The EMBO Journal (2004) 23, 255–259 | © 2004 European Molecular Biology Organization | All Rights Reserved 0261-4189/04
www.embojournal.org

THE EMBO JOURNAL

Mini Review

Unravelling natural killer cell function: triggering and inhibitory human NK receptors

Lorenzo Moretta[1,2,3,*] and Alessandro Moretta[2,3]

[1]Istituto Giannina Gaslini, Genova-Quarto, Italy, [2]Dipartimento di Medicina Sperimentale, Università degli Studi di Genova, Genova, Italy and [3]Centro di Eccellenza per le Ricerche Biomediche, Università degli Studi di Genova, Genova, Italy

Natural killer (NK) cells represent a highly specialized lymphoid population characterized by a potent cytolytic activity against tumor or virally infected cells. Their function is finely regulated by a series of inhibitory or activating receptors. The inhibitory receptors, specific for major histocompatibility complex (MHC) class I molecules, allow NK cells to discriminate between normal cells and cells that have lost the expression of MHC class I (e.g., tumor cells). The major receptors responsible for NK cell triggering are NKp46, NKp30, NKp44 and NKG2D. The NK-mediated lysis of tumor cells involves several such receptors, while killing of dendritic cells involves only NKp30. The target-cell ligands recognized by some receptors have been identified, but those to which major receptors bind are not yet known. Nevertheless, functional data suggest that they are primarily expressed on cells upon activation, proliferation or tumor transformation. Thus, the ability of NK cells to lyse target cells requires both the lack of surface MHC class I molecules and the expression of appropriate ligands that trigger NK receptors.

The EMBO Journal (2004) **23**, 255–259.doi:10.1038/sj.emboj.7600019; Published online 18 December 2003
Subject Categories: immunology
Keywords: cytolytic T lymphocytes; killer Ig-like receptors; MHC class I-specific inhibitory receptors; natural cytototoxicity; natural killer cells; NK coreceptors

Introduction

Natural killer (NK) cells have long been arbitrarily defined using imprecise phenotypic and functional criteria. They were first identified on a functional basis, that is, their ability to kill certain tumors in the absence of prior stimulation. Different from T or B lymphocytes, NK cells do not express clonally distributed receptors for antigen (Trinchieri, 1990; Moretta *et al*, 1994). They appear to play a role in the control of tumor growth *in vivo* by preventing the dissemination of metastatic tumors, at least in a murine model (Kim *et al*, 2000). NK cells are also fundamental in defenses against highly cytopathic viruses, primarily herpesviruses (Biron *et al*, 1999).

Despite their relevance in defense mechanisms, major questions surrounding their mode of action and their precise nature remained unanswered. However, this has dramatically changed in recent years and we now have a fairly accurate perception of the general mechanisms that regulate NK cell activation and function. Indeed, the general molecular strategies that allow NK cells to spare normal cells and kill tumor or virally infected cells have now been clarified. The major surface NK receptors contributing to the process of negative or positive signaling during NK cell-mediated immune responses will be briefly illustrated.

