3 activation would enable us to better understand ILC3 functions in steady state and during inflammatory conditions.

CONCLUSION

In conclusion, ILC3 are important innate effectors involved in the defense against extracellular pathogens as well as in the maintenance of the epithelial barrier. ILC3 can be activated directly after engagement of environmental sensors and/or in response to epithelial and APC-derived cytokines. Subsequently, they produce their signature cytokine IL-22 as well as other mediators, thus being able to modulate both, immune- as well as epithelial cell functions at mucosal interfaces (**Figure 1**).

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Role of exosomes released by dendritic cells and/or by tumor targets: regulation of NK cell plasticity

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Exosomes are endosomal-derived nanovesicles released by normal and tumor cells, which transfer functionally active proteins, lipids, and nucleic acids between cells. They are important mediators of intercellular communication and act on the adjacent stroma as well as in the periphery. Recently, exosomes have been recognized to play a pathophysiological role in various diseases such as cancer or infectious diseases. Tumor cell-derived exosomes (Tex) have been shown to act as tumor promotors by educating non-malignant cells to provide a tumor supporting microenvironment, which helps to circumvent immune detection by the host and supports metastasis. However, Tex with anti-tumor, immune-activating properties were also described reflecting the complexity of exosomes. Here, we assess the role of extracellular microvesicles/exosomes as messengers affecting NK cell function in health and disease and discuss the molecular basis for the differential impact of exosomes on NK cell activity. The molecular composition/load of exosomes and the mechanisms regulating their release remain unclear and need to be further analyzed to facilitate the development of new treatment options targeting the exosomal machinery.

Keywords: exosomes, NK cell regulation, tumor derived vesicles, dendritic cell-derived exosomes, microvesicles

INTRODUCTION

Intercellular communication is a prerequisite for a functional immune surveillance in order to obtain a healthy organism. Virus infected or malignant cells are identified and eliminated by the immune system via membrane bound or secreted molecules.

Besides these well-known pathways of cell–cell communication the impact of endosomal-derived microvesicles, so called exosomes, has emerged as another important mechanism to regulate the immune response.

The function of exosomes was originally attributed to the elimination of obsolete proteins, however, it became quite clear that these vesicles are important mediators of intercellular communication. Exosomes are a homogeneous group of nanovesicles with a size of approximately 50–100 nm. They are budded into the limiting membrane of endosomes to form multivesicular bodies (MVB). The fusion of the MVB with the plasma membrane leads to the release of the intraluminal vesicles into the extracellular space.

Exosomes are specifically loaded with membrane and cytosolic proteins as well as nucleic acids, depending on the originating cell and its homeostatic state. This results in the formation of functionally distinct exosomes and their activity ranges from immune activation to immune suppression or induction of tolerance. However, the mechanisms that regulate the exosomal release and the sorting of specific molecules in exosomes are far from being understood. In this perspective, we focus on the interaction of exosomes with NK cells.

DENDRITIC CELL-DERIVED EXOSOMES: POTENT ACTIVATORS OF NK CELLS

The ability to activate NK cells has been primarily reported for exosomes derived from dendritic cells (DCs). Dendritic cell-derived exosomes (Dex) can directly trigger NK cell activation in mice and cancer patients. The interaction of TNF expressed on Dex with the corresponding TNF receptors on NK cells induced interferon-γ (IFNγ) secretion from NK cells. These data demonstrate that Dex can mediate essential innate immune functions – a feature that was previously ascribed to DCs themselves (1). The use of Dex in clinical phase I trials was reported to trigger NK cells in half of the patients (2–4), but the underlying mechanisms for the Dex bioactivity on NK cells remained unclear. In 2009, Viaud et al. showed that membrane bound ligands for NKG2D as well as IL-15Ra seemed to play an essential role in Dex-induced activation of NK cells in humans and mice (5). Thereby, Dex triggered an IL-15Ra and NKG2D-dependent NK cell proliferation and activation in secondary lymphoid organs in mice. A first phase I clinical study with 15 melanoma patients supported the notion that Dex vaccines significantly enhanced the number of circulating NK cells and restored their NKG2D expression levels (5). Furthermore, exosomes released from DCs expressed BAG6, a ligand for the activating NK cell receptor NKp30. BAG6-expressing exosomes induced NK cell-mediated cytokine release and cytotoxicity, indicating a role for exosomes released from DCs to initiate the innate function of NK cells (6). The exosomal release of danger signals that alert NK cells may be considered as priming signal and supports a two-step activation model of human NK cells involving a priming and a triggering event.

As Dex also express functional MHC molecules and T cell co-stimulatory molecules such as CD40, CD80, and CD86 (4), they are also potent T cell activators (reviewed elsewhere). In addition, Dex can directly mediate tumor cell killing via Fas-L and TRAIL (1). This combination of direct anti-tumor and immune stimulatory activity seem to make Dex ideal tools for cancer treatment. Nevertheless, Dex therapies were so far not expanded into the

clinical routine. This might be attributed to technical difficulties to produce sufficient amounts of exosomes with defined properties. A better basic understanding of the regulation, biosynthesis, and specific protein and RNA cargo of exosomes from different cell types and environments would definitely inspire the development of novel therapies, which might in the end be based on *in vivo* modulation of exosome release and function. This holds also true for exosomes released from tumor cells (Tex). Tumor-derived exosomes are predominantly described as immune-suppressing vesicles, however, there are also reports of anti-tumor immune-activating Tex. As soon as we understand the signals directing the formation of functional distinct exosomes, we can proceed and modify the exosomes and their release therapeutically to retain their anti-tumor activity.

TUMOR CELL-DERIVED EXOSOMES: BENEFIT OR DANGER?

Tumor cells develop a number of mechanisms to escape or suppress an active immune response such as down-regulation of surface MHC molecule expression (7), secretion of immune-inhibitory cytokines (8) or by regulating stromal components to generate a tumor growth promoting microenvironment (9).

More recently, the impact of tumor cell-derived exosomes on immune surveillance has been discussed. While the effect of tumor cell-derived exosomes (Tex) on T cells is extensively investigated, little is published on the direct impact of Tex on NK cell function. Unlike for Dex, Tex are discussed to be immune-activating as well as immune-inhibitory, although reports on Tex with immune-stimulating function are clearly outnumbered by studies indicating an inhibitory effect on the immune response.

TEX AS IMMUNE STIMULATORS

As Tex have the ability to express tumor-associated antigens, they play a role in cancer immunology, such as transport of antigens to DCs to initiate an anti-tumor immune response via cross-presentation (10, 11). HepG2 and PLC/PRF/5 cell lines were used as models to study heat shock protein (Hsp)-bearing exosome secretion by hepatocellular carcinoma cells under stress conditions (12). Their results showed that incubation of NK cells with Hsp-bearing exosomes augmented cytolytic activity against K562 or HepG2 target cells through granzyme B release; up-regulation of activating receptors CD69, NKG2D, and NKp44; and down-regulation of inhibitory receptor CD94. This seemed to be dependent not only on exosome concentration but also on Hsp expression, with notably higher Hsp expression on HepG2-released exosomes after treatment with chemotherapeutics. Interestingly, treatment with resistant anti-cancer drugs seemed to enhance Hsp expression on exosomes more efficiently than sensitive chemotherapeutics, leading to a more pronounced NK cell activation (12). This is in line with findings of Gastpar and colleagues, who showed that NK cells were stimulated by human pancreas and colon carcinoma sublines-derived exosomes, depending on their capacity to present heat shock protein 70 (Hsp70)/Bag-4 on their membranes. Natural killer cells were stimulated selectively by Hsp70/Bag-4 surface-positive exosomes; an effect that could be blocked with Hsp70-blocking antibody (13). BAG6, another Hsp70-interacting protein, is known to engage the activating NK cell receptor NKp30 and BAG6-expressing exosomes trigger NK cell-mediated cytokine release and cytotoxicity (14, 15). Moreover, Tex may induce the up-regulation of granzyme B, IL-2, IFNγ, TNFα, CD25, and reduce CD95L expression in NK cells (16), further arguing for Tex-supported NK cell activation.

Still, Tex are mostly described as inhibitors of the immune system supporting tumor immune evasion, indicating that the formation of NK cell-activating exosomes may depend on the tumor cell type, state of tumor progression, and the microenvironment, factors that still need to be defined.

TEX AS IMMUNE INHIBITORS

Several reports state an immune suppressive effect of tumor cell-derived exosomes on NK cells. This effect is frequently associated with an altered surface protein expression. Often, ligands for the activating NK cell receptor NKG2D are being identified as crucial factors.

Clayton et al. demonstrated that NKG2D is down regulated on CD3-positive peripheral blood leukocytes following exposure to tumor-derived exosomes, resulting in an impaired cytotoxic effector function of the $CD8^+$ T cells (17). This effect was dependent on the exosomal expression of NKG2D ligands (NKG2D-L) and could be abolished by blocking with corresponding NKG2D ligand antibodies. Yet, in this study the effects of Tex were only detected on T cells, but not proven for CD3-negative PBL. Subsequent publications indicate that NKG2D-L-bearing exosomes can have similar effects on NK cells. For instance, incubation of NK cells with MICA-containing exosomes provokes an NKG2D-dependent reduction of NK cell cytotoxicity independent of NKG2D-L expression by the target cell (18). In a further study, NKG2D-L-expressing tumor-derived exosomes showed a direct interaction with NK cells and $CD8^+$ T cells leading to a significant reduction in cell surface NKG2D expression (19). This was at least partly dependent on NKG2D-L; however, in this study the impact of exosomal TGFbeta1 on the observed effects was much stronger. Exosome-mediated down-modulation of NKG2D correlated with poor functional responses, as the interaction resulted in an impaired cytolytic function of NK cells. Thus, the efficiency of Tex might reflect their specific feature to display multiple inhibitory signals at once and by this being able to even overcome pro-inflammatory signals. This is in line with findings reporting an inhibition of IL-2-mediated up-regulation of CD25 by tumor exosomes selectively in NK cells and $CD8^+$ T cells (20). At the same time, $CD4^+CD25^+$ Treg cells remained IL-2 responsive and their inhibitory function was enhanced by tumor exosomes. Liu and colleagues showed that exosomes derived from murine mammary carcinoma cell lines inhibit NK cell cytotoxic activity *ex vivo* and *in vitro*. Key features of NK cell activity were inhibited, including release of perforin but not granzyme B, as well as the expression of cyclin D3 and activation of Jak3-mediated pathways. Human tumor cell lines were also found to produce exosomes that were capable of inhibiting IL-2-stimulated NK cell proliferation. Thus, it was proposed that tumor exosomes contribute to the growth of tumors by blocking IL-2-mediated activation of NK cells and their cytotoxic response to tumor cells (21).

Clinical observations revealed that sera of acute myeloid leukemia (AML) patients have higher levels of microvesicles with an explicit molecular profile expressing membrane-associated

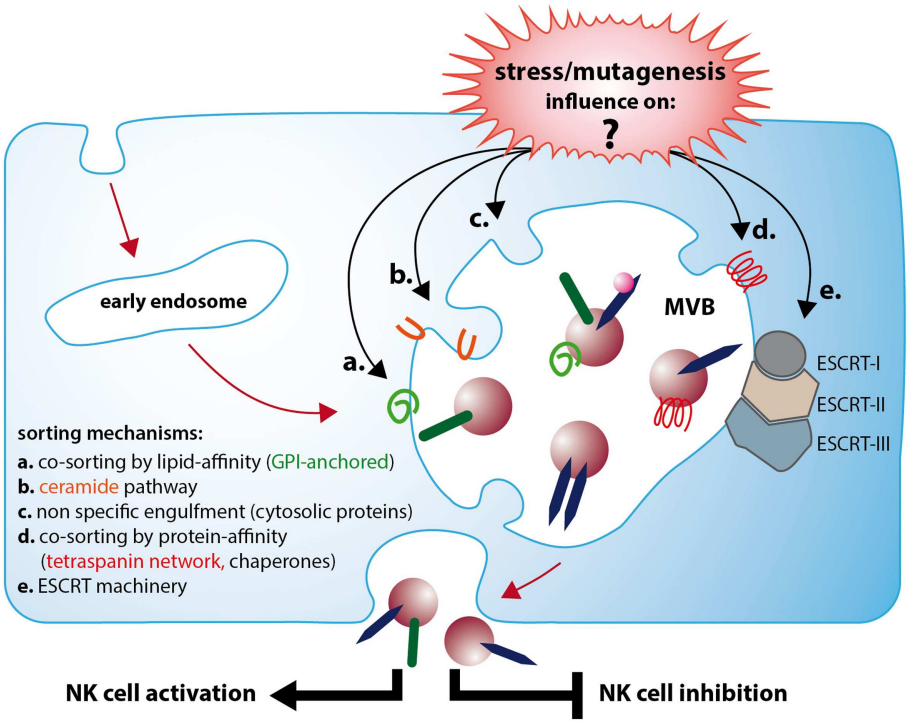

FIGURE 1 | Exosomes released from stressed or neoplastic cells can lead to NK cell activation or inhibition, which depends on a differential molecular composition. Immune-activating exosomes (e.g., Dex) often express TNF and can induce INFγ secretion and enhance cytolytic activity of NK cells. In contrast, immune suppressive exosomes often contain TGFbeta1 and NKG2D ligands and inhibit NK cell cytotoxicity. Cellular stress and mutagenesis affect the biogenesis of functional distinct exosomes. Although several features attributed to one or the other effect are described, the mechanisms leading to the respective exosome formation are still poorly understood. A major question remaining to be answered is how stress and mutagenesis affect the different sorting mechanisms responsible for the genesis of immune-activating or -suppressive vesicles. As soon as the determining pathways and their regulation are comprehensively explored, the exosome cargo will become more predictable and allow the development of new, astute strategies to employ exosomes as therapeutic tools.

TGFbeta1, MICA/MICB, and the myeloid blast markers CD34, CD33, CD117 (22). Again, TGFbeta1 seemed the prominent factor to induce immune suppression by decreasing NK cell cytotoxicity and down-regulation of NKG2D expression. In this setting, treatment with IL-15 protected NK cells from adverse effects of tumor-derived microvesicles. Findings on exosomes/microvesicles isolated from solid tumors or AML patient sera and their modulatory functions on different immune cells were recently summarized and comprehensively reviewed (23). In gastric cancer patients, elevated Fas levels have been detected on tumor-infiltrating as well as circulating NK cells. This was closely related to a significantly increased NK cell apoptosis (24). Thus, exosomes expressing death receptor ligands might not only be able to kill T cells (25–27) but it seems quite likely that they will have similar effects on NK cells.

