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Review

Surface receptors and functional interactions of human natural killer cells: from bench to the clinic

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Abstract. The past 10 years have witnessed dramatic progress in our understanding of how natural killer (NK) cells function and their role in innate immunity. Thanks to an array of inhibitory receptors specific for different HLA class I molecules, human NK cells can sense the decrease or loss of even single alleles at the cell surface. This represents a typical condition of a potential danger, i.e. the presence of tumor or virally infected cells. NK cell triggering and lysis of these cells is mediated by several activating receptors and coreceptors that have recently been identified and cloned. While normal cells are usually resistant to NK-mediated attack, a remarkable exception is represented by dendritic cells (DCs). In their immature form they are susceptible to NK-mediated lysis because of the expression of low levels of surface HLA class I molecules. The process of DC maturation (mDCs) is characterized by the surface expression of high levels of HLA class I molecules. Accordingly, mDCs become resistant to NK cells. A recent major breakthrough highlighted the role played by donor NK cells in allogenic bone marrow transplantation to cure acute myeloid leukemias. 'Alloreactive' NK cells derived from donor hematopoietic precursors not only prevented leukemic relapses, but also prevented graft rejection and graft-versus-host disease.

Key words. NK cell; dendritic cell; NK receptor; HLA class I allele; hematopoietic transplantation.

Introduction

Natural killer (NK) cells represent a distinct lymphoid population characterized by unique phenotypic and functional features and account for 5–20% of peripheral blood lymphocytes. NK cells were originally identified on a functional basis, the denomination being assigned to lymphoid cells capable of lysing tumor cell lines in the absence of prior stimulation in vivo or in vitro [1]. Both their origin and the mechanism(s) mediating their function remained mysterious until recently. Regarding their origin, NK cells derive from a precursor common to T cells and expressing the $CD34^+CD7^+$ phenotype. In addition, functional NK cells can be obtained in vitro and in vivo from $CD34^+$ hematopoietic precursors isolated from several different sources [2–6]. Cell maturation in vitro has been shown to require appropriate feeder cells and/or interleukin (IL)-15. The molecular mechanisms underlying the ability of NK cells to discriminate between normal and tumor cells, predicted by the 'missing self hypothesis' [7],

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have been clarified only during the past decade. NK cells recognize MHC class I molecules through surface receptors delivering inhibitory, rather than activating, signals. Accordingly, NK cells lyse target cells that have lost (or express low amounts of) MHC class I molecules. This event occurs frequently in tumors or in cells infected by some viruses such as certain Herpesviruses or Adenoviruses. In addition to providing a first line of defense against viruses, NK cells release various cytokines and chemokines. These induce or modulate inflammatory responses, hematopoiesis, control the growth and function of monocytes and granulocytes and influence the quality of subsequent T cells responses, either directly or by their effect on the maturation and function of dendritic cells (DCs). This leads to a preferential polarization toward Th1 responses.

NK cells can be inactivated via MHC-specific inhibitory receptors

Human NK cells express a wide array of HLA class I-specific inhibitory receptors (iNKRs). Those referred to as killer Ig-like receptors (KIRs) recognize shared allelic determinants of HLA class I molecules, while ILT2 (LIR1) is characterized by a broad specificity for different HLA class I molecules and CD94/NKG2 A recognizes HLA-E molecules [8–12]. Of note is that each iNKR is expressed only by a fraction of (and not all) NK cells [9]. Another important notion is that all mature human NK cells express at least one receptor specific for self HLA class I molecules. The coexpression of two or more self-reactive iNKRs occurs less frequently. This would mean that the whole NK cell pool of a given individual can sense the loss of even a single class I allele on autologous cells [9]. Upon cross-linking, iNKRs recruit and activate SHP-1 and SHP-2 phosphatases through immunoreceptor tyrosine-based inhibition motifs (ITIMs) [11]. This mechanism, utilized by different inhibitory receptors, blocks the activating signals delivered upon engagement of different triggering NK receptors.