Inactivating NK cells by MHC class I-specific inhibitory receptors

In the early 1990s, parallel studies in humans (Moretta *et al*, 1990, 1992, 1993; Ciccone *et al*, 1992; Colonna *et al*, 1993) and mice (Ljunggren and Kärre, 1990; Yokoyama and Seaman, 1993) revealed that NK cells recognize major histocompatibility complex (MHC) class I molecules via surface receptors that deliver signals that inhibit rather than activate NK cell cytotoxicity. Accordingly, lack of interaction of these receptors with MHC class I molecules may result in the killing of target cells (Moretta *et al*, 1996). This occurs when target cells have lost or express insufficient amounts of MHC class I molecules, as frequently occurs during tumor transformation or infection by certain viruses. Compared to mouse, human NK cells evolved a much more complex set of inhibitory receptors. A group of these, termed killer immunoglobulin (Ig)-like receptors (KIRs), recognize different allelic groups of HLA-A, -B or -C molecules (Moretta *et al*, 1996; Lanier, 1998; Long, 1999). ILT2 (or LIR-1) receptors are more 'promiscuous', as they recognize a large number of HLA class I alleles, while CD94-NKG2A (Lopez-Botet *et al*, 1997) recognize HLA-E, an HLA class I molecule with a limited polymorphism. It is well known that various HLA class I alleles provide signal sequence peptides that bind HLA-E and enable it to be expressed on the cell surface. Importantly, each type of KIR is expressed only by a subset of NK cells (Braud *et al*, 1998). Moreover, each NK cell expresses at least one receptor specific for self HLA class I molecules, while the coexpression of two or more self-reactive receptors is rare. This type of receptor distribution allows the whole NK cell pool to detect the loss of even a single HLA class I allele on self cells, a frequent event in tumor transformation (Garrido *et al*, 1997). A common characteristic of the various HLA class I-specific inhibitory receptors is the presence, in their cytoplasmic tail, of immunoreceptor tyrosine-based inhibitory motifs that enable them to recruit and activate SHP-1 and SHP-2

*Corresponding author. Istituto Giannina Gaslini, Largo Gerolamo Gaslini 5, 16147 Genova-Quarto, Italy. Tel.: +39 010 5636 806/807; Fax: +39 010 3730 671; E-mail: lorenzomoretta@ospedale-gaslini.ge.it

Received: 14 August 2003; accepted: 4 November 2003; Published online: 18 December 2003