Taken together, via diverse mechanisms acting in concert, Tex potentially contribute to an immunosuppressive environment, emphasizing their physiological importance for tumor immune escape. The activity of NKG2D-L on Tex is obviously contradictory to the NKG2D-L activity on Dex, which are known to activate NK cells. This reflects the context-dependent impact of a given molecule on exosomes – which further strengthens the need for a better basic understanding of exosome regulation and formation.

EXOSOME CARGO FORMATION

In summary, the composition of exosomes with proteins and nucleic acids seems to be decisive regarding their ability to inhibit or promote an immune response. Most likely, the sorting is not solely modulated by the transported protein itself, as NKG2D-L are nicely targeted to exosomes in the breast cancer cell line T47d, whereas exosomes of the strongly NKG2D-L positive T cell lymphoma line Jurkat are completely NKG2D-L negative (17). Ashiru et al. stated a putative sorting mechanism for the NKG2D-L MICA. They speculate that the specific translocation of MICA*008 into exosomes is in contrast to other MICA/B molecules due to its difference in the predicted transmembrane and cytoplasmic domains (18). Recently, the group was able to demonstrate that GPI modification of MICA*008 is responsible for the recruitment into exosomes (28). Nevertheless, today it is still little known about the mechanisms of protein sorting into exosomes. Some exosomal proteins are sorted by the ubiquitin pathway (29, 30). Current findings suggest that tetraspanin-enriched microdomains may act as compartmentalizing platforms that aid the selection of some intracellular components toward exosomes, as genetic deletion of the tetraspanin CD81 impaired the inclusion of a number of selective CD81-binding proteins into exosomes (31). Furthermore, the contribution of the ESCRT (endosomal sorting complex

required for transport)-machinery is discussed (32–34). It was also stated that the formation of proteolipid protein-containing exosomes does not require the ESCRT machinery, but sphingolipid ceramide and the activity of the neutral sphingomyelinase (35). Proteins expressed on exosomes do not share a general exosome-targeting amino acid motif, but are rather characterized by a broad range of sequence motifs that confer plasma membrane binding and higher-order oligomerization (36). This might be different for the specific loading of RNA species into exosomes. Recently it was reported that A2B1 (heterogeneous nuclear ribonucleoprotein hnRNPA2B1) specifically recognizes and binds sequence motifs present in miRNAs to control their packaging into exosomes (37).

However, the signaling pathways that direct the specific loading of proteins and nucleic acids of different cell types in response to the cell status and environment remain largely unknown (see **Figure 1**). Recently, it was shown that viral stress signals may alter the composition of exosomes and by this can change their immune-modulatory capacity. The activation of the cytosolic pattern recognition receptor (PRR) RIG-I with its respective ligand leads to an enhanced release of NK cell-activating exosomes from melanoma cells. Thus, former tumor-promoting exosomes were transformed to immune-activating, anti-tumor immune agents. This pathway establishes a novel and unexpected link of a PRR and the release of exosomes (Dassler and Reiners, unpublished work). In this context, the impact of TLR activation on exosome release and characteristics seems an interesting topic that deserves to be studied in more detail.

CONCLUSION

The multiple functions of exosomes make them somewhat double edged features for new therapeutic strategies: on the one hand, they appear to be especially suitable, as they may be selectively loaded with optimal drug combinations, have an excellent biodistribution and biocompatibility and can act with different mechanisms at once. On the other hand, to employ exosomes as anti-tumor reagent, a better understanding on exosomes target selection is required to improve therapeutic exosome application. Exosomes are suggested to bind to and be taken-up by selected targets (18). For instance, exosomes are rich in tetraspanins and some studies suggest exosomal tetraspanin–integrin complexes to be involved in target cell binding (38). Rana et al. were able to show that even minor differences in exosomal tetraspanin-complexes strongly influence target cell selection *in vitro* and *in vivo*. Their report on the contribution of exosomal tetraspanins to target cell selection is one step toward the ability to predict potential target cells.

As long as we do not thoroughly understand the mechanisms of protein (and nucleic acid) sorting into exosomes, the outcome of any new therapeutic strategy involving exosomes will be sparsely predictable. Only the basic knowledge on how the molecular composition of exosomes is determined and how exosome biogenesis is influenced by cell status, the environment or signaling will enable us to develop new, innovative strategies with exosomes as tools or targets.

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Cross-talks between natural killer cells and distinct subsets of dendritic cells

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In recent years, the essential role of bi-directional cross-talk between natural killer (NK) and dendritic cells (DC) during immune responses has been clearly elucidated. In particular, this cross-talk results in the development of an efficient innate response, through DC-mediated NK cell activation, and a potent adaptive immune response, through NK-mediate DC editing and maturation. Recently, some novel human DC subsets have been identified: migratory DCs in afferent lymph and draining lymph nodes; CLEC9A$^+$/BDCA3$^+$ (CD141) DCs in interstitial dermis, liver, lung; *inflammatory* DCs in several inflammatory fluids. At the same time, it has been shown that also human NK cells are present in these compartments. Here, we will review the most recent findings on NK/DC cross-talk and we will discuss the necessity of acquiring more complete knowledge about these interactions in view of the new information available on both DC and NK cell subsets.

Keywords: natural killer cells, dendritic cells, humans, cross-priming, Th1 cells

INTRODUCTION

Natural killer (NK) cells were originally identified as lymphocytes that can spontaneously kill certain tumor target cells in the absence of previous stimulation *in vivo* or *in vitro* (1). NK cell activation results from the balance of signals produced by activating (2) and inhibitory (3) receptors. CD16 (FcRIIIa) is one of these activating NK cell receptors and binds human immunoglobulins, therefore mediating antibody-dependent cellular cytotoxicity (ADCC) of opsonized target cells. However, many other innate receptors acting upstream of the adaptive immunity have also been discovered. Among these, the first to be identified were natural cytotoxicity receptors (NCR) termed NKp46, NKp44, and NKp30 (2). NK cells also express additional activating receptors such as NKG2D and DNAM-1, which are partially shared with T lymphocytes, 2B4, NTBA, and NKp80 which promote NK cell triggering during the process of natural cytotoxicity (4). Activating NK cell signals are therefore mediated by several receptors and it is widely accepted that the ligands for NK cell activating receptors are mainly expressed on "stressed" cells, hence favoring killing of both tumor or infected cells (4). Nevertheless, an important exception to this rule is the ability of NK cells to kill normal autologous dendritic cells (DCs) (5, 6) as well as other immune cells such as macrophages and T lymphocytes (7–9).

On the other hand, human NK cells also express different inhibitory receptors recognizing human leukocyte antigen (HLA) class I molecules: killer immunoglobulin (Ig)-like receptors (KIRs) are specific for allelic determinants of HLA class I molecules, the Ig-like transcript (ILT)-2 receptor is characterized by a specificity for different HLA class I molecules, and CD94/NKG2A recognizes non-classical HLA class I molecules HLA-E (4). Therefore, cells that have lost HLA class I molecules such as tumor or virus-infected cells fail to deliver inhibitory signals to NK cells.

Peripheral blood NK cells in humans can be divided into two main subsets according to CD56 expression, namely CD56^{dim} and CD56^{bright}, characterized by distinct functional and phenotypic properties. It has been established that a division of labor exists among these two subsets: CD56^{dim}, expressing CD16, KIRs, and high levels of perforin, have enhanced killing activity, whereas CD56^{bright} cells, characterized by low levels of perforin and CD16, no KIRs and high expression of NKG2A, can secrete large amounts of cytokines (e.g., IFN-γ, GM-CSF, TNF) but not kill target cells. Nevertheless, with the appropriate stimulus, also CD56^{dim}CD16$^+$ NK cells are abundant cytokine producers (10, 11).

In the last few years, the functional links between NK cells and DCs have been widely investigated and different studies have demonstrated that reciprocal activations ensue upon NK/DC interactions. More recently, the anatomical sites where these interactions take place have started to be identified together with the related cell subsets involved.

Dendritic cells were identified for the first time in 1973 by Ralph Steinman as accessory cells in mice spleen. During the last two decades, it has been established that DCs are professional antigen presenting cells (APCs), uniquely skilled to attract and activate CD4$^+$ and CD8$^+$ T cells. Most of our knowledge on DCs comes from studies of blood and skin DCs. However, improvements of both flow cytometric and genomic approaches have recently allowed the identification of several distinct subsets of DCs. Despite their heterogeneity, there are some features common to all DC subsets, both in mice and humans. Immature DCs act like sentinels efficiently sampling antigenic material. Upon pathogen encounter, they undergo a complex maturation process that leads to professional antigen presentation, cytokine production, and T cell stimulatory capacities. During the maturation process, they upregulate distinct molecules on their surface

such as major histocompatibility complex (MHC) class II, CD80, CD83, CD86, and CD40 essential for antigen presentation and interaction with T cells; at the same time, they migrate from the periphery to secondary lymphoid organs (SLO) where they can induce $CD8^+$ and $CD4^+$ T cell response (12).

Two main populations of DCs have been described in humans: $BDCA2^+$ (CD303)/$CD123^+$ plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) (13). The latter includes several subsets identified in distinct tissues, thus resulting in a high level of heterogeneity; peripheral blood contains two main DC subsets: $BDCA1^+$(CD1c) DCs and $CLEC9A^+$/$BDCA3^+$ (CD141) DCs (14, 15); as they are both also present in lymph nodes and tonsils, they have been described as blood-derived lymphoid organ-resident DCs (14–17).

Besides peripheral blood, also the skin includes distinct and well-characterized DC subsets: the epidermis contains Langerhans cells (LCs) and the dermis at least three subsets: dermal $CD1a^+$ DCs, dermal $CD14^+$ DCs, and $CLEC9A^+$/$BDCA3^+$ DCs (18–20). All these DC subsets can migrate through the lymph to draining lymph nodes (21). Finally, in several inflammatory conditions such as atopic dermatitis, psoriasis, rheumatoid arthritis, and tumor ascites, a different DC subtype, referred to as "inflammatory DC," has lately been described (22). Transcriptomic analysis revealed that they likely derive from monocytes that differentiate at the site of inflammation. Interestingly, it has been recently suggested that, in inflamed tissues, $CD56^{bright}$ NK cells may induce differentiation of monocytes into inflammatory DCs (23).

This broad heterogeneity corresponds to distinct specialized functions in terms of tissue distribution, cytokine release, antigen presentation, and regulation of T cell response. These different features of the distinct DC subsets will be reviewed here and discussed in the context of possible interactions of NK cells with different DC subsets.

DISTRIBUTION OF HUMAN NK CELL SUBSETS

In the last few years, it has become evident that NK cells are not exclusively found in peripheral blood and SLO but can populate different non-lymphoid tissues (24). In mice, where investigating NK cell localization is more straightforward than in humans, the presence of NK cells in many organs has been revealed (25) and distribution seems to be subset-specific, as different NK cell subsets showed organ-specific localizations. Lately, some light has also been shed regarding the distribution of human NK cells in solid tissues, showing that NK cells populate, and may re-circulate through most human peripheral tissues, and that organ-specific chemokine expression patterns can drive the homing of functionally distinct NK cell subsets to the various human body compartments, both at steady-state and pathology (26). In particular, $CD56^{bright}$ NK cells selectively accumulate in several organs, including SLO, liver, visceral adipose tissues, and gastrointestinal tract. Moreover, in a large variety of human malignancies, $CD56^{bright}$ NK cells represent the majority of NK cells infiltrating the tumor. Recently, we have reported that seroma, an accrual of fluid subsequent to surgical procedures such as axillary lymph node dissection, represents an accumulation of afferent lymph, drained from upstream tissues during the interval of time needed for lymphatic vessels to re-anastomose with the efferent ducts (27). Seroma accumulates without major contamination by either surgery-induced exudate or leaky blood-derived cells, thus confirming the lymph-associated origin of the cells contained in seroma fluids (21). Interestingly, only $CD56^{bright}$/$CD16^{low/neg}$/KIR^{neg} non-cytotoxic NK cells were detectable in afferent lymph from seroma fluids and appear therefore able, similarly to naïve T cells, to re-circulate via afferent lymph. NK cells are also present in human efferent lymph (28) suggesting that they can re-circulate from solid tissues to peripheral blood through lymphatic circulation and SLO.

The evidence that $CD56^{bright}$ NK cells are, in most solid tissues, more abundant than in peripheral blood (which contains only around 2% of human body total lymphocytes) (29, 30), suggests they might probably outnumber $CD56^{dim}$ NK cells in the human body. The functional role of such an abundant non-cytolytic, but cytokine-secreting, NK cell subset in solid organs remains to be fully clarified. Interestingly, it has been shown that human DCs primarily activate this NK cell subset (31) promoting IFN-γ release and proliferation.

DISTRIBUTION OF HUMAN DC SUBSETS

Thanks to improvements in both flow cytometric and genomic techniques, it is now clearer and clearer that human DCs represent a heterogeneous cell population and that each DC subset is often characterized by specific functional properties. $BDCA1^+$ DCs have recently been described as the most potent human IL-12-producing APCs (32), suggesting a potential key role in promoting IFN-γ release by NK cells, and therefore Th1 polarization. $CLEC9A^+$/$BDCA3^+$ DCs, originally identified in peripheral blood and lymph nodes, have recently been detected also in other human organs such as skin, liver, lung, and intestine, where they show a more mature phenotype compared to $CLEC9A^+$/$BDCA3^+$ DCs observed in either blood or lymph nodes, indicating that they may represent a mature stage of differentiation (18). Moreover, $CLEC9A^+$/$BDCA3^+$ DCs are characterized by the peculiar ability to cross-present antigens from dead cells better than other DC subtypes (18) but they seem equally able to cross-present soluble antigens (33) when compared to other DCs. It can be hypothesized that different DC subsets need distinct TLR stimulation to efficiently cross-present exogenous antigens. Cross-presentation represents a key process for specific CTL response against most tumors and viruses that do not infect APCs. The antigen forms, as well as the activation signals received by DCs, are likely to be critical in determining the efficiency of cross-presentation. Moreover, it has been shown that $CLEC9A^+$/$BDCA3^+$ DCs have the dual capacity to produce both IL-12 and type I IFN (34), thereby enabling both NK cell activation and Th1 polarization, which could be significant for a protective immune response against viral infections. In particular, type I IFN can enhance NK cell cytotoxicity while IL-12 promotes IFN-γ secretion and Th1 polarization.

Dendritic cells in human skin also show distinct patterns of functional capabilities: dermal $CD1a^+$ DCs have been described as immature cells capable of inducing T cell response only upon stimulation, while in steady-state they might be tolerogenic (35). On the other hand, dermal $CD14^+$ DCs seem to play a critical role in the regulation of humoral immunity and LC in the induction of CTL response (20). LCs also secrete IL-15 and are therefore potentially able to activate both $CD8^+$ T cells and NK cells (36).