Switching NK cells 'on': the activating receptors involved in natural cytotoxicity

The need for inactivating NK cells to prevent damage to HLA class I^+ normal self cells implies the existence of an 'on' signal when NK cells interact with target cells. Activating receptors that are specific for HLA class I molecules and display a high homology with the corresponding inhibitory receptors have been identified in a fraction of NK cells [13–15]. However, these cannot explain why NK cells can lyse HLA class I-negative target cells. Indeed, the major receptors involved in NK cell triggering in the process of natural cytotoxicity do not recognize HLA class I molecules. Our group described NKp46, NKp30 and NKp44 molecules, collectively referred to as 'natural cytotoxicity receptors' (NCRs). They represent the first identified and molecularly characterized receptors mediating NK cytotoxicity [16, 17]. Of note is that the widely used NK cell markers CD16 and CD56 do not precisely identify NK cells [1, 5]. In contrast, NCRs are highly NK specific [16, 17]. Thus, NKp46 and NKp30 are expressed by all resting and activated NK cells [18–20]. NKp44 is selectively expressed by NK cells upon activation [21, 22], and may contribute to the higher efficiency of IL-2-cultured NK cells in tumor cell lysis. NCRs play a major role in the lysis of most tumor cell lines, as shown by monoclonal antibody (mAb)-mediated receptor-blocking experiments. Moreover, a direct correlation exists between the surface density of NCRs on a given NK cell and the intensity of the NK-mediated cytotoxicity [23]. NCRs belong to the Ig superfamily with no homology to each other and a low degree of identity with known human molecules [19, 20, 22]. Human NK cells can lyse murine tumor cells however, this interaction involves only a single NCR: NKp46. In line with these data, an NKp46 homolog cDNA has been found in mice [24]. An additional receptor, NKG2D, is present in both NK cells and cytotoxic T lymphocytes (CTLs). NKG2D is specific for the stress-inducible MICA/B [25, 26] or for ULBP proteins [27, 28]. Remarkably, MICA/B are expressed predominantly, but not exclusively, by cells of epithelial origin. NKG2D is involved in NK-mediated cytotoxicity against certain tumors that express at least one of the above ligands [26, 28]. In contrast, the cellular ligands recognized by NCR have not been identified so far; however, the available information is compatible with the concept that these ligands may also be expressed primarily by activated or proliferating cells. If this holds true, the lack of ligands for NCRs (and for NKG2D) would prevent NK cell activation upon interaction with normal tissues. This, as discussed below, could be particularly relevant in allogeneic, mismatched hemopoietic transplantation. Other activating surface molecules including 2B4, NTB-A and NKp80 [29–33] may contribute to NK cell triggering during the process of natural cytotoxicity. However, their role may be that of coreceptor, i.e. to amplify the NK cell triggering induced by NCRs or NKG2D [16].

How NK cells have evolved to cooperate with adaptive immunity

NK cells have long been considered as 'primitive' effector cells. However, today, our understanding of these cells is substantially different. Thus, NK cells have evolved to adapt to various mechanisms of specific immunity. For example, they have acquired Fcγ receptors that allow them

to eliminate with greater efficiency IgG-coated target cells or pathogens. Moreover, they release cytokines and chemokines that regulate T cell function. In this context, early activation of NK cells during defense against pathogens may influence the quality of the subsequent T cell response by inducing T cell polarization toward Th1 cells. As illustrated below, NK cells can also interact with DCs. This cross-talk may not only influence the functional capability of both cell types, but may also affect the subsequent induction of specific immunity. In addition, as discussed above, NK cells have developed a mechanism leading to the rapid detection and elimination of potentially dangerous cells characterized by low expression of MHC class I antigens consequent to tumor transformation or viral infection (e.g. Herpesviruses). Remarkably, this mechanism is rather sophisticated, as human KIRs can detect allelic determinants of HLA class I molecules. In addition, it is a recently evolved mechanism since KIRs are absent in mice in which a similar function is mediated by structurally different receptors [8]. Moreover, major differences in the KIR expression also exist in chimpanzees, i.e. a species that diverged from humans only approximately 5 million years ago [34]. These findings clearly indicate that KIRs have evolved recently, in parallel with the rapid evolution of the HL class I molecules.