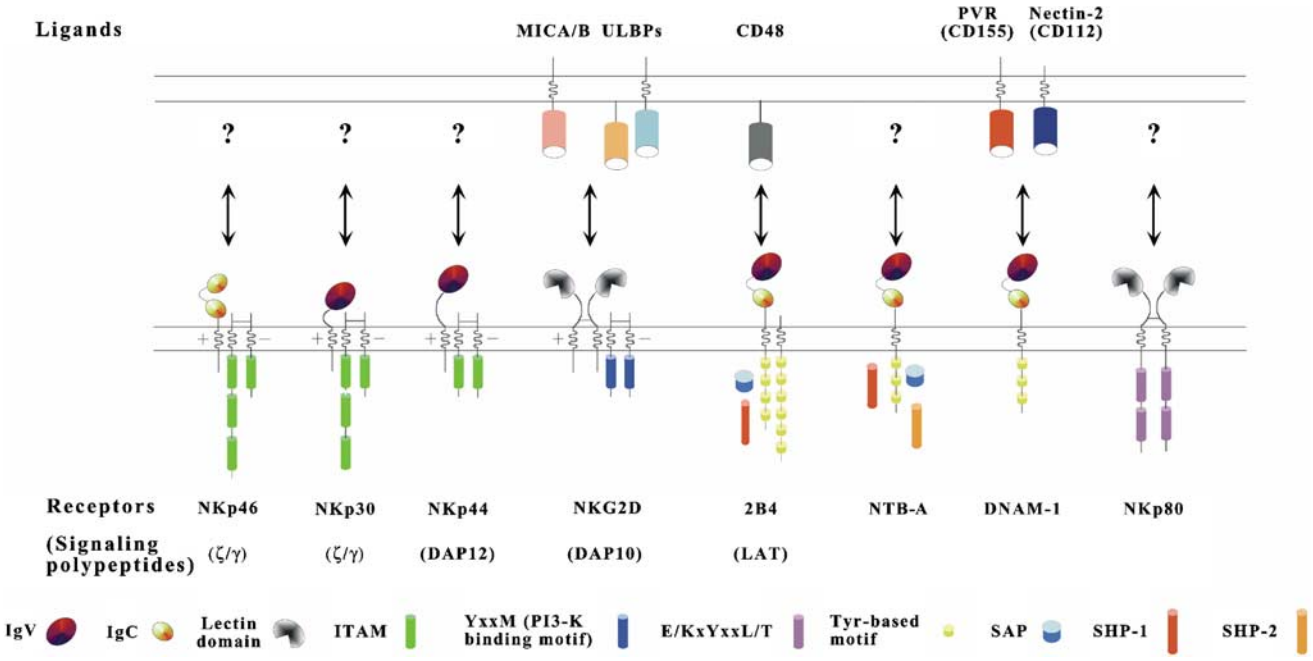

Figure 1 Activating NK receptors and coreceptors and their cellular ligands. This figure illustrates the molecular structure of the NK receptors NKp46, NKp30, NKp44 and NKG2D as well as of the NK coreceptors 2B4, NTB-A, DNAM-1 and NKp80. Their interaction with signaling polypeptides or with relevant cytoplasmic molecules is also shown. The known cellular ligands are illustrated in a simplified form.

phosphatases (Moretta *et al*, 1996; Lanier, 1998; Long, 1999). In turn, these phosphatases switch off the activating signaling cascade initiated by the various activating receptors.

NK cell triggering: the activating receptors and their ligands

Provided that turning NK cells 'off' represents the major fail-safe device to prevent the NK-mediated attack of normal HLA class I^+ autologous cells, an 'on' signal must be generated upon interaction of NK cells with potential target cells. This signal is extinguished whenever appropriate interactions occur between inhibitory receptors and MHC class I molecules. On the other hand, the 'on' signal can be readily detected when NK cells interact with target cells that lack MHC class I molecules. The receptors responsible for NK cell activation in the process of natural cytotoxicity remained elusive until recently, when three novel surface molecules were identified and molecularly characterized. Collectively termed natural cytotoxicity receptors (NCRs), NKp46 (Sivori *et al*, 1997; Pessino *et al*, 1998), NKp44 (Vitale *et al*, 1998; Cantoni *et al*, 1999) and NKp30 (Pende *et al*, 1999) possess limited homology with known human molecules and no homology to each other (Moretta *et al*, 2000, 2001) (Figure 1). As their expression is restricted to NK cells, they represent the most accurate surface markers for human NK cell identification. NCRs play a major role in the NK-mediated killing of most tumor cell lines, as revealed by monoclonal antibody (mAb)-mediated receptor-masking experiments (Moretta *et al*, 2001). Moreover, their surface density on NK cells correlates with the magnitude of the cytolytic activity against NK-susceptible target cells (Sivori *et al*, 2000). The ligands recognized by NCRs are still not molecularly defined. However, as revealed by cytolytic assays, they are expressed by cells belonging to different histotypes (Moretta *et al*, 2000, 2001; Sivori *et al*, 2000; Costello *et al*, 2002; Pende *et al*, 2002). While NKp46 and NKp30 enable a precise identification of all NK cells, regardless of whether these cells are resting or activated (which is not true for other widely used NK cell markers including CD56 and CD16), NKp44 is selectively expressed by activated NK cells (Vitale *et al*, 1998; Cantoni *et al*, 1999; Moretta *et al*, 2001). This might explain, at least in part, the higher levels of cytolytic activity of activated NK cells cultured in IL-2.

NKG2D, a surface receptor of the NKG2 family (type II membrane proteins characterized by a lectin-like domain) (Cerwenka and Lanier, 2001; Diefenbach and Raulet, 2001), also plays a role in NK-mediated cytolysis. NKG2D is not restricted to NK cells, but is also expressed by cytolytic T lymphocytes. NKG2D is specific for the stress-inducible MICA and MICB or ULBP proteins (Sutherland *et al*, 2001). These ligands are expressed predominantly, but not exclusively, by cells of epithelial origin.

Activating coreceptors and their ligands

Other triggering surface molecules expressed by NK cells (but shared by other leukocyte types) appear to function primarily as coreceptors (Figure 1). That is, their ability to signal depends on the simultaneous co-engagement of one or another triggering receptor (Moretta *et al*, 2001). They may function to amplify signaling by true receptors. Two such coreceptors, 2B4 (Parolini *et al*, 2000) and NTB-A (Bottino *et al*, 2001), appear to serve a dual and opposite function, depending on the availability of downstream regulating elements in their signaling pathways. They are both members of the CD2 subfamily, although their amino-acid identity is low (Bottino *et al*, 2001). The cytoplasmic domains of 2B4 and NTB-A bind a small protein termed SAP. This molecular association is crucial for 2B4 or NTB-A to deliver signals