In general, immature (non-activated) DCs act as sentinels in peripheral tissues; upon activation through danger signals they initiate the maturation process that allows them to migrate to lymph nodes via afferent lymph. In many experimental animal models, DCs have been shown to be able to continuously migrate from intestine or from skin to SLO (12, 37, 38). Most recently, DCs in human afferent lymph have also been characterized (21). Besides dermal CD1a$^+$ DCs, dermal CD14$^+$ DCs and LC, afferent lymph also includes CLEC9A$^+$/BDCA3$^+$ DCs and CD1a$^+$ CD14$^+$ DCs, the latter likely representing an immature stage of differentiation from CD14$^+$ DCs to CD1a$^+$ DCs.

NK CELL CROSS-TALK WITH DENDRITIC CELLS

The cooperative interaction between DCs and NK cells plays a key role in triggering immune response against pathogens. This dialog results in a bi-directional activation and has effects also on the subsequent adaptive immune response, influencing the development of Th1 cells and CTLs, both essential for an effective anti-tumor and anti-viral immune response.

DCs INDUCE NK CELL ACTIVATION

Dendritic cells promote the release of cytokines by NK cells (mainly TNF and IFN-γ) and enhance NK cell proliferation and cytolytic activity. DC-mediated NK cell activation occurs mainly through the release of soluble factors (**Figure 1**) although cell-to-cell contacts play a relevant role during NK/DC interaction, as better specified below.

It has been shown, in different mice models, that NK cell pre-activation by DCs is required for an efficient immune response against viral infections (39–41) and tumors (42). A large variety of microbial stimulation and signaling via TLRs can induce DC maturation and secretion of several cytokines which can in turn activate NK cells. IL-12, mainly secreted by mDCs (in particular from BDCA1$^+$ DCs), efficiently stimulate IFN-γ secretion by NK cells. IL-18 can potentiate the effect of IL-12 by inducing the expression of IL-12R on NK cells. Moreover, IL-18 synergizes with IL-12 for enhancing NK cell cytolytic activity (43).

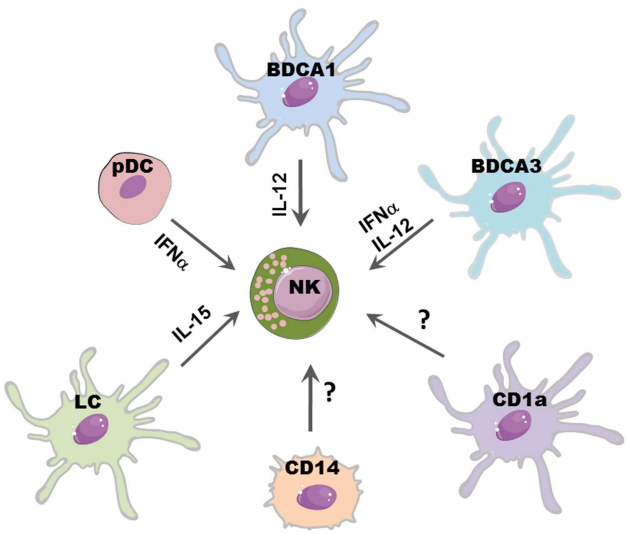

FIGURE 1 | DCs subsets may differently affect NK cell function. Distinct DC subsets reflect different capability to promote NK cell function: IL-15 released by LC may promote NK cell proliferation; IL-12, mainly produced by CLEC9A$^+$/BDCA3$^+$ DCs and BDCA1$^+$ DCs, induces IFN-γ release and subsequent Th1 polarization of T cells; NK cytolytic activity may be boosted by IFN-α secreted by pDCs and CLEC9A$^+$/BDCA3$^+$ DCs.

Also pDCs might activate NK cells, most likely via the release of type I IFN, which has been shown sufficient to boost NK cell cytotoxicity (44). Indeed, a protective NK cell response during infection with the murine cytomegalovirus (MCMV) was found to be type I IFN-dependent (45). Of note, the recently described CLEC9A$^+$/BDCA3$^+$ DC subset can also release high amounts of INF-α, suggesting that, upon viral infection, they may play a key role in promoting NK cell cytotoxicity in peripheral tissues, such as skin, liver, lung, and intestine (34).

Another relevant cytokine for NK cell development and functions is IL-15, which is also produced by DCs. This cytokine can be presented by DCs via its binding to IL15R alpha or as transmembrane protein; it can stimulate NK cell proliferation, survival, and priming of protective NK cell response (44). In particular, it has been shown that LC can support NK survival via IL-15 (46). Besides the membrane-bound form of IL-15, it has been established that also other contact-dependent mechanisms are involved in NK–DC cross-talk. In general, the formation of stimulatory synapses between DCs and NK cells plays a critical role during NK cell activation induced by DC-derived cytokines, including IL-12 (47). Also, the interaction of CXC3CL1 expressed on DCs with CX3CR1 on NK cells results in IFN-γ release by NK cells (48) and it has been shown that influenza virus-infected DCs can support IFN-γ production by triggering the activating receptors NKp46 and NKG2D (49).

Most of the studies on NK/DC interactions in humans are based on DCs derived from monocytes, which are generated after several days of culture with different cytokines. On the other hand, the interactions between *ex vivo* isolated human DCs and NK cells have been poorly investigated so far and, despite the clear heterogeneity of human DC subsets, only peripheral blood DCs have, to some extent, been investigated (50–52). In these studies, it has been shown that both plasmacytoid and myeloid peripheral blood DCs are capable of activating NK cells, enhancing their cytolytic activity and inducing IFN-γ release in response to influenza virus or dsRNA.

Among human NK cell subsets, CD56^{bright} NK cells were found to be particularly responsive to activation by DCs (31, 53). Interestingly, they are enriched in SLO and in most solid tissues (26). Their presence in afferent lymph also suggests that they may re-circulate from peripheral solid tissues to SLO; thus, it is conceivable that, *in vivo*, NK–DC cross-talk may occur either in peripheral tissues or in lymph nodes, where, in both cases, NK cells can encounter distinct myeloid DC subsets. Recent reports indicate that DC heterogeneity may also correspond to the induction of different functions in NK cells: BDCA1$^+$ DCs may be important for IL-12 secretion in SLO, favoring IFN-γ secretion and consequent Th1 polarization of T cells; CLEC9$^+$BDCA3$^+$ DCs may be relevant in peripheral tissue where, upon virus infection, they can induce NK cytolytic activity by releasing IFN-α.

In conclusion, the activation of NK cells ensuing upon interaction with DCs has important consequences not only for the lysis of tumor or virus-infected cells, but it can also boost ongoing adaptive responses by the release of IFN-γ, which promotes type 1 polarization of T cells (**Figure 2**). Moreover, once activated, NK cells can edit DCs, by eliminating the more immature, allegedly tolerogenic DCs, as further discussed below. At the same time, NK cells can also shape adaptive immune responses by causing DC activation.

NK CELLS INDUCE ACTIVATION AND EDITING OF DCs

Activation of NK cells can occur via triggering of activating receptors by target cells or by stimulation of soluble factors released by accessory cells. Following activation, NK cells release large amounts of TNF and IFN-γ, which are known to affect DC maturation. TNF enhances the expression of costimulatory molecules on DCs and, synergizing with IFN-γ, contributes to DC production of IL-12 (54, 55). Moreover, exposure of NK cells to innate cytokines such as IL-12 and IL-18 (both released by mDCs) can promote Th1 polarization [**Figure 2** and Ref. (56)]. INF-γ can also induce the expression of a membrane-bound form of IL-15 on DCs, thus sustaining both T and NK cell survival and activation (57).

Besides soluble factors, it has been shown that engagement of the NK activating receptor NKp30 can mediate DC maturation (58). Thus, recognition of target cells by NK cells can induce an additional mechanism of DC maturation, which might be particularly relevant in tumor immunity, where the absence of danger signals precludes DC maturation via the engagement of pattern recognition receptors (59, 60).

During initiation of an anti-viral or anti-tumor immune response, the microenvironment is influenced by a peculiar cytokine milieu, which includes cytokines released following NK cell activation (61, 62). It must be noted that NK cell triggering often occurs upstream of T cell activation, providing both a first line of defense and an early production of cytokine, critical for the subsequent development of the adaptive immune response. Although it is generally accepted that CTL response needs helper signals provided by $CD4^+$ T cells, interactions occurring between DCs and NK cells can bypass these helper signals by leading to the production of IFN-γ, which, in turn, can stimulate IL-12 production by DCs, thus eventually leading to a protective CTL response (57, 63, 64).

While the helper role of NK cells in inducing DC-mediated generation of Th1 polarized T cells and CTLs has been well documented, an issue not exhaustively elucidated so far is the ability of NK cells to promote DC cross-priming. Nevertheless, some reports suggest a role for NK cells in promoting antigen cross-presentation by DCs. It has been shown that DCs can take up dying cells killed by NK cells and present them on MHC class I molecules (65, 66). Obviously, NK cell ability to lyse virally infected or tumor cells could help uptake and cross-presentation of antigens by DCs but whether NK cells also play a direct role in favoring DC cross-presentation is still not clear. In a human *in vitro* system, it has

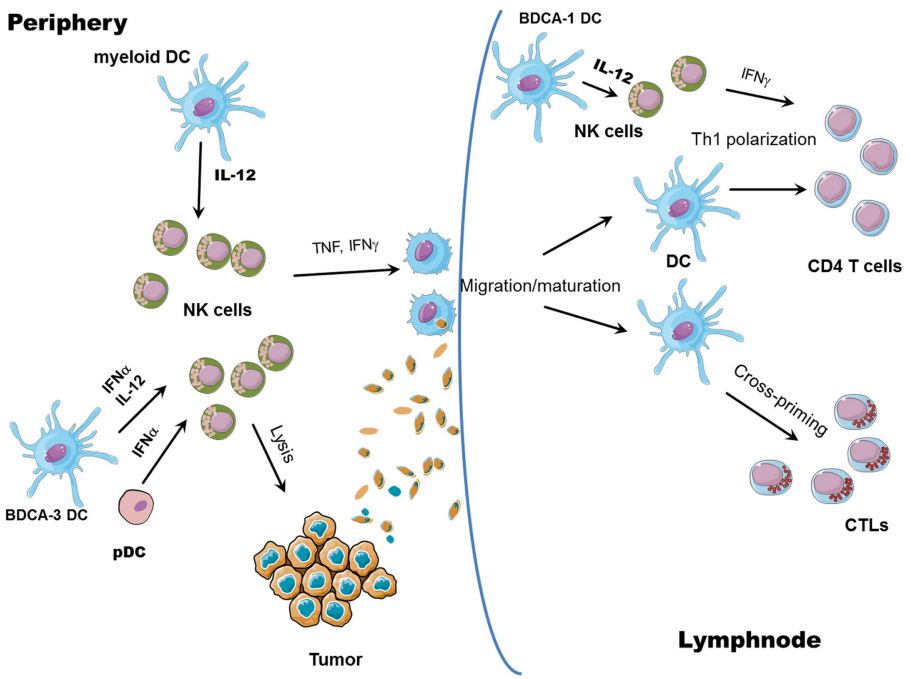

FIGURE 2 | NK/DC cross-talk. The interaction between NK cells and DCs results in reciprocal activation: mature DCs release cytokines able to promote NK cell activation (all myeloid mature DCs can produce IL-12 whereas pDCs and $CLEC9A^+$ $BDCA3^+$ DCs can release large amounts of IFN-α); in turn, IFN-γ released by activated NK cells promotes Th1 polarization and, together with TNF, DC maturation, and migration to draining lymph nodes. Activated NK cells can also lyse tumor cells, leading to the generation of tumor antigenic material; tumor debris are then engulfed by DCs and tumor antigens are processed and presented by mature DCs to both $CD8^+$ (cross-priming) and $CD4^+$ T cells.

been demonstrated that cross-presentation of antigens to $CD8^+$ T cells by DCs requires NK cells: capture of tumor cells and maturation status of DCs are not sufficient to induce cross-priming of T cells without further NK-mediated activation and IL-18 release (67). Moreover, the capability of mono-derived DCs, generated in the presence of IFN-α (IFN-DCs), to prime $CD8^+$ T cells against human tumor antigens is dependent on NK cells; NK cell removal indeed leads to generation of IFN-DCs with no priming activity of tumor Ag-specific T cells (68). *In vivo*, in a mice model of melanoma, tumor regression resulted from an immune cascade initiated by activated pDCs and involving NK cells, mDCs, and T cells. It was shown that CpG-activated pDCs can recruit NK cells at the tumor sites via chemokine production (CCL3, CCL4, and CCL5), and enhance their cytolytic activity through IFN-α release. Activated NK cells, in turn, can kill tumor cells, induce mDC maturation, and migration to draining lymph nodes, where mDCs can cross-present tumor antigens to CD8 T cells (69). Again, in this study, cross-priming of $CD8^+$ T cells is exclusively NK cell-dependent, as NK cell depletion results in complete abrogation of $CD8^+$ T cell priming. Therefore, it is likely that NK cells can favor cross-presentation by DCs, although the specific abilities of different DC subsets, as well as the mechanisms involved are still to be clearly identified. It is conceivable that NK cell killing of tumor cells could provide antigens subsequently taken up, processed, and cross-presented by DCs; at the same time, activation of NK cells is associated to the secretion of cytokines, such as TNF or IFN-γ, potentially able to help cross-priming of specific CTLs (**Figure 2**).

Thus, NK cells, upon interaction with DCs, can induce the activation of specific functions on DCs. Nevertheless, the capability of NK cells to induce DC activation is not the only mechanism by which NK cells may influence DC functions. Once activated by DCs, NK cells acquire the capability of killing immature, but not mature, mDCs (5). It has been proposed that more mature, activated DCs, by upregulating their surface expression of MHC class I molecules, would be protected from NK cell lysis. Conversely, immature DCs, expressing lower levels of MHC class I molecules, are more susceptible to NK cell killing. DCs that fail to express sufficient amounts of MHC class I molecules would induce inappropriate, low affinity T cell priming resulting either in Th2 response or in the induction of tolerance (70, 71). For these reasons, it was hypothesized that NK-mediated DC killing might represent a mechanism of DC selection for the control of downstream adaptive immune response (DC editing) (70). While *in vivo* evidence for DC activation by NK cells has extensively been provided, the direct demonstration that DC editing by NK cells also occurs *in vivo*, as well as its putative role in promoting an efficient immune response, has only recently been proven (72). In an experimental model of cancer cell vaccination, NK cells were necessary for removing less immunogenic DCs by a perforin-dependent mechanism, leading to an improved capability of residual DCs to induce anti-tumor CTL response and mice survival.