Interactions between NK cells and DCs

Until recently, no information existed on the possible interactions between NK cells and DCs. Owing to the expression of iNKRs, NK cells do not kill normal, MHC class I^+ cells. However, recent data revealed an important exception. DCs are susceptible to lysis by autologous NK cells despite the surface expression of significant levels of surface MHC class I molecules [35]. This suggested that DCs may have a unique susceptibility to NK-mediated lysis. This property is of major interest since DCs represent the most important-antigen presenting cells (APCs) and play a crucial role in the initiation and maintenance of the immune response [36–39]. In their immature form (immDCs), myeloid DCs are particularly efficient in antigen capture and in releasing proinflammatory cytokines and chemokines [40, 41]. Among the antigens captured by immDCs, there may be a wide spectrum of invading pathogens as well as dying cells that are present in the tissue microenvironment. ImmDCs are inefficient in antigen presentation and their further maturation is required for optimal antigen presentation. DC maturation is characterized by the up-regulation of surface MHC molecules, de novo expression of costimulatory molecules, as well as by a parallel down-regulation of surface receptors involved in antigen capture and by changes in the pattern of expression of chemokine receptors [36–41].

In recent studies, the effect of the interaction between NK cells and DCs has been analyzed [42]. A remarkable finding was the occurrence of strong NK cell proliferation [35, 42] and the up regulation of NK cell cytotoxicity [35]. The iNCRseased NK cytotoxicity was directed not only toward tumor cells, but also against immDCs themselves (fig. 1). Another remarkable finding was that the NK-mediated lysis of DC was mediated by the NKp30 NCRs, while other activating receptors or coreceptors played either a marginal role (NKp46) or no role at all [35].

T cell costimulatory molecules such as CD80 and CD86 have also been reported to mediate NK cell activation and to be involved in DC recognition and lysis [43–45]. Although these molecules might play a role in the activation of NK cells by DCs, they do not appear to control DC susceptibility to NK-mediated lysis. Notably, in the case of involvement of CD80 and CD86, one would expect an increased susceptibility to lysis of mDCs. In fact, the high expression of these costimulatory molecules is one of the hallmarks of mDCs as compared to immDCs. However, in different studies, mDCs were consistently resistant to autologous NK cell-mediated lysis [35, 45, 46]. On the other hand, down-regulation of ligands recognized by triggering NK receptors (in particular NKp30) upon DC maturation does not occur. Experiments of mAb-mediated masking of the HLA class I-specific inhibitory receptors (or of HLA class I molecules) indicated that the resistance of mDCs to NK-mediated lysis reflects the marked up-regulation of HLA class I molecules on these cells. Indeed, under these experimental conditions, the lysis of mDCs was restored to a level comparable to that of their immDC counterpart. These data also imply that mDCs express levels of ligands for NKp30 sufficient to induce NK cell activation [47].

Conceivably effective DC/NK cell interactions may occur primarily during infections. In this context, the effect of live bacteria on the cross-talk between DCs and NK cells was analyzed. The extracellular bacteria *Escherichia coli*, or the intracellular mycobacterium BCG, have been used [47, 48]. In both systems, bacterial infection of DCs resulted in rapid NK cell activation. These activated NK cells could efficiently lyse autologous immDCs. However, those immDCs that had been exposed to bacteria became rapidly resistant to NK-mediated cytotoxicity due to their maturation and up-regulation of HLA class I molecules (fig. 1). This observation is relevant because it provides information on DC/NK cell interactions that might occur in the course of bacterial infection in vivo. In addition, exposure of immDCs to either bacteria resulted in the expression of CD80 and CD86 coreceptors, HLA molecules and the chemokine receptor CCR7 (allowing DC migration to lymph nodes) [41]. This would mean that immDCs, upon encountering pathogens, undergo rapid maturation, can function as efficient APCs and migrate to secondary lymphoid organs where they can interact with

