



Review

The Social Life of NK Cells

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Abstract. Natural killer (NK) cells represent a distinct population of lymphocytes originally identified by its ability to kill transformed cell lines *in vitro*. It is now clear that these cells also play an important role in the innate immune response against a variety of pathogens, such as virus, bacteria and parasites. In the past few years, different protocols have been developed to activate NK cells *ex vivo*, allowing a detailed molecular analysis of the interaction of these cells with their cellular targets. NK activity is regulated by signals generated by both inhibitory and stimulatory receptors expressed by target cells. Indeed, recent results indicate that, while major histocompatibility complex class I molecules expressed on target cells inhibit NK lytic activity by engaging surface inhibitory receptors, costimulatory molecules, such as B7-1, B7-2 and CD40, are able to actively trigger NK activity. This review discusses the most recent findings on the role of costimulation on NK activation and foresees the possible consequences of the interaction between NK cells and dendritic cells on the development of an adaptive immune response.

Key words: NK cells; costimulation; dendritic cells; B7-1; B7-2; CD40.

More than Just “Killer Cells”

The spontaneous cytolytic activity observed in spleen cells of naive mice tested against the YAC-1 tumor cell line was the first indication of the presence of cells endowed with a “natural” killer activity^{23, 29}. More than 10 years later, KÄRRE²⁸ and LJUNGGREN and KÄRRE³⁷ proposed the “missing self” hypothesis, which suggested a link between natural killer (NK) cell function and major histocompatibility complex class I (MHC-I) expression. According to this hypothesis, NK cell-mediated killing occurs whenever NK cells interact with cells with aberrant or absent expression of MHC-I molecules. Indeed, experiments performed with transgenic and gene-deficient mice have provided conclu-

sive evidence that the expression of MHC-I molecules by target cells controls the specificity of NK activity²⁴. In addition to their role in eliminating cells not expressing the appropriate self MHC molecules, NK cells have also been implicated in the innate response against cells infected by viruses, intracellular bacteria and parasites^{6, 53}. These findings led to the idea that recognition and killing of target cells with absent or inappropriate expression of MHC-I molecules was the only ability of NK cells and that NK cells were exerting this function independently from the activity of other cells of the innate and the adaptive immune systems.

Since then, a cross-talk between NK cells and other components of the immune system has been suggested by the identification on the surface of activated NK

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cells of a number of interleukin