CONCLUDING REMARKS

Studies performed in the last few years have clearly shown that, during immune response, different leukocytes act by not only displaying their own protective functions, but also interacting with each other to optimize the response against microorganisms and cancer cells. Recent identification of different DC subsets in the human system is leading to new insights in the field of innate cell interactions, particularly for the cross-talk occurring between these DC subsets and NK cells. As DC subsets show a specific distribution in human tissues, their interactions with NK cells should now be better dissected. Noteworthy, some light has also been recently shed regarding the distribution and trafficking of NK cells in the human body, thus allowing a more complete depiction of where these two cell types could physically interact. Interestingly, DC subsets are now emerging as cells endowed with peculiar functions, either in terms of specific cytokine secretion or of signals provided to other neighboring cells through distinctive surface molecules during cell-to-cell contacts. Therefore, NK/DC interactions should no longer be considered as the cross-talk between two homogeneous populations of innate cells but rather as a more complex network of cell subset cooperation acting in discrete regions of the body to fulfill complementary tasks.

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NK cell autoreactivity and autoimmune diseases

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Increasing evidences have pointed out the relevance of natural killer (NK) cells in organ-specific and systemic autoimmune diseases. NK cells bear a plethora of activating and inhibiting receptors that can play a role in regulating reactivity with autologous cells. The activating receptors recognize natural ligands up-regulated on virus-infected or stressed or neoplastic cells. Of note, several autoimmune diseases are thought to be linked to viral infections as one of the first event in inducing autoimmunity. Also, it is conceivable that autoimmunity can be triggered when a dysregulation of innate immunity occurs, activating T and B lymphocytes to react with self-components. This would imply that NK cells can play a regulatory role during adaptive immunity; indeed, innate lymphoid cells (ILCs), comprising the classical $CD56^+$ NK cells, have a role in maintaining or alternating tissue homeostasis secreting protective and/or pro-inflammatory cytokines. In addition, NK cells display activating receptors involved in natural cytotoxicity and the activating isoforms of receptors for HLA class I that can interact with healthy host cells and induce damage without any evidence of viral infection or neoplastic-induced alteration. In this context, the interrelationship among ILC, extracellular-matrix components, and mesenchymal stromal cells can be considered a key point for the control of homeostasis. Herein, we summarize evidences for a role of NK cells in autoimmune diseases and will give a point of view of the interplay between NK cells and self-cells in triggering autoimmunity.

Keywords: NK cells, autoreactivity, autoimmunity, NKG2D, DNAM1, regulatory NK cells, mesenchymal stromal cells, LAIR1

INTRODUCTION

Natural killer (NK) cells are one of the main components of innate immunity [reviewed in Ref. (1–7)]. It is thought that they provide the body with a strong defense against microorganisms, such as viruses and bacteria, together with their efficient action in limiting neoplastic cell growth (1). The functional definition of NK cells, that is their ability of killing other cells without any prior stimulation, implies that different cell populations can have the functional characteristics of NK cells without sharing a common phenotype. The large majority of the surface markers able to identify this cell population are actually expressed by other kinds of lymphocytes leading to an intrinsic difficulty in defining a cell as an NK cells on the basis of phenotype. As several other components of the innate arm of the immune system, NK cells can secrete cytokines and chemokines. Both activation of cytolytic machinery and secretion of regulating soluble factors are dependent on a wide number of surface and intracellular receptors that, interacting with the appropriate ligand, can lead to activation or inhibition of a given cell function. As always in a biological system, the balance between these opposite signals is responsible for the final outcome in the microenvironment; thus, NK cells can influence and regulate the activities of adaptive immune responses, including T cells [reviewed in Ref. (8)] and dendritic cells (DCs) (9, 10) through well identified surface receptors. Recent findings have pointed out that NK cells may play important roles in autoimmune disorders; indeed, a genetic correlation between NK cell expression of HLA-I receptors and autoimmune diseases has been shown. In addition, it appears that NK cells may play opposite roles with both regulatory and inducer activity in some autoimmune diseases (11–25).

FUNCTIONAL BALANCE BETWEEN ACTIVATING AND INHIBITING SIGNALS IN NK CELLS

It is well known that the functional behavior of NK cells can be regulated by positive and negative signals. A detailed analysis of positive and negative NK cell receptors is reported elsewhere (6). Roughly, two main systems of molecular regulators are expressed on NK cells: the first one is represented by invariant NK cell receptors for HLA-I while the second one is composed of several receptors which do not bind HLA-I. The molecular and functional characteristics of NK cell receptors for HLA-I have been extensively analyzed (26–28): briefly, killer immunoglobulin-like inhibitory receptors (KIRs) and C-lectin-type-inhibitory receptors (CLIRs) can recognize either unique or several HLA-I alleles blocking NK cell function. Some members of these receptors can be expressed on NK cells also in an activating isoform that, in the extracellular portion, is apparently identical to the inhibiting one, indicating that the same HLA-I allele product can be positively recognized as well. Furthermore, in some instances, only the activating form of a member of KIR family has been identified, although it is not still defined unequivocally its corresponding HLA-I ligand. All these findings would render the scenario of NK cell receptors for HLA-I much more complicated than it was supposed in the late 90s (29, 30). Regarding the non-HLA-I receptors present on NK cells, some are of the activating type such as CD69, NKp30, NKp44, NKp46,

NKG2D, and DNAM1 (31–33), while others are of the inhibiting type as LAIR1 (34). It should be noted that the peculiar behavior of the 2B4 receptor, which can deliver an activating signal when the signal transducer called SAP/SH2D1A is present in the cytoplasm; but in some instances it can deliver an inhibiting signal also in the presence of this transducer [reviewed in Ref. (35, 36)]. It is commonly thought that NK cells do not aggress self-cells because the balance between negative and positive signals is always in favor of the negative regulation: this balance is broken when self-cells do not express HLA-I (as during viral infections) or up-regulate natural ligands for activating receptors as it happens during tumor transformation (10, 37).

EVIDENCE FOR THE RECOGNITION OF SELF-CELL BY NK CELLS

Like T lymphocytes, NK cells should not recognize autologous cells, unless autoreactivity is triggered, potentially leading to an autoimmune disease. Based on the original definition of NK cells (1), in principle a self-cell can be killed by NK cells without any previous stimulation. To avoid this damage, a self-cell is equipped with two major molecular mechanisms: (a) strong expression of HLA-I antigens able to deliver inhibiting signals to NK cells; (b) low levels or lack of expression of surface ligands essential for triggering NK cell activation [reviewed in Ref. (8, 37)]. In the latter context, also the down-regulation of ligands for co-receptors of NK cell activation can play a key role in avoiding self-aggression [reviewed in Ref. (4, 38)].

It is becoming evident that NK cells can recognize self-cells, which express ligands for activating receptors (8); indeed, NK cells can aggress both T and antigen presenting cells (APCs) upon triggering with toll-like receptor (TLR) or stimulation with IL2 or IL15 cytokines. These stimuli lead to the up-regulation of NKG2D receptor or to the neo-expression of CD69 and NKp44, which in turn can trigger cytolytic activity and cytokine production (31, 39). On the other hand, several stimuli conceivably acting through the T cell receptor/CD3 complex, such as phytohemoagglutinin (PHA), alloantigens, superantigens, and antigenic peptides, can induce the neo-expression of NKG2D ligands (NKG2DL) on $CD4^+$ and $CD8^+$ T lymphocytes [reviewed in Ref. (8)]. Moreover, also microorganisms as HIV or *Mycobacterium tuberculosis* can trigger NKG2DL expression on $CD4^+$ T cells and T regulatory (Treg) cells (40, 41). The NKG2DL are represented by stress-induced MHC class I-related molecules, such as MICA/B, or the UL16 binding proteins (ULBPs), that are indeed recognized not only by NK cells but also by a large number of "unconventional" T lymphocytes, as γδ T and NKT cells (11, 12, 42–44). It is conceivable that even $CD8^+$ memory T cells could be triggered through NKG2DL; all these cell populations can lead, acting alone or together, to autoreactivity (11). Indeed, the duty of innate immunity is to clear the body from a specific pathogen or impede the development of cancer; thus, one can consider autoimmunity as a drawback of a defective lymphoid stress surveillance that does not limit properly the dissemination of infected or malignant cells and does not maintain tissue integrity, leading to an altered adaptive immune response. In addition, also the poliovirus receptor (PVR) or nectin-2, both ligands for DNAM1 (45) can be expressed on activated or HIV-infected $CD4^+$ T cells possibly leading to NK cell recognition through the DNAM1 activating receptor. To our knowledge, no reports are present so far in the literature on the possible interactions between activated T cells and NK cell receptors, such as natural cytotoxicity receptors and/or 2B4, although the 2B4 ligand CD48 can be expressed on T, B, and NK cells [reviewed in Ref. (46)]. It has been shown in a mouse model that blocking of 2B4 with a 2B4-fusion protein inhibits the generation of autoimmune hepatitis (AIH) suggesting that a still undefined $2B4^+$ lymphocyte subset can be involved (47). This deserves further studies in humans to better clarify the molecular mechanisms of NK cell-T lymphocyte cross-talk. Nevertheless, these findings strongly indicate that NK cells can strikingly regulate T cell responses influencing adaptive immunity. In the adaptive immune response, APCs take a key role; indeed, APC can adequately expose the peptide antigen to allow its recognition by T cells (48). Different kinds of APC, with a reported different capacity of presenting the peptide antigen, can be identified (49–51). Focusing our analysis on monocyte and monocyte-derived dendritic cells (moDCs), it is known that NK cells can actively interact with these APC that produce interleukin 12 (also known as NK stimulating factor), which triggers both proliferation and cytolytic activity of NK cells (52). In turn, NK cells can produce cytokines, as TNFα, which contribute to DC cell maturation. Several reports have shown that IL2-activated NK cells can lyse self-APC and that NK–APC interaction may lead to cytokine production (9, 10, 49, 53, 54). Importantly, this interaction can be mediated by different activating receptors, including some natural cytotoxicity receptors, and by NKG2D or DNAM1 (9, 54–59). In addition, ligands for NKG2D can be up-regulated on APC upon stimulation with TLR-ligands, further supporting the idea that microbial infections can evoke an autoreactive response that leads to a limited adaptive immune response. Indeed, the NK cell-mediated elimination of a given APC before antigen presentation to T cells should conceivably impede an optimal T cell activation [reviewed in Ref. (10, 49)]; thus, also the second player of the adaptive immune response can be shut down by NK cells. Finally, on epithelial and mesodermal-derived cells, as well as on leukocytes, adhesive ligands such as the intercellular adhesion molecule-1 (ICAM1) can be up-regulated upon triggering by TLR or inflammatory cytokines, including IFNγ and TNFα (60). The counter receptor of ICAM1 is the lymphocyte function associated antigen-1 (LFA1), which is a major player of leukocyte-to-cell adhesion and NK cell activation [reviewed in Ref. (60–64)]. Of course, stress signals can up-regulate the ligands for NK cell activating receptors also on this cell population, favoring the NK cell-mediated self-aggression [reviewed in Ref. (37)]. These findings strongly suggest that the interaction between NK and self-cells during infection and/or inflammation should be the rule and not the exception; in addition, NK cells together with the so-called T cells with NK activity (primarily NKT and γδT cells) can down-regulate or even impede the generation of an adaptive immune response (43, 65, 66). It is conceivable that this interaction does not happen in the peripheral blood but within tissues or in the lymph nodes, at least in the case of organ-specific autoimmune diseases. In this context, several evidences have been reported on the presence of NK, NKT, or γδT lymphocytes, expressing NKG2D and DNAM1, among tissue infiltrating cells during autoimmune diseases; in the same

tissues NKG2D and/or DNAM1 ligands are detectable. Indeed, these cells have been found in psoriatic, blistering diseases, and alopecia areata (AA) skin lesions (16, 22, 67–71), central nervous system (CNS) in multiple sclerosis (MS) patients (23, 25, 72–78) and synovial fluid in rheumatoid arthritis (RA) (17, 79–83).

MESENCHYMAL STROMAL CELLS AS A TARGET FOR NK CELLS

Within tissues NK cells can interact with other cells of innate immunity as monocyte-derived macrophages and dendritic cells, mesodermal cells, and extracellular-matrix components (EMCs) besides NKT and γδT cells (**Figure 1**). In particular, mesenchymal stromal cells (MSCs) are fibroblast-like cells responsible for the production of several extracellular-matrix proteins as collagen, vitronectin, fibronectin, and laminin, through which parenchymal cells can maintain both shape and functional interactions in a given organ. Among MSC, mesenchymal stem cells can undergo differentiation to stromal cells typical of connective tissues, including osteocytes, adipocytes, and chondrocytes [reviewed in Ref. (84)]. According to some experimental findings, the property to differentiate is not limited to cells of mesodermal origin but is also shared by ectodermal cells as neurons. Although conflicting results are reported in the literature, it is becoming evident that MSC can be a source of pluripotent stem cells that can be employed in tissue repair and regeneration. In addition, a functional common feature of MSC is the ability of regulating immune responses [Ref. (85); reviewed in Ref. (84, 86)]. Indeed, it has been shown that MSC derived from different tissues can down-regulate the activation of the immune system both *in vitro* and *in vivo* murine models. More importantly, these cells have been proposed as an additional therapeutic tool to control graft versus host disease (GVHD) in particular in children (87, 88). MSC can have a role in regulating autoreactivity through the modulation of cell-to-cell interactions and the production of extracellular-matrix proteins, cytokines, and enzymes [Ref. (85); reviewed in Ref. (84)]. The prevailing point of view of the literature is that MSC have a regulatory inhibiting role on several T and NK cell-mediated activities (87, 89). This regulation is reported to be mediated by soluble factors, such as TGFβ, HGF, IDO, and PGE2, which affect lymphocyte functions upon lymphocyte–MSC interaction [Ref. (85); reviewed in Ref. (84, 86, 89)]. On the other hand, it appears that NK and T cells can aggress MSC recognizing NKG2D and DNAM1 ligands, leading to MSC killing and release of pro-inflammatory cytokines (57, 90–92). This property is mainly confined to cytokine-activated NK cells, as *ex vivo* peripheral blood isolated NK cells are not efficient in MSC killing (90–93). It is of note that the regulatory role of MSC on NK cell functions is found *in vitro* at well defined MSC:NK cell ratios, ranging from 1:1 to 1:4, while at lower MSC–NK cell ratios the inhibiting effect is barely or not detectable and an activating effect is found (90). Due to *in vitro* culture conditions, at this ratios MSC grow as a monolayer covering the culture well, with lymphocytes seeded on them; thus, both extracellular-matrix proteins, as