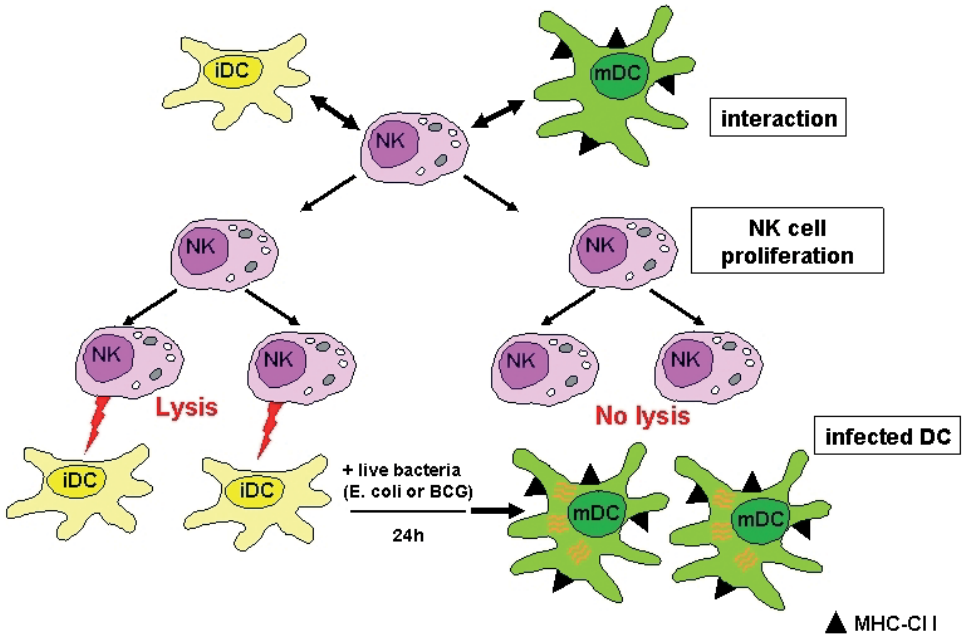

Figure 1. Schematic representation of the interactions between NK cells and DCs and the effect of infection with live bacteria. When NK cells interact with either immature (i) or mature (m) DCs they undergo rapid proliferation and acquire strong cytolytic activity. Activated NK cells can efficiently lyse iDCs that express low levels of MHC class I molecules. In contrast, mDCs are resistant to lysis due to the high surface expression of MHC class I molecules. In the presence of live bacteria (e.g. E. coli or BCG), iDCs undergo rapid maturation into mDCs, thus becoming resistant to NK cell cytotoxicity. In addition, they become capable of migrating to secondary lymphoid organs and to function as efficient APCs. The rendezvous between NK cells and DCs can occur in vivo during infection and represents a mechanism to amplify innate immune responses against pathogens. Moreover, selective killing of iDCs may represent a regulatory mechanism by which unnecessary (see text) cells are removed.