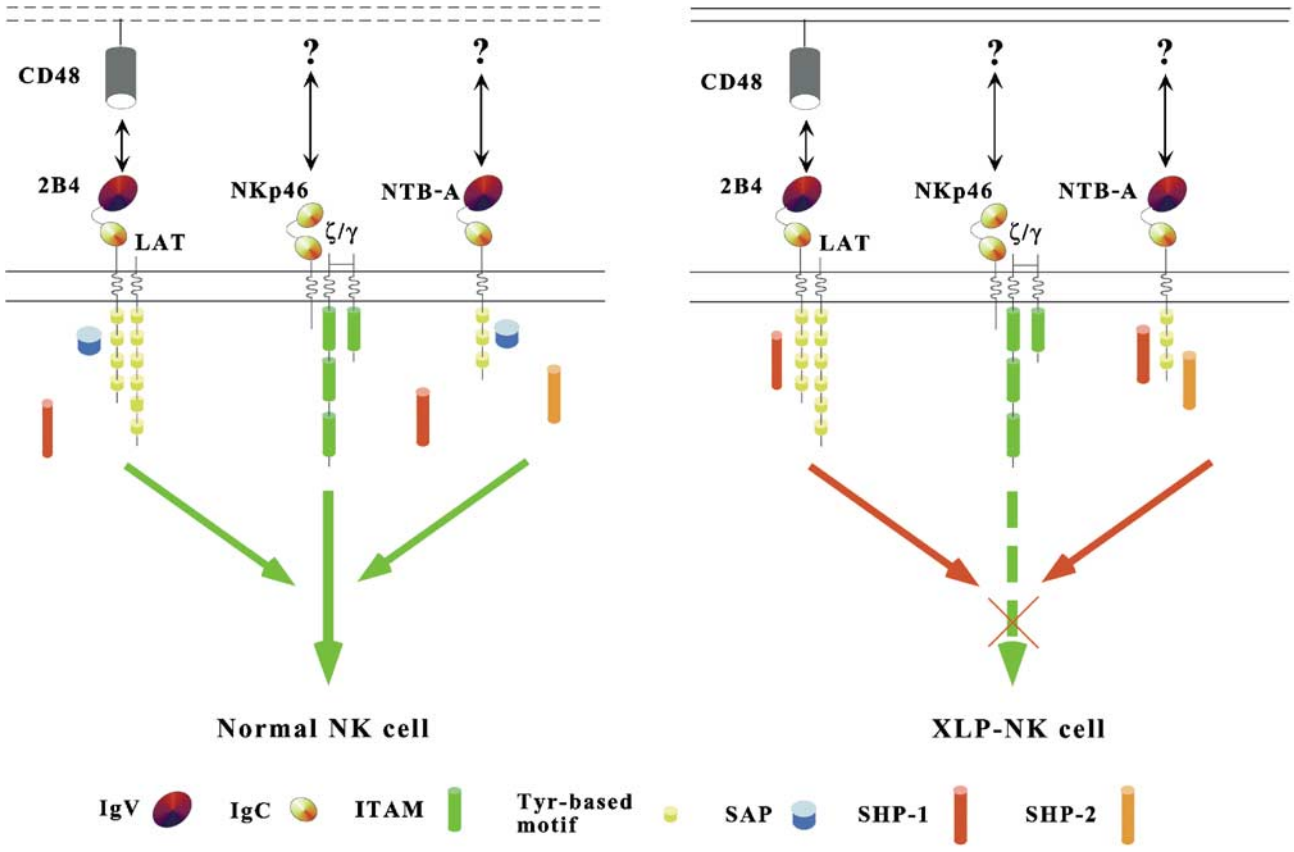

Figure 2 Altered function of 2B4 and NTBA surface receptors in XLP. In normal NK cells (left), both 2B4 and NTBA function as coreceptors cooperating with triggering receptors (here NKp46 is shown) to induce optimal NK cell activation upon interaction with EBV-infected B cells. B-EBV express high levels of CD48, the ligand for 2B4, as well as still undefined ligands for NKp46 and NTBA. NK cell triggering via 2B4 (upon interaction with CD48) requires the recruitment of SAP, an intracytoplasmic polypeptide, which likely prevents the generation of inhibitory signals mediated by SHP-1 phosphatase. The triggering signals delivered via NKp46 and amplified by 2B4 and NTBA lead to NK cell activation and induction of cytolysis of B-EBV cells. The molecular defect in XLP patients is represented by the lack of functional SAP molecules. As a consequence, the lack of their association with 2B4 and NTBA leads to recruitment and activation of SHP-1. This in turn extinguishes the triggering signals delivered via NKp46 or other activating receptors. As a result, NK cells fail to lyse EBV-infected B cells and/or to release cytokines relevant in the control of viral infection. In over 70% of patients, the clinical outcome is represented by fulminant infectious mononucleosis and death.

that activate NK cells. Indeed, in the absence of SAP, both molecules transduce inhibitory signals resulting in NK cell inactivation (Parolini *et al*, 2000; Bottino *et al*, 2001). This effect is due to SHP-1 phosphatase binding to the cytoplasmic domain of 2B4 or NTB-A (Figure 2). Thus, recruitment and activation of SHP-1, occurring in the absence of SAP, leads to dephosphorylation of downstream elements involved in NK cell activation. The devastating effects of the lack of SAP, due to critical mutation in the SAP-encoding gene, have been documented in the severe inherited immunodeficiency known as X-linked lymphoproliferative disease (XLP) (Parolini *et al*, 2000) (Figure 2). Genotypically affected males display apparently normal immunological functions until they are exposed to Epstein–Barr virus (EBV). This leads, in over 70% of such patients, to lethal infectious mononucleosis. In these patients, the interaction with EBV-infected cells (which express both CD48, that is, the 2B4 ligand, and the still undefined NTB-A ligand) induces NK cell inactivation and failure to lyse EBV-infected cells (Parolini *et al*, 2000; Bottino *et al*, 2001) (Figure 2). Importantly, blockage of 2B4 and NTB-A with specific mAbs leads to restoration of NK-mediated lysis of EBV cell lines, thus offering an important clue for the development of new therapeutic tools in the treatment of XLP patients (Bottino *et al*, 2001).