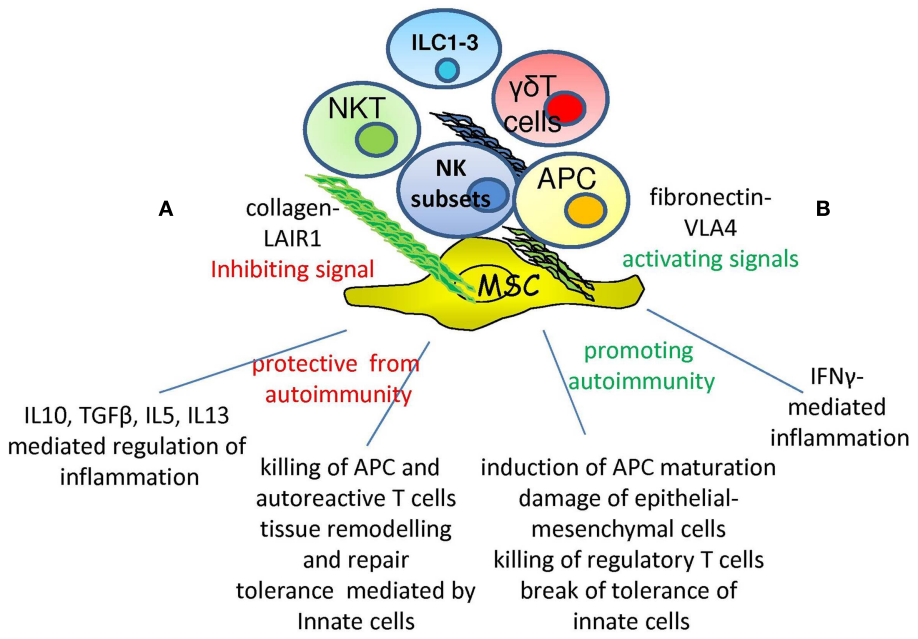

FIGURE 1 | Opposite roles of NK cells in autoimmunity. Within microenvironment the interaction of specific NK cell receptor with extracellular-matrix can deliver different signals depending on the type of receptor involved: LAIR1–collagen interaction would lead to inhibition while VLA4-fibronectin engagement to activation. Depending on the type of NK cell subset involved, NK cells show the ability of protecting from the occurrence of autoimmunity **(A)** through the secretion of immune-regulating cytokines as IL10, TGFβ, IL5, and IL13. In addition, they can eliminate APC and autoreactive T cells through the triggering of activating receptors or regulate tissue homeostasis. On the other hand, NK cells can aggress tissues inducing inflammation through IFNγ production **(B)**, favoring the maturation of APC with the consequent triggering of adaptive immune response. Killing and damaging parenchymal, epithelial, and mesenchymal cells eventually lead to an altered tissue homeostasis and then to autoimmunity. ILC1-3, NKT, and γδT cells are involved and may regulate the NK cell–microenvironment interactions.

collagen and fibronectin, and inhibiting cytokines, as TGFβ, can be concentrated to the MSC surface and in the extracellular medium facilitating the delivery of an inhibiting signal to lymphocytes. On the other hand, it is still to be determined what happens during the interaction of a single MSC and an NK cell: it is conceivable that within connective tissues MSC–NK interactions take place in the presence of several extracellular-matrix proteins whose receptors are expressed on NK cells.

EXTRACELLULAR-MATRIX PROTEIN RECEPTORS ON NK CELLS AS REGULATORS OF NK CELL FUNCTIONS: FOCUS ON THE LEUKOCYTE ASSOCIATED Ig-LIKE RECEPTOR 1

Natural killer cells can express different extracellular-matrix protein receptors as well as matrix metalloproteinases responsible for matrix degradation (94, 95). Some of these receptors are called very late antigen (VLA) as they are expressed on long-term cultured cells (**Figure 1**). However, some are constitutively expressed at the NK cell surface, such as VLA4 (96–98), and can also be up-regulated upon stimulation. Several different effects of NK cell interaction with the matrix proteins fibronectin, laminin, vitronectin, osteopontin, and collagen are reported in the literature (99–105) and their deep analysis is beyond the scope of this review. As an example, the engagement of VLA4 can induce activation of NK cells (98, 106–109). Herein, we focus on the leukocyte associated Ig-like receptor 1, LAIR1 or CD305 (110, 111), that has been shown to be a receptor for the Gly-Prol-Hyp common motif of collagens type I, II, III, XIII, XVII, and XXIII (112–115) (**Figure 1**). Importantly, LAIR1 is able to deliver an inhibiting signal which down-regulates NK cell activation through the CD16 receptor, reducing calcium mobilization, and the cytolytic activity triggered through this molecule (110, 116, 117). The LAIR1-mediated inhibiting signal occurs through the recruitment, by its cytoplasmic tail equipped with immunoreceptor tyrosine inhibiting motif (ITIM), of the SHIP1 phosphatase; this, in turn, impedes the phosphorylation and consequent activation of the immunoreceptor tyrosine activating motif (ITAM) present in the intracellular domain of several activating NK cell receptors (2, 118). LAIR1 can be expressed as different isoforms (LAIR1a, b, and c) or as a soluble form termed LAIR2; it is conceivable that the interaction of NK cells with collagens delivers a negative signal that may be impaired in the presence of soluble (s) LAIR (119–121). No direct evidence for the interaction of LAIR1 expressed by NK cells and collagen is reported so far; however, that indeed cross-linking of collagen can trigger an inhibiting signal in lymphocytes upon LAIR1 engagement has been demonstrated for T and B cells (117, 122–124), APCs (125, 126), and tumor cells (127–130). Altogether, these findings suggest that collagen produced by MSCs may be involved in the negative regulation of NK cell function. It is still to be defined which stimuli can regulate LAIR1 expression on NK cells. It is of note that LAIR1 is present on almost all leukocytes and it appears to be associated with the leukocyte common antigen (LCA) tyrosine phosphatase (CD45) on NK cells (131); thus, LAIR1 could regulate NK cell activation by itself and/or through the association with CD45. Interestingly, the lack or lower expression of LAIR1 is associated with an impaired inhibiting signal delivered upon LAIR1 engagement in B cells isolated from systemic lupus erythematosus (SLE) patients or B cell chronic leukemia (129, 132) supporting the idea that down-regulation of LAIR1 expression can be associated with autoimmune or neoplastic diseases.

NK CELL SUBSETS AND INNATE LYMPHOID CELLS AS PLAYERS AND REGULATORS OF AUTOIMMUNITY

It is generally thought that autoreactivity and autoimmune diseases are based on an altered adaptive immune response determining the generation of T and B cell-mediated aggression of self-cells (133–136). This can be the result of a too strong reaction to self-antigen due to altered central or peripheral tolerance of autoreactive T and B cell clones. Treg cells are the main effectors of tolerance and several evidences have demonstrated that the lack of an optimal regulation of the adaptive immune response may be a consequence of their impaired function (137). NK cells can influence tolerance by eliminating Treg cells (15, 138, 139) or by acting as regulatory cells themselves (14, 21, 140–144). Indeed, upon engagement of activating receptors, NK cells can release several regulating cytokines, such as TGFβ and IL10, which are considered mediators of tolerance for T cells (5, 145). For instance, during viral infections, it is conceivable that the interaction of NK cells with infected self-cells results in the secretion of TGFβ and IL10, which in turn modulate T and B cell responses; of note, TGFβ is a strong down-regulator of NK cell-mediated activation and proliferation (146–149). Interestingly, secretion of functional TGFβ can be elicited in NK cells upon triggering with soluble HLA-I molecules that interact with the corresponding counter-receptors, as CD8 and/or the activating isoforms of KIRs and/or CLIRs (150, 151). An increment of sHLA-I can be detected in the sera of patients suffering from different autoimmune diseases; thus, one could suggest that sHLA-I can down-regulate NK cell activation. In addition, together with TGFβ, NK cells can release FasL (152); in turn, soluble FasL, interacting with Fas at the surface of lymphocytes, can lead to their cell death. Thus, the NK cell-mediated down-regulation of immune response may occur both by blocking activation with TGFβ and triggering cell death via FasL–Fas interaction (152). Recently, several distinct NK cell subsets have been found in different tissues playing opposite functional roles in immune response (**Table 1**). Briefly, it is commonly accepted that $CD56^{dull}$ and $CD56^{bright}$ NK cells present in the peripheral blood have distinct phenotype and functional activities. Indeed, $CD16^{+}KIR^{+}CD56^{dull}$ NK cells are primarily cytotoxic while the $CD16^{-}KIR^{dull}$ $CD56^{bright}$ produce huge amounts of cytokines. It is not clear whether $CD56^{dull}$ posses the plasticity to become $CD56^{bright}$ and viceversa. Also, human NK cells can be subdivided on the basis of CD27 and CD11b expression (153, 154): the minority of peripheral NK cells is $CD27^{+}$ (about 5%), while this population is more represented in the bone marrow and further in the spleen and tonsils. $CD27^{+}$ NK cells, either $CD11b^{+}$ or $CD11b^{-}$, can produce high amounts of cytokines while among the $CD27^{-}$ NK cells those expressing CD11b are highly cytotoxic (**Table 1**). Of note, early during pregnancy the majority of human decidual lymphocytes are characterized by unique phenotype: $CD16^{-}CD11b^{-}CD56^{bright}$ either expressing or not CD27, CD9, and CD151 tetraspanning family members. Some of these cells can produce IL22 and express immunomodulatory molecules as galectin-1 and progestagen-associated protein 14 (155). Importantly, decidual NK (dNK) $CD56^{bright}CD27^{+}$ cells

Table 1 | Features of NK cell subsets in peripheral blood and tissues.

NK cell type	Cytotoxic	Regulatory/tolerant
Prototype examples	Peripheral NK cells	Decidual NK cells Liver NK cells Tissue infiltrating NK cells
Phenotype	$CD56^{dim}CD27^{-}CD11b^{+}$	$CD56^{bright}CD27^{-}CD11b^{-}$ $CD56^{brigth}$ or $CD27^{+}$
Cytokine produced	Mainly IFNγ and TNFα	Several different cytokines (TGFβ, VEGF, IL10, IL17, IL22)
Main activity	Cytolysis	Vascular remodeling Maternal-fetal immune regulation
Tissue localization	Peripheral blood, bone marrow	Lung, uterus, liver, and gut
Immunity against	Viruses and tumor immunosurveillance	Maintenance of tissue homeostasis
Role in autoreactivity	Triggering or protective effect	Mainly protective effect

Schematically, NK cells can show two different functional behaviors (a) cytolytic NK cells (cNK) express high levels of lytic granules and kill spontaneously tumor cells; (b) regulatory/tolerant NK cells producing several soluble factors which are relevant in regulating tissue homeostasis. Cytotoxic NK cells may exert a key role in inducing inflammation and they can down-regulate adaptive immunity acting on antigen presenting cells. Regulatory/tolerant NK cells are involved in controlling tissue homeostasis playing a protective role aimed to maintain and reconstitute the healthy conditions during tissue reparation.

suppress Th17 through an IFNγ-dependent pathway and this population is lost in women with spontaneous abortion. Additional NK cell subsets, as NK2, NK3, NKr, and NK22 specifically involved in the secretion of immune-regulatory cytokines have been recently identified [reviewed in Ref. (21, 156–160)]. Subsets with a protective role in autoimmunity are NK2 cells, predominant in allergic disease, producing high amounts of IL4, IL5, and IL13 (161), NK3 cells which release IL10 (162), together with secreting TGFβ NKr cells which are involved in maternal-fetal immune tolerance (80) while NK22 cells limit inflammation and protect gut mucosal integrity through the action of IL22. To further complicate this scenario, innate lymphoid cells (ILCs, **Table 2**) distinct from NK cells, has been identified in mucosa associated lymphoid tissue (163). To uniform this variegate picture, it has been suggested to include NK cells within the ILC1 subset and it has been proposed that the CD56 molecule can be considered the best marker to distinguishing between NK and other lineage negative lymphoid cells (**Table 2**) as both kind of cells can express NKp46 and NKp44 receptors. More importantly, ILC1, ILC2, and ILC3 subsets express peculiar transcription factors as T-bet or RORα or RORγT (**Table 2**) resembling Th1, Th2, or Th17 T cell subsets respectively. Of note, ILC1, ILC2, and ILC3 cells are present in the gut and display a pro-inflammatory or a protective role depending on the main cytokine produced (**Table 2**). Finally, the $NKp46^{-}NKp44^{+}ROR\gamma T^{+}CD127^{+}$ NK cells show a protective role in autoimmunity but they may be counteracted by $NKp46^{+}NKp44^{-}ROR\gamma T^{-}CD127^{-}$ NK cells which appear to be pathogenic through the production of IFNγ (164). Altogether these findings suggest that both different NK cell subsets and ILC are primarily involved in either host defense against viruses and tumor immunosurveillance or in regulating tissue homeostasis and autoimmunity. Furthermore, it is still to be determined the "plasticity" of an NK cell or ILC subset as it has been demonstrated for some T cell subsets [reviewed in Ref. (165, 166)].

NK CELLS AND GENETIC CORRELATION WITH AUTOIMMUNE DISEASES

Natural killer cell development and function is strictly related to genetic elements: the genetic background, particularly the defects and variations of KIR/HLA genotypes, can influence the function of a given NK cell receptor in target cell recognition and impair NK cell activation as well as self-tolerance. This influence is supposed to be related to autoimmunity (167); indeed, several findings have pointed out associations between risk of systemic or organ-specific autoimmune diseases and KIR/HLA genotypes, which indicate that self-tolerance may be broken with inappropriate receptor and ligand pairs or with the interrupted signal balance (38, 168–177). In general, the presence of an activating receptor for HLA-I associated with the lack or reduction of inhibitory pairs has been shown in several autoimmune diseases [reviewed in Ref. (21, 178)] suggesting that an imbalance in favor of activating receptors for HLA-I is associated with autoimmunity (**Figure 2**). This is in line with the findings observed in bone marrow transplantation where the expression of activating KIRs can override the regulating signals generated through inhibiting KIRs and/or CLIRs (179–182).

In the following paragraphs, the findings regarding the potential role of NK cells in different autoimmune diseases will be listed and discussed.