T cells and induce a prompt specific immune response against infecting bacteria. In turn, DCs induce a rapid up-regulation of the NK cell function, thus potentiating another important effector arm of innate immunity. Although NK cells, under normal conditions, reside in blood, bone marrow and spleen, in the course of inflammatory responses they can be addressed by different chemokines to interact with DCs in tissues or lymph nodes [49]. One may ask why should NK cells kill immDCs, thus depleting an important source of APCs. A conceivable answer is that immDCs, upon exposure to bacteria, rapidly acquire resistance to NK cell cytotoxicity. Accordingly, they escape killing and migrate to lymph nodes (via CCR7). The mechanism by which DCs induce a rapid increase in cytolytic activity in NK cells, may conceivably be consequent to the production of large amounts of IL-12 by DCs following infection [50]. As to the proliferative responses of NK cells upon interaction with DCs, this could reflect the production of cytokines such as IL-2 [51] and IL-15 [52]. The potent NK cell activation induced by DCs may possibly explain why NK cells are required for effective BCG immune therapy [53]. Thus, the NK cell cytotoxicity that is rapidly induced by BCG-activated DCs could provide an explanation for the still unclear anti-tumor effect of BCG therapy. Of note is that the NK cell cytotoxicity does not mediate any substantial direct anti-bacterial effect. Why, therefore, is NK cytolytic activity greatly increased during bacterial infections? A possible explanation is that NK cells, upon DC-induced activation, may play a regulatory role in the homeostasis of the immune response in the course of bacterial infections. In view of their ability to distinguish between infected and non-infected DCs, the NK-mediated lysis of uninfected DCs could represent a means to eliminate an excess of immDCs that have not engulfed bacteria to be presented to T cells [47]. The elimination of immDCs could have several implications for the homeostasis of the immune response. For example, immDCs recruited to inflamed regions produce high amounts of proinflammatory cytokines [such as IL-1α, IL-1β and tumor necrosis factor (TNF)-α] [54, 55]. A prolonged presence of immDCs might thus represent a threat due to the sustained production of these cytokines. NK-mediated lysis of immDCs might also represent a powerful feedback mechanism to limit the excess of antigen presentation in secondary lymphoid organs and consequent overinflammation. Possible advantages of eliminating immDCs relate also to the recent information that immDCs may induce IL-10-producing regulatory T cells [56–58]. These T cell, by the suppression of effector T cells functions, could be coun-

terproductive to the clearance of pathogens. Thus, removal of an excess of immDCs might be useful to prevent the induction of antigen-specific suppressor T cells at the site of inflammation. Another important outcome of immDC removal by NK cells might be related to the quality of T cell responses upon antigen presentation. Recently, polarizing signals for type 1 or type 2 T cell responses have been suggested to be initiated by DC-delivered signals in secondary lymphoid organs [55, 59]. In particular, impaired DC maturation could lead to an increased T helper 2 (Th2) response [54, 55, 60]. However, a definite role for immDCs in shaping a preferential Th2 response has not yet been clearly established. A further mechanism by which the interaction between NK cells and DCs may indeed influence T cell polarization is the release of significant amounts of interferon (IFN)-γ by NK cells following interaction with autologous DCs [35]. In vitro studies in both murine and human systems have demonstrated the importance of IFN-γ in the induction of type 1 immune responses [61, 62]. IFN-γ can mediate this effect through different mechanisms, including priming for IL-12 production by DCs and induction of the IL-12Rβ on T cells [63, 64]. Interestingly, in a murine model of skin graft rejection, the recognition of donor APCs by host NK cells had a dramatic effect on alloreactive Th1/Th2 cell development: turning off host NK cells was sufficient to skew the alloresponse to the Th2 pathway [65].

In conclusion, during infections, the presence in tissues and lymph nodes of activated NK cells capable of discriminating between infected and non-infected DCs and producing relevant cytokines upstream of the adaptive immune response suggests that NK cells may play an important regulatory role at the interface between innate and specific immunity.