Another molecule that functions as a triggering coreceptor in NK cells was identified very recently as a result of attempts to identify the cellular ligands of triggering receptors. In this study, mAbs specific for putative surface ligands expressed on the cell surface of NK-susceptible target cells were selected on the basis of their ability to downregulate the NK-mediated cytolytic activity. The surface molecules recognized by the selected mAbs were found to be the Poliovirus receptor (PVR, CD155) and Nectin-2 (CD112), that is, two members of the nectin family (Figure 1). Analysis of the surface reactivity of PVR-Fc and Nectin-2-Fc soluble hybrid molecules indicated that both molecules represent specific ligands of DNAM-1 (Bottino *et al*, 2003) DNAM-1 is a transmembrane protein involved in lymphocyte adhesion and signaling, characterized by two extracellular Ig-like domains and by a cytoplasmic portion containing three tyrosine residues (Figure 1). In addition to NK cells, T cells, monocytes and a small subset of B lymphocytes also express the protein. As a result of mAb-mediated crosslinking, DNAM-1 transduces activating signals

resulting in tyrosine phosporylation of DNAM-1 itself, enhancement of cytotoxicity and cytokine production in both T and NK cells. The role of DNAM-1 in NK-mediated killing varies in the different target cells analyzed thus far, suggesting differences in the expression of the DNAM-1 ligands. Indeed, carcinomas and hemopoietic cell lines express PVR and Nectin-2, and their lysis involves DNAM-1. By contrast, most B-EBV cell lines analyzed do not express either PVR or Nectin-2, and their lysis does not involve DNAM-1 (Bottino *et al*, 2003). This correlation strongly suggests that PVR and Nectin-2 represent the major (if not the only) ligands of DNAM-1. As for other triggering receptors, the NK cell activation via DNAM-1 is controlled by HLA class I-specific inhibitory receptors. As a consequence, normal cells are usually protected from lysis. Interestingly, in normal, non-'stressed' tissues, molecules belonging to the nectin family are mostly unexpressed. However, they are localized in the intercellular borders of endothelial and epithelial cells. This specific localization may have relevant implication in the process of leukocyte recirculation in which leukocytes must pass between endothelial cells (as occurs during inflammatory responses).