FEATURES OF NK CELLS IN SKIN DISEASES

Typical autoimmune diseases of the skin are psoriasis, pemphigus vulgaris (PV), and AA; herein, we will not deal with skin specific manifestations of SLE which can be considered as a systemic autoimmune disorder with involvement of the skin. It has been reported that NK cells represent about 5–8% of infiltrating lymphocyte in psoriatic lesions and these cells are $CD56^{bright}CXCR3^{+}CCR5^{+}$ cells (67) expressing the activation antigen CD69. These cells produce IFNγ after IL2 stimulation; in

Table 2 | Innate lymphoid cells characteristics.

Characteristic	ILC group 1	ILC group 2	ILC group 3
Cell type	NK cells ($CD56^{dim/bright}$ $NKp46^{+}$) ILC1 cells not cytotoxic	$IL1R^{+}$ $IL23R^{+}$	ILC3 and LTi cells Some $CD56^{+}$ cells
Main cytokine produced	IFNγ	IL5 and IL13 triggered through IL25 or IL33	IL22 and/or IL17 IFNγ
Cytolytic activity	Yes (NK cells)		
Main transcription factor expressed	T-bet Eomes	RORα GATA3	RORγt
Peculiar phenotypic features	c-kit^{-} (CD117) $IL12R\beta2^{+}$	c-kit^{-} $IL12R\beta2^{-}$ Subunits of IL25R and IL33R	c-kit^{+} $IL12R\beta2^{-}$
Common phenotypic features	$IL7R\alpha^{+}$ (CD127) $NKRP1a^{+}$ (CD161)	$IL7R\alpha^{+}$ $NKPRP1a^{+}$	$IL7R\alpha^{+}$ $NKRP1a^{+}$
Immune function	Viral infections, tumor surveillance NK IFNγ Inflammation (ILC1)	Tissue defense/homeostasis	Inflammation ($IL17^{+}IFN\gamma^{+}$ colitis) Protection (*Citrobacter rodendrium* ILC3 $IL22^{+}$) Gut barrier, wound healing, and epithelial proliferation Lymph node formation ($IL17^{+}$)
Main tissue localization	Lymphoid organs, uterus, lung, gut, liver	Lung, adipose tissue, gut	Gut lamina propria and cryptopatches, mesenteric lymph nodes, palatin tonsil
Role in autoreactivity	IBD ($CD56^{bright}$ NK cells)	Murine models of gut parasitic infections Human IBD	Murine models of colitis Chron disease

Innate lymphoid cells (ILCs) are mucosa associated lymphoid cells which can express some markers of NK cells. It has been proposed to include NK cells within the ILC1 subset of ILC. NK cells are $CD56^{+}$ and display strong cytolytic activity while ILC produce a set of different cytokines depending on the subset they belong to. This dichotomy is not so well defined as some ILC3 cells can express CD56 and thus it is still debate whether NK and some ILC subsets may be inter-converted due to intrinsic plasticity. IBD, inflammatory bowel disease.

turn IFNγ can upregulate the HLA-I antigens on cheratinocytes and trigger activation of these cells as well. These findings would suggest that $CD56^{+}$ NK cells can favor the development of psoriasis inducing local inflammation and amplify T cell autoimmune reactivity. This notion is further supported by the finding that $CD56^{bright}CXCR3^{+}CCR5^{+}$ NK cells from psoriatic lesions can trigger cheratinocytes to produce CCL5 and CXCL10 chemokines which in turn favor NK cell chemotaxis. Of note, NK cells can also release IL22, a cytokine mainly produced by Th1 and Th17 T cells (183–185). Cheratinocytes incubated with IL22 can proliferate upon interaction with IL22 receptor (186) and this leads to parakeratosis and acanthosis, typical features of psoriatic lesions (187). Furthermore, genomic deletion of the activating receptor NKG2C is significantly increased in psoriatic patients compared to healthy matched controls (70, 188, 189). Thus, the lack of recognition by NK cells of autoreactive T cells may lead to exacerbation of psoriasis (**Figure 2**). In this context, the finding that NK cells bearing the inhibiting NK receptor NKG2A are incremented in skin psoriatic lesions can suggest that the imbalance between $NKG2C^{+}$ and $NKG2A^{+}$ NK cells may favor the expansion of autoreactive T cells (70). In AA, it has been found that $CD56^{+}NKG2D^{+}$ NK cells are mainly localized around and within the anagen hair follicles in prominent aggregates possibly leading to aggression of hair follicles favoring the collapse of the relative immune privilege of this cutaneous region (69). Finally, in PV it has been reported that peripheral NK cells display a Th2 type-biased phenotype (190) as they express high mRNA for IL10, a decrement of IL12Rβ, and produce IL5 *in vivo*, exclusively in patients with active disease compared to healthy control. Furthermore, NK cells may function as APCs for desmoglein three antigens to $CD4^{+}$ T cells, suggesting also the possibility of a role for NK cells in inducing the tissue damage associated to PV (191).

NK CELLS IN MULTIPLE SCLEROSIS

Multiple sclerosis is a CNS inflammatory autoimmune disease involving as target the myelin associated with neuronal axons; MS eventually leads to a progressive disability and host death due to the impairment of vital CNS functions. A potential pathogenic role of NK cells in MS is supported mainly in relapsing remitting MS patients (RRMS) [reviewed by Chanvillard et al. (23)]; indeed, NK cells can directly aggress and damage oligodendrocytes which produce myelin and NK cells are increased in MS lesions (192, 193). On the other hand, NK cells can directly affect the life of autoreactive T cells or APCs; in MS patients treated with IFNβ (194) or with the anti-CD25 antibody daclizumab, there is a selective expansion and activation of $CD56^{bright}$ NK cells and this correlates

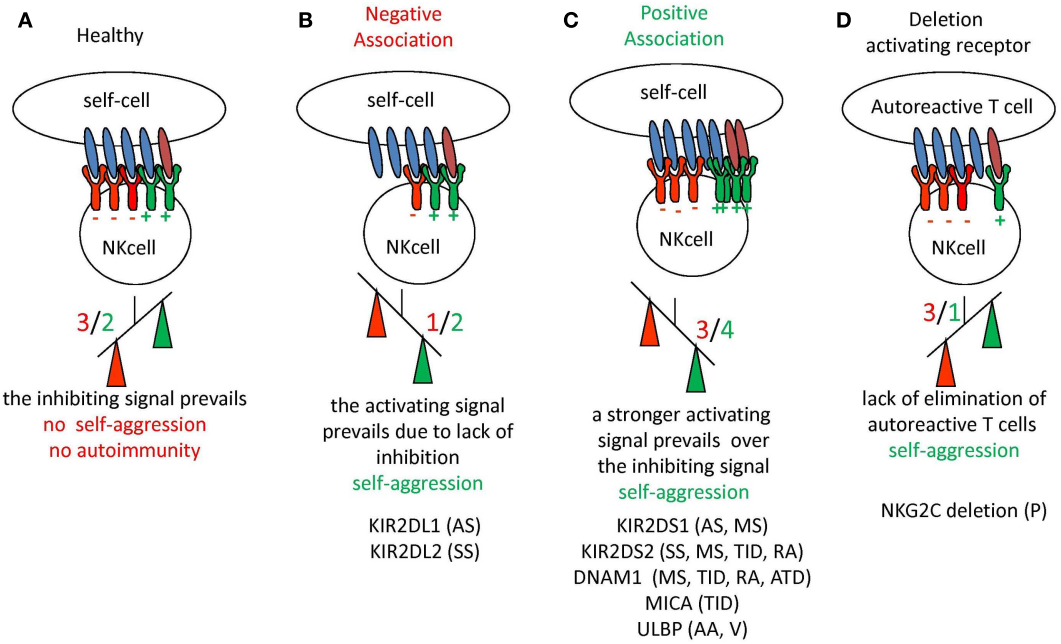

FIGURE 2 | Schematic representation of the imbalance between inhibiting and activating receptors on NK cells with the occurrence of autoimmunity and autoimmune disease. **(A)** In healthy conditions the negative signal (in red) delivered through inhibiting receptors for HLA-I can prevail on the positive signal (in green) induced by the engagement of activating receptor. The net effect is that no damage to self-cells is induced, thus there is no autoreactivity. **(B,C)** The down-regulation of expression of inhibiting receptors **(B)** or an increment of activating receptors **(C)** determines in any case the prevalence of the activating signal on the inhibiting one, leading to self-reactivity (indicated as prevalence of positive signal shown in green). Although not shown, these two situations may also be found together. **(D)** In this case the lack of activating receptors can lead to impaired blocking of autoreactive T cell clones favoring autoreactivity. For each situation are listed the autoimmune diseases where an alteration of NK receptors have been reported. KIR2DS1 and KIR2DS2 are the activating isoforms of the NK receptor for HLA-C alleles. The KIR2DL1 and KIR2DL2 are the inhibiting isoforms of NK receptors for HLA-C alleles. NKG2C is the activating isoform of CLIR. DNAM1 is an activating receptor present on NK cells and MICA and ULBP are the ligands of the NKG2D surface receptor involved in the recognition of either infected or tumor transformed cells. AA, Alopecia areata; AS, ankylosing spondylitis; ATD, autoimmune thyroid disease; MS, multiple sclerosis; P, psoriasis; PV, pemphigus vulgaris; RA, rheumatoid arthritis; SS, systemic sclerosis; TID, type I diabetes; V, vasculitis.

with a down-regulation of T cells activation and inhibition of inflammation (195, 196), suggesting that CD56^{bright} NK cells are relevant in the control of MS lesions. Importantly, this NK cell subset appeared to kill T cells through granzyme K and A, which activate the mitochondrial pathway of apoptosis. The expansion of CD56^{bright} NK cells can be dependent on their relative higher expression (compared to CD56^{dull} NK cells) of the intermediate affinity receptor for IL2. Thus, during MS therapy CD56^{dull} NK cells should be shut down through the blocking of the CD25 receptor by daclizumab; on the other hand, the expansion of CD56^{bright} NK cells is favored because daclizumab does not impair their proliferation. Furthermore, in MS the NK2 cell subset is responsible for the production of IL5 and IL13, which may actively suppress the activity of self-reactive T cells. These cells disappear in MS patients when an exacerbation of the disease is present, while they re-appeared during the remission phase, suggesting that NK2 cells may be relevant for the disease control (197, 198).

NK CELLS AND TYPE I DIABETES

A reduction of peripheral NK cells has been reported in early diagnosed type I diabetes (TID) while the amount of NK cells is mostly similar to healthy controls in long-standing TID patients; more importantly, long-standing TID display lower amounts of IFNγ and lower expression of some natural cytotoxicity receptors (199) associated with high levels of glycosylated hemoglobin, suggesting that the impairment of NK cells could be a consequence of the disease. It is of note that some NK cells have been identified also within the pancreas, nearby to β pancreatic islets (200), although this finding has not been confirmed (201). In a murine diabetes model, it has been reported that NK cell are essential in abolishing the onset of the disease in NOD mice through a TGFβ-dependent mechanism that interferes with the activation of β-islet specific T cells (202, 203).

NK CELLS IN RHEUMATOID ARTHRITIS

It has been reported that NK cells producing IL22 and TNFα are increased in the synovial fluid of RA patients. It is of note that culture supernatants from these IL22-producing NK cells can trigger the proliferation of synovial fibroblast-like synoviocytes and this proliferation is inhibited using anti-IL22 and anti-TNFα antibodies (79). In addition, NK cells from synovial fluid are mainly CD56^{bright}, express high levels of activation antigens and produce IFNγ. Furthermore, they can induce monocyte differentiation to dendritic cells, which in turn can trigger NK cells (204). Altogether

these findings would suggest an active role of NK cells in sustaining inflammation in RA patients.

NK CELLS IN INFLAMMATORY BOWEL DISEASE

Inflammatory bowel diseases are represented by ulcerative colitis (UC) and Chron disease (CD): these two illnesses are characterized by the inflammation of gut accompanied by diarrhea and impairment of absorption of nutrients. It is commonly accepted that IL17A-producing lymphocytes are extremely relevant in IBD (205); among the different cell populations residing and colonizing (Th17, Th1-Th17, NKT, γδT cells) bowel mucosa in UC or CD, NK cells, and the group 3 of ILC3 (see **Table 2**) are good producer of IL17A. These cells can release IL17A immediately upon engagement with pathogen associated molecular patterns (PAMPs) and/or cytokines as IL23 (163). More importantly, both NK cells and ILC3 producing IFNγ and IL17 are abundant in inflamed CD mucosa while it is debated whether ILC3 secreting IL22 cells are increased or decreased in IBD (164, 206). It appears that some ILC of the subgroup 3 (**Table 2**) are relevant in the generation of the gut-associated lymphoid tissues and the maintenance of healthy conditions. In this context, the fine tuning of the respective functional role of colitogenic ILC producing IFNγ (ILC1 and some ILC3) and protective ILC3 secreting IL22 should be relevant in the generation of IBD.

NK CELLS IN AUTOIMMUNE LIVER DISEASES

Natural killer cells present in the healthy liver are different from those found in the peripheral blood; indeed, the former are mainly $CD56^{dull}$ and about a half do not express CD16. Furthermore, these cells are more prompt to be stimulated with IL2 and, unexpectedly, do not lyse autologous hepatocytes, although these cells do not bear HLA class I antigens [reviewed in Ref. (207)]. Autoimmune diseases that hit the liver are mainly represented by AIH, primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC). AIH is characterized by the progressive destruction of the liver parenchyma which eventually leads to cirrhosis and in several instances to hepatic failure and host death. NK cells, together with γδT cells, play a role in the physiopathology of the AIH (208, 209). This is confirmed also in a murine model where administration of poly immune complexes (IC) can induce a strong production of type I IFN and consequent activation of liver NK cells leading to liver destruction with similar histopathologic features found in human AIH (210). In PBC, besides $IL17^+$ cells infiltrating damaged bile ducts, hepatic NK cells active against biliary epithelial cells are found, but it is to be determined whether they are directly involved in the break of immune tolerance characteristic of this disease (207, 211–213). PSC is characterized on one hand by the reduced frequencies of some alleles of inhibiting receptors for HLA-I (214) and on the other by the expression of peculiar alleles of the NKG2D ligand MICA (215); both these molecular events might regulate the NK cell-mediated immune interaction with cholangiocytes.