NK cells in mismatched hematopoietic transplantation

Recently, a major breakthrough highlighted the possible role of NK cells in the cure of the life-threating acute myeloid leukemias (AMLs). While in an autologous setting, all NK cells are inhibited because they express iNKRs specific for self alleles, in an allogeneic setting, some NK cells may lyse allogeneic cells provided they express HLA class I alleles that are not recognized by their iNKRs [9]. Because each KIR actually recognizes allotypic determinants that are shared by different HLA class I alleles (table 1), an HLA mismatch between NK cells and allogeneic target cells does not necessarily lead to NK-mediated killing [9]. Another relevant point is that the KIR repertoire of NK cells is predictable on the basis of the HLA class I typing of the donor [66]. This information, together with the HLA typing of the allogeneic mismatched target cells, will allow us to predict the existence of 'alloreactive' NK cells. In addition, adequate information on the degree of alloreactivity of a given NK cell population can be obtained by assessing the magnitude of the NK-mediated cytolysis against the allogeneic target cells examined. Notably, 'alloreactive' NK cells are confined to NK cells that use KIRs as a source of iNKRs for self HLA class I molecules (table 1) [9]. KIR-HLA class I mismatches occur in a fraction of leukemic patients undergoing haploidentical bone marrow transplantation (in which donor and recipient pairs are identical for one HLA haplotype and incompatible at the HLA loci of the unshared haplotype). All these cases are obviously at high risk of T cell-mediated alloreactions both in the host-versus-graft (HvG) and graft-versus-host (GvH) direction [66]. These responses can be controlled to a large extent by immunosuppression therapy and T cell depletion of the graft [to prevent GvH disease (GvHD)]. In patients undergoing haploidentical hematopoietic transplantation, the occurrence of KIR-HLA class I mismatched ('alloreactive') NK cells had important consequences in the clinical outcome. Indeed, 'alloreactive' NK cells could prevent severe complications such as leukemic relapses, GvHD and graft rejection [67]. The AML patients undergoing haploidentical hematopoietic transplantation displayed a survival rate as high as 60% (at 5 years) in the presence of KIR-HLA mismatches (and, thus, of alloreactive NK cells). In the absence of such mismatches, the survival rate was only 5% [67]. A likely explanation of these effects, supported by experimental data in vitro as well as by a murine model [67], is the following. Donor NK cells originate from stem cells transplanted into leukemic patients (who have previously received chemotherapy and radiotherapy). These NK cells not only eliminate residual leukemic cells, thus preventing leukemic relapses, but can

Table 1. Specificity of the main HLA-class I-specific inhibitory NK receptors (iNKRs).

iNKR	HLA class I specificity
(KIR2DL1) p58-1	'Group 2' HLA-C alleles (-Cw2, -Cw4, -Cw5, -Cw6) (Asn77, Lys80)*
(KIR2DL2/3) p58.2	'Group 1' HLA-C alleles (-Cw1, -Cw3, -Cw7, -Cw8) (Ser77, Asn80)*
(KIR3DL1) p70	HLA-Bw4 alleles (e.g. HLA-B27)
(KIR3DL2) p140	HLA-A3, -A11
ILT2 (LIR1)	various HLA class I alleles
CD94/NKG2A	HLA-E

* Note that the two groups of HLA-C alleles can be distinguished on the basis of alternative amino acid sequence motifs at position 77 and 80. Site-directed mutagenesis unequivocally demonstrated that these residues are crucial for KIR-mediated recognition.

also act on DCs of the patients [66, 67]. Notably, DCs are responsible for donor T cell priming thus inducing GvHD**.** Indeed, in a murine model, alloreactive NK cells accelerated the loss of bone marrow, spleen and gut DCs, as compared to mice receiving non alloreactive NK cells [67]. The same study convincingly demonstrated that the elimination of recipient APCs was responsible for the protective effect against GvHD mediated by alloreactive T cells.

In addition, NK cells kill the residual lymphohematopoietic cells of the patients, including T lymphocytes which are responsible for graft rejection. Despite their alloreactivity, donor NK cells do not damage normal, non-hemopoietic tissues of the host (patient). This may be explained by the fact that the ligands for the major triggering NK receptors are not expressed by resting, normal cells (at least not those of non-hemopoietic origin) [66]. The results of these studies highlight the possibility of exploiting NK cell alloreactivity in the therapy of AML and, possibly, other tumors. In this context, donor selection, at least in the case of high-risk AML, could now involve a deliberate search for a 'perfect mismatch' [68] at certain HLA class I loci. Moreover, mature alloreactive NK cells could be selected from healthy donors, expanded in vitro and deliberately infused to prevent leukemic relapses and GvHD.

In conclusion, NK cell alloreactivity combines all the favourable features that make these cells uniquely suited for transplantation in the therapy of AML and, possibly, other tumors.

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