The recognition of inducible self ligands by multiple receptors that mediate a coordinate activation of NK cells represents an important strategy that is frequently employed by the innate immune system. A similar strategy is utilized by dendritic cells (DC), which become activated upon engagement of an array of different Toll-like receptors, each responsible for recognition of distinct, pathogen-associated non-self ligands (Banchereau and Steinman, 1998; Beutler, 2003). Importantly, the recognition of inducible self ligands by NK cells together with the recognition of various non-self ligands by DC not only leads to the induction of potent effector mechanisms (mediated by cells of the innate immunity) but also contributes to shaping and/or regulation of adaptive immune responses.

Functional interactions between NK and DC, two important players in innate immunity

Recent data have highlighted the importance of NK/DC cooperation during the early phases of innate immune responses. These observations prompted the suggestion that NK cells may be involved in the editing process of DC. Thus, they would control the fitness of DC undergoing maturation before they migrate into secondary lymphoid compartments. This occurs thanks to the selective NK-mediated killing of DC still at an immature stage (Moretta, 2002). In parallel, in inflamed tissues, DC promote NK cell activation and enhancement of their cytolytic activity. A further interaction between NK and DC might take place in certain secondary compartments to which a subset of NK cells, expressing the chemokine receptor CCR7, migrate together with (CCR7 +) DC. The final outcome of these various NK/DC interactions is the selection of the 'most fitting' DC, that is, cells that are characterized by optimal expression of HLA molecules and by high levels of costimulatory ligands that will optimize their ability to prime T cells.

Thus, the availability of multiple activating receptors and coreceptors enables NK cells not only to sense the expression of distinct self ligands *de novo* expressed by potentially harmful cells but also to interact with particular normal cells including DC (via the NKp30 receptor) (Ferlazzo *et al*, 2002) or endothelial cells (via the DNAM-1 coreceptor) (Bottino *et al*, 2003). The expression of HLA class I in these potential target cells is likely to modulate the threshold of NK cell activation induced via triggering receptors.

Concluding remarks

Although many core questions regarding NK cell receptors have been answered in recent years, there are still relevant issues that need to be solved in order to better understand NK cell physiology. One major problem is with regard to the identification of the cellular ligands for the major triggering receptors (NKp46, NKp30 and NKp44) as well as for other receptors or coreceptors (see Figure 1). Available information is compatible with the concept that, similar to MICA/B, PVR or Nectin-2, they may also be represented by molecules primarily expressed by cells that have been 'stressed' by cytokine activation, proliferation, high temperature, viral infection or tumor transformation. If this holds true also for the ligands recognized by the major activating NK receptors, it would imply that two major checkpoints control NK cell activation, the first represented by the ligands for the various activating receptors that would be *de novo* expressed primarily by potentially harmful cells, and the second represented by MHC class I molecules and their interaction with the inhibitory receptors, which allows sensing the possible loss of MHC class I on cells. According to this hypothesis, NK cells would be activated only when needed, that is, when they encounter 'stressed' cells expressing inducible self ligands that, in addition, have lost MHC class I molecules. In an autologous setting, these cells always represent a danger that needs to be promptly removed.

Acknowledgements

We thank Mr Stefano Canu for editing the manuscript. This work was supported by grants awarded by Associazione Italiana per la Ricerca sul Cancro (AIRC), Istituto Superiore di Sanità (ISS), Ministero della Salute, Ministero dell'Istruzione dell'Università e della Ricerca (MIUR), Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST) and Compagnia di San Paolo.

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