NK CELLS IN LUPUS ERYTHEMATOSUS SYSTEMICUS

Systemic lupus erythematosus is a systemic autoimmune disease characterized by tissue damage mediated mainly through type II and III hypersensitivity. Several autoantibodies are present in SLE patients and it is evident that interaction with cellular antigens can deliver an activating signal to leukocytes bearing Fcγ receptors, as NK cells and monocyte–macrophages, which eventually leads to cell damage and inflammatory cytokine production. In SLE, a reduction of the absolute number of NK cells with an impaired cytolytic activity is reported (20, 216–222) with an imbalance between $CD56^{dull}$ and $CD56^{bright}$ peripheral blood NK cell subsets (223) characterized by an increase of cytokines production (220) and a lower lymphokine activated killer cell activity (219). It is of note that in different systemic autoimmune disorders, as systemic sclerosis (SSc) and anti-neutrophil cytoplasmic antibody-associated vasculitis, the number of $CD3^-CD56^+$ NK cells are markedly reduced (224). These findings could be interpreted either as a consequence or as a pathogenic player of the autoimmune disorder. In addition, the NK cell subsets found in the peripheral blood may be considered as the results of the localization of effector cells within target tissues, mainly in the case of systemic autoimmune diseases (24, 76). Recently, it has been reported a prominent reduction of NK cells expressing the DNAM1 activating receptor together with an up-regulation of DNAM1 ligand on plasmocytoid dendritic cells (pDCs) which in turn can mediate NK cell death through type I IFNα (20). Of note, in the MRL-lpr/lpr mice model kidney-infiltrating NK cells express activation antigens and high content of cytotoxic granules, suggesting a possible role in the kidney tissue damage associated with SLE (20). The presence of autoantibodies to inhibitory NK cell receptors and NKG2A (225) can promote excessive NK cell function leading to increased levels of autoantigens and further stimulating autoimmune reactions. Of note, in SLE an increase of CS1/CD319 activating receptor of the SLAM family on NK and pDCs could be detected upon triggering with RNA-IC (225); in addition, expression of CS1/CD319 on B cells of SLE patients increased. Altogether, these findings would suggest a role of CS1/CD319 homophylic interaction among pDC, NK, and B cells in SLE (226, 227). It is still to be determined whether these interactions are involved in the pathogenesis of SLE and whether NK cells may be protective or not in this disease.

AUTHORS' VIEWPOINT

It is clear that antigen unspecific autoreactivity can occur, before the onset of an autoimmune disease or in healthy individuals that will not develop any illness: cytotoxic NK (some ILC1 cells), NKT, and γδT cells, are the main active players of this phenomenon while regulatory/tolerant NK cells and ILC2 and ILC3 are mainly involved in maintaining tissue homeostasis. The complex cellular network composed of effector lymphocytes, MSCs, and APCs is the place where the fate of antigen unspecific reactivity determines whether adaptive immune responses will take place or not. One could hypothesize that a strong innate immunity can impede the generation of adaptive immunity as infectious agents are cleared before specific T and B lymphocyte can respond. On the other hand, a low innate response chronically triggers specific T and B cells favoring the establishment of an autoimmune disease due to persistence of the antigen. Finally, an adequate innate response can lead to an optimal B and T cell response that definitively clear the antigen without self-aggression as a consequence (**Figure 3**). If this idea is true, to cure an autoimmune disease

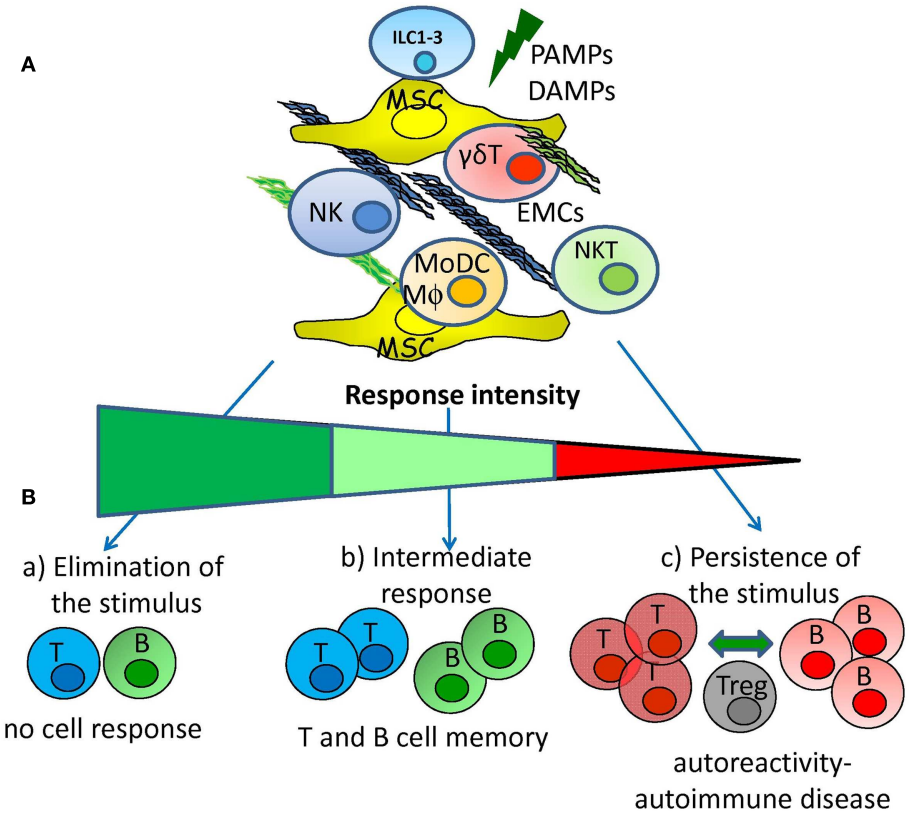

FIGURE 3 | Hypothesis for the generation of adaptive autoreactivity and autoimmunity. **(A)** Pathogen associated molecular patterns and/or damage associated molecular patterns (PAMPs and DAMPs) can activate innate immunity interacting with receptors expressed on innate lymphoid cells (NK, ILC subsets, NKT, and γδT lymphocytes). The activation of innate immunity can be regulated by reciprocal interactions among mesenchymal stromal cells (MSC), extracellular-matrix components (EMCs), lymphoid cells, monocyte-derived macrophages (MΦ), and dendritic cells (MoDCs). **(B)** Innate response elicited by NK, ILC subsets, NKT, and γδT lymphocytes interacting with MSC and EMCs can lead to: (a) rapid elimination of the danger signal that avoids the triggering of adaptive immune cell response; (b) intermediate innate response that leads to the triggering of adaptive immunity with the generation of memory T and B cells; (c) low innate response that determines the persistence of the danger signal leading to generation of autoreactive T and B cells. Autoreactive T and B lymphocytes are controlled by regulatory cells (Treg) but chronic stimulation tends to break the tolerance leading to autoimmune disease.

one should trigger innate immunity instead of down-regulate adaptive immunity. However, any therapeutic treatment should take into account that both innate and adaptive immune responses can be regulated through MSCs and EMCs besides lymphocytes and APC.

AUTHOR CONTRIBUTIONS

Both the authors have equally contributed to the preparation and reviewing of this manuscript.

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The role of natural cytotoxicity receptors in various pathologies: emphasis on Type I diabetes

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Natural killer (NK) cells are innate immune lymphocytes that function mainly as immune sentinels against viral infection and tumorigenesis. NK cell function is governed by inhibitory and activating signals arising from corresponding receptors. A prominent group of activating NK receptors is the natural cytotoxicity receptors (NCRs), which includes NKp30, NKp44, and NKp46. These receptors bind various diverse ligands of pathogenic, tumor, and even self origin. Type 1 diabetes mellitus (T1D) is a multifactorial autoimmune disease, in which insulin-producing beta (β) cells are ablated by the immune system. This killing of β cells is carried out mainly by T cells, but many other immune cells have been implicated in the pathogenesis of this disease. Importantly, NK cells were shown to be key participants in the initial autoimmune attack. It was shown that all β cells from humans and mice, healthy or sick, express an unknown ligand for the activating NKp46 receptor. In this review, we describe the role played by the NCRs in various pathologies with an emphasis on Type I diabetes.

Keywords: NK cells, NCR, NKp46, beta cells, diabetes

Natural killer (NK) cells are innate immune cells that differentiate, like T- and B-lymphocytes, from the common lymphoid progenitor, in the bone marrow. In 1975, NK cells were described to have an intrinsic capacity to rapidly kill tumor cells (1, 2). Subsequent research revealed that these cytotoxic responses could also be elicited by virally infected non-tumor cells (3). Additionally, NK cells have been recognized to be major producers of Interferon-γ, in both pathological and physiological states. NK cells also produce a variety of other cytokines, both pro-inflammatory (such as tumor necrosis factor-α) and immunosuppressive (such as interleukin-10, IL-10) (4). Indeed, in addition to their role in eliminating pathogen-infected and -transformed cells, NK cells have also been implicated in autoimmune responses, primarily as immune-regulators that can limit or enhance the autoimmune response, both via cytokine signaling and direct interactions with other immune cells. The role of NK cells has been investigated in several different autoimmune conditions including: systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, and Type I diabetes (T1D) (5–10).

Natural killer cells, which serve as innate immune sentinels, are found throughout the body in both lymphoid and non-lymphoid tissues. While they reside mainly in the peripheral blood (where they represent 2–18% of the lymphocytes in humans), liver (where NK cells can represent up to 50% of the resident lymphocytes), and spleen, they are also found in the skin, in mucosal tissues such as the lung, in the uterine decidua (11) (where they play a key roles in promoting trophoblast invasion and arterial growth) as well as many other tissues. In addition to their functional role in peripheral non-lymphoid tissues, NK cells can also proliferate and undergo maturational processes in these locations (7, 11–15).

Human NK cells are generally classified into two subgroups based on the expression of CD56 and CD16, which roughly correspond to their activation state. Most circulating NK cells are $CD56^{dim}CD16^{+}$ and are considered to be activated. In this state, when NK cells recognize their target cells, they respond by either killing the target or by producing and releasing cytokines. In contrast, $CD56^{hi}CD16^{-}$ NK cells, which represent the major population of NK cells found in peripheral lymphoid tissues (16) and in the decidua, respond to stimulation by pro-inflammatory cytokines by producing large amounts of cytokines and acquire cytotoxicity only after prolonged activation. It has been well established that the "immature" $CD56^{hi}CD16^{-}$ NK cells differentiate into the more active $CD56^{dim}CD16^{+}$ state, which in turn can differentiate and mature further (7, 17–24).

Natural killer cell activity is governed by a delicate balance between activating and inhibitory signals, which arise from corresponding activating and inhibitory receptors. Unlike the antigen-specific, somatically recombined receptors found on T- and B-lymphocytes, NK cell receptors are germline encoded and the activating receptors are of limited repertoire (25).

The inhibitory NK receptors, which predominantly recognize major histocompatibility complex 1 (MHC1, or human leukocyte antigen – HLA) proteins, consist of several receptor subgroups. The largest of these groups is the killer-cell immunoglobulin-like receptor (KIR) in humans, in which two or three immunoglobulin-like domains in the extracellular portion of the receptors recognize specific MHC1 alleles differentially. The KIR genes are organized in a highly polymorphic, multigene family that displays considerable allelic polymorphism and though both activating and inhibitory KIRs exist, the inhibitory alleles are more prevalent (26). Other MHC1 recognizing receptors include

the leukocyte immunoglobulin-like receptor subfamily B member 1 (LIR1) and the NKG2A-CD94 receptor complex. Moreover, NK cell tolerance toward normal cells is achieved through the expression of MHC1-binding inhibitory receptors by the NKs. Thus, cells that evade cytotoxic CD8+ T-cell recognition and killing by downmodulating the expression of MHC1, render themselves potential targets for NK cell mediated elimination. In addition, several other inhibitory receptors, which bind to non-MHC1 ligands exist, including CEACAM1, CD300a, and TIGIT (26–31). All the different inhibitory receptors contain one or more intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which mediate the downstream inhibitory signals by recruiting protein tyrosine phosphatases (32). MHC1 molecules also educate the NK cells to become functionally competent, and NK cells function poorly in the absence of MHC1 (33).

The activating NK cell receptors recognize tumor-, pathogen-, stress-induced, and self-ligands. Unlike the inhibitory receptors, many of the activating NK receptors lack intracellular signaling motifs. Instead, upon binding to their various ligands, these receptors, which belong to various receptor groups, recruit immunoreceptor tyrosine-based activating motif (ITAM)-containing adapters, such as: DAP10, DAP12, FcεRIγ, or CD3ζ, which in turn mediate tyrosine-kinase based downstream signaling (28, 31). The most prominent NK cell activating receptors are the natural cytotoxicity receptor (NCR) family, which includes three Ig-like proteins: NKp30 (34), NKp44 (35), and NKp46 in humans (36, 37). Of these, NKp46 and NKp30 are constitutively expressed by all NK cells, while NKp44 is expressed only after activation. The gene coding for NKp46 is located in the leukocyte-receptor complex on human chromosome 19, while the genes for NKp44 and NKp30 are located on chromosome 6. Upon binding their respective ligands, the NCRs recruit different ITAM-containing adapters to initiate signal transduction: NKp46 can recruit FcεRIγ and CD3ζ, the latter of which is also recruited by NKp30, while NKp44 recruits the adapter DAP12 (28, 38, 39).

Like many of the other activating NK receptors, the NCRs recognize tumor and self-ligands, yet, to-date they are the only NK receptors which have been found to directly recognize pathogen-derived molecules (28). Indeed, the first NCR ligands recognized were the influenza virus hemagglutinin (HA) and the Sendai virus HA-neuraminidase, which are recognized by NKp46 and NKp44 (28, 40). NKp46 also recognizes unknown ligand/s expressed by *Fusobacterium nucleatum* (41) and *Mycobacterium tuberculosis*, NKp44 recognizes the E-protein of Dengue virus and West Nile virus as well as bacterial cell wall components of *Pseudomonas aeruginosa, Nocardia* spp., and *Mycobacteria* spp. (42). NKp30 recognizes the PfEMP-1 protein of *Plasmodium falciparum* (43) and has recently been shown to bind and mediate the killing of various fungal species (44). Poxvirus HA has also been recognized as a target NKp46 and NKp30 (45). Yet, in the case of NKp30, the poxvirus HA serves as an inhibitory ligand, as is also the case with the pp65 protein of HCMV (46). In addition to recognizing pathogen expressed ligands, the NCRs also recognize several other known ligands, including Heparan sulfates, which are recognized differentially by the different NCRs, (47) as well as BAT3 (expressed by stressed cells) and B7-H6 (expressed by tumor cells), which are recognized by NKp30, (48, 49) and MLL5, which has recently been identified as a tumor expressed protein ligand of NKp44 (16). In addition, all the NCRs recognize unknown ligands expressed constitutively by several types of hematopoietic cells (granulocytes, monocytes, and dendritic cells). The reciprocal interaction between these cells and NK cells can result in their mutual activation or, alternatively, this interaction can sometimes lead to NK cell-mediated killing of immature dendritic cells (50–53).

NKp46 is unique amongst the NCRs and is considered to be the most specific NK cell marker. NKp46 is also distinct in that it is the only NCR that has a murine ortholog, named NCR1 (54–56). As such, NKp46 and NCR1 are the most studied of the NCRs. Mice knockouts (KO) for the *NCR1* gene were generated through the insertion of a reporter gene, encoding green fluorescent protein (GFP), into the *Ncr1* locus. While the heterozygous $Ncr1^{+/gfp}$ NK cells are haplo-sufficient and display a wildtype (wt) phenotype in the homozygous $Ncr1^{gfp/gfp}$ mice, *Ncr1* is knocked out and their NK cells lack NCR1 dependent functions (54, 57). Yet, despite this powerful, commercially available tool, the tumor and cellular ligand(s) for NKp46/NCR1 remain unknown.

To address the issue of NKp46 ligand expression, when such ligands are unknown, fusion proteins containing the extracellular portion of NKp46 fused to the Fc portion of human IgG1 have been used for cell and tissue staining. By applying this technique to murine and human tissue samples, it was found that both human and murine insulin-producing beta cells (β cells) constitutively express an NKp46 ligand (57, 58). In addition to β-cells, only two more normal tissues were found to express an NKp46 ligand(s), the salivary glands and hepatic stellate cells (57, 59). However, NKp46 and the other NCRs have also been found to bind to as of yet unknown ligands, of cellular, bacterial, fungal, and viral origin. Although the identities of these ligands remain largely unknown, experimental evidence suggests that each of the NCRs interacts with several distinct ligands (28).

In addition to the NCRs, NK cells express several other activating receptors: CD16 which mediates antibody-dependent cell-mediated cytotoxicity (ADCC); NKG2D which recognizes several stress-induced ligands expressed by cancerous, virally infected, and other stressed cells; as well as several receptors including NKp80, 2B4, DNAM1, NKG2C, and some short tailed KIRs that recognize ligands expressed physiologically on different cell types. In this regard it is important to note that all the activating NK receptors, with the exception of CD16, have been shown to be insufficient on their own in stimulating NK cell cytolytic functions (27, 28, 31).

Thus, NK cell populations, which variably express different activating and inhibitory receptors, may respond differentially upon encountering a potential target cell. However, the underlying principles that control NK cell activation remain the same: activating signals emanating from their corresponding receptors (mediated by tyrosine-kinase based signal transduction pathways), are integrated with repressive signals from inhibitory receptors (mediated by protein phosphatases), culminating in either target cell killing or in unresponsiveness (27, 60).

In the following segments, we will describe the involvement of NK cells in general and NKp46 specifically in T1D to exemplify the complexity of studying the roles of NCRs in human disease. T1D is a multifactorial autoimmune disease in which insulin producing

β cells, which reside in the islets of Langerhans in the pancreas, are attacked and killed predominantly by autoreactive T lymphocytes. Indeed, adoptive transfer experiments of T cells from diabetic mice to non-diabetics, confers diabetes via the killing of β cells in the recipient (61). However, other immune cells have also been implicated in diabetes pathogenesis (**Figure 1**): antibody producing B-lymphocytes have been implicated in maintaining T-cell reactivity toward β cells (62); macrophages, which are recruited to the islets of Langerhans in non-obese diabetic mice (NOD, the most studied animal model for T1D), prior to T-cell arrival, have been shown to be critical for diabetes progression (63); in addition, NK cells have also been implicated in T1D pathogenesis.

The first reports linking NK cells to diabetes came in the 1980s and early 1990s. These reports found that the NK cells from diabetic and diabetic prone rats were more cytotoxic than their counterparts from diabetic resistant rats, from the same genetic backgrounds (65–67). The next reports linking NK cells to diabetes came when experiments demonstrated that antibody-dependent depletion of NK cells (using an anti-asialo GM1 antibody, which depletes NK cells, with "off-target" effects on T cells and basophils (68)) could prevent diabetes development in two separate chemically induced diabetes models: low-dose streptozotocin (LDST) and cyclophosphamide (69, 70). Regardless, in both cases the experiments were conducted in mice, thus widening the scope and validity of the original findings: NK cells were not only capable of killing islet cells, but also seemed to be important in diabetes development in two distinct experimental models. When NOD mice from two genetic backgrounds were studied, one of which develops severe insulitis (inflammation in the islets of Langerhans) that rarely develops into diabetes and the other, which develops insulitis that rapidly progresses to diabetes, it was found that the main difference was a higher proportion of NK cells in the early insulitis immune infiltrates of the diabetes prone mice (71).

As for activating NK receptor presentation in T1D patients, while reduced NKp46 and NKp30 levels were observed only in patients with long-standing disease, a reduction in NKG2D was observed in both newly diagnosed and long-standing patients (72, 73). Moreover, the prevalence of activating KIR genes was higher in T1D patients when compared to HLA-matched controls. As the KIR repertoire expressed by NK cells determines self-tolerance and affects T-cell function, it would indeed be reasonable that the KIR genes influence susceptibility to T1D (73–75). Thus, NK cells were implicated in diabetes development both in animal models and human patients.

The first mechanistic link between NK cells and diabetes was the discovery that β cells from healthy mice, NOD mice, *Psammomys obesus*, and humans all express an NKp46 ligand (57, 58, 76). Although the identity of this ligand still remains unknown, some insights into the biology of the ligand and its interactions with NKp46 and NCR1 have been gained. It was found that two glycosylated amino acid residues located in the extracellular segment of NKp46, Thr^{125} and the Asn^{216}, are critical for binding this ligand (58). Importantly, binding of NKp46 to influenza virus HA is dependent on a different glycosylated residue – Thr^{225}. With regards to the expression kinetics of the β cell ligand for NKp46, it was shown that in both adult and young, mice and humans, the

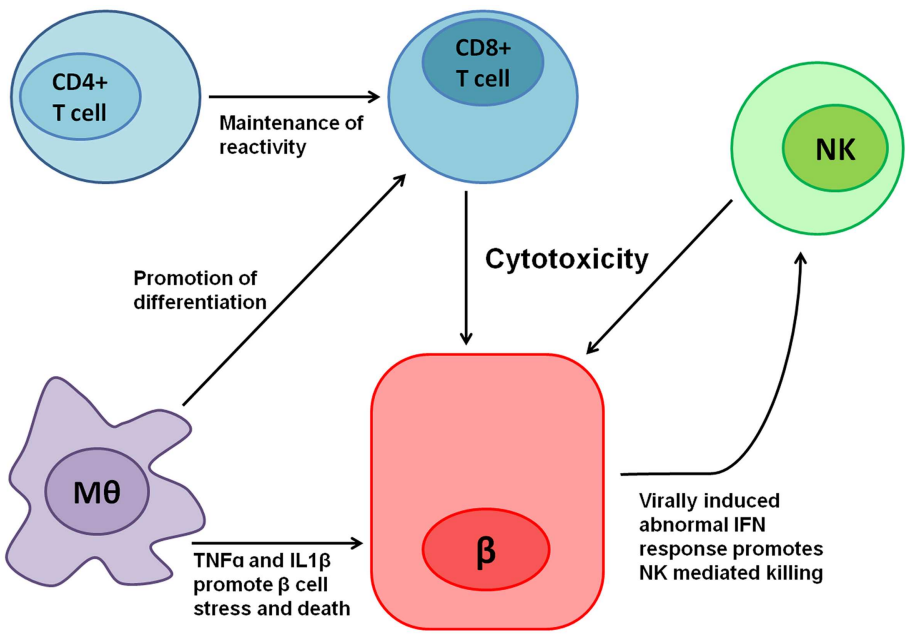

FIGURE 1 | Various immune cells are involved in T1D pathogenesis. Cytotoxic CD8+ T cells mediate most of the direct β cell killing, while CD4+ T cells help in maintaining their reactivity. Macrophages (Mθ) release tumor necrosis factor-α (TNFα), IL1β, and other cytokines, which cause β cell stress and promote cell death, in turn contributing to the local inflammation. Moreover, Mθ promote CD8+ T-cell differentiation mediated both by direct interactions and in Trans by cytokines. Local inflammation, β cell stress, and the expression of an unknown ligand for NKp46 contribute to NK cell reactivity toward β cells, which have been shown to kill β cells. In addition, β cells have been shown to exhibit abnormal interferon (IFN) responses when infected by viruses (64), thus potentially contributing even further to NK mediated killing.

ligands are expressed constitutively. However, while human β cells display ligand expression even at the earliest embryonic stages in which insulin is expressed, the murine ligand is not expressed by embryonic β cells. Nonetheless, following birth (as early as day 1 post-partum) the NKp46 ligand is expressed by all murine β cells. One possible explanation for the differences in ligand expression between human and murine β cells is that the NKp46 ligand is somehow associated with functional maturation of the β cells, which occurs relatively earlier during human pregnancy (58).

Moreover, the β-cell ligand expression was found to be closely correlated with β cell function. It was found to be present mainly in the insulin granules and to be released to the plasma membrane as β cells degranulate and release insulin. It was also shown that when β cell function starts to falter, as it happens during Type 2 diabetes development, the β cell NKp46 ligand expression is mostly lost, only to return to normal levels if the Type 2 diabetic phenotype is reversed (76).

The expression of the NKp46 ligand is stable in functional β cells and is maintained in the β cells that survive the immune attack in pre-diabetic and diabetic NOD mice. Ligand expression is also maintained during experimental ablation of β cells: it is maintained in the β cells that survived cytotoxic death mediated by diphtheria toxin A and is also seen during subsequent β cell regeneration. Thus, because of the expression of the NKp46 ligand is stable and is seen in all functional β cells examined, β cells are constantly at risk of being attacked by NK cells via NKp46-mediated activation (58, 77).

Indeed, the outcome of β cell recognition by NK cells was exemplified in a series of experiments (58, 59). In agreement with earlier reports from rat NK cell (65), the incubation of human or mouse β cells with their corresponding NK cells caused NK cell degranulation as well as direct killing of the target β cells, as assessed by radioactive killing assays. Importantly, this β cell induced NK degranulation was NKp46 dependent as it was significantly lower in NK cells derived $Ncr1^{gfp/gfp}$ when compared to NK cells from control $Ncr1^{+/gfp}$ mice. Moreover, in agreement with earlier results, human NK cells killed human β cells and this killing was impeded by blocking NKp46 (using anti-NKp46 sera). Thus human and murine β cells are indeed *in vitro* targets for isolated NK cells, and are killed NKp46 dependently (58).

Additionally, NK cells and NKp46 were also implicated in T1D pathogenesis *in vivo*. NK cells isolated from pre-diabetic NOD islet immune infiltrates, stained for CD107a expression, which is a marker for recent NK cell degranulation (78). Moreover, immunization of NOD mice using NKp46-Ig and NCR1-Ig fusion proteins significantly reduced diabetes incidence, without depleting the NK cell population (57). NKp46 involvement in T1D was not specific to NOD mice: when diabetes was induced in WT and $Ncr1^{gfp/gfp}$ KO mice using a LDST protocol, diabetes development was significantly impaired in mice lacking NCR1. Thus, NK cells and their NKp46 activating receptor facilitate diabetes development *in vivo*, both in induced and genetic models of the disease.

Thus, all functional β cells express a ligand for NKp46 and NK cells kill them, when encountering β cells. However, diabetes does not develop in all individuals because NK cells never encounter β cells under normal conditions [(58) and **Figure 2**].

Natural killer cell involvement in diabetes and the intrinsic NKp46 dependent capacity of NK cells to kill β cells highlight some key aspects of NK cell biology and the roles of the NCRs. Despite powerful tools such as transgenic mice and fusion proteins, many cellular ligands remain unknown, some of which are directly associated with the disease. The identification of the NCR cellular ligands has so far remained very difficult. This is due in part to the fact that each NCR has several different ligands, expressed differentially by different cells and tissues. It remains to be seen whether and how other diseases are influenced by NCRs. Indeed, in the case of liver stellate cells, NK cell recognition of their cognate NKp46

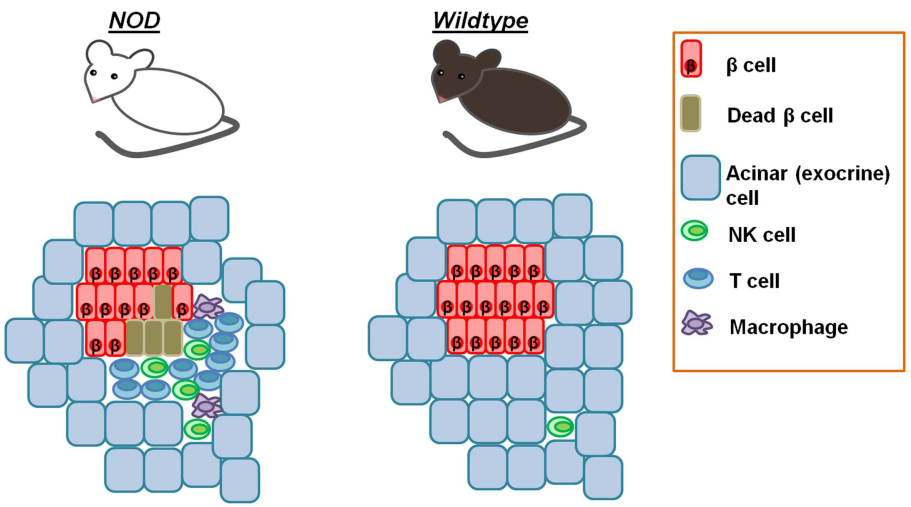

FIGURE 2 | Pancreatic NK cells in homeostasis and diabetes. Whereas NK cells are rarely found in the pancreases of normal healthy mice, in the peri-islet immune insulitis infiltrates of NOD mice, NK cells are found starting at the earliest stages of insulitis. This explains why diabetes does not develop in all individuals, despite the intrinsic ability of NK cells to kill β cells.

ligand inhibits liver fibrosis (59). Thus, depending on the context the same NCR can serve to exacerbate disease in one setting and ameliorate it in another. Presumably, this has to do with different ligands expression, though this issue remains unclear. Additionally, as the identification of target cells by NK cells is dependent on multiple receptors and different combinations of receptor engagements elicit different responses by the NK cells, the issue of NK cell response to target recognition is vastly complicated.

An early view of NK cells and their receptors identified them as a primordial mechanism of immunity to infectious pathogens and subsequently, the NK cell receptors were speculated to be pattern recognition receptors for pathogens (44, 79). However, the finding that activating NK cell receptors, in general, and the NCRs specifically, bind several distinct ligands of endogenous origin implicates NK cells to be more general immune mediators. It appears that the NCRs are thus general "danger" pattern recognizers that confer complex activities to NK cells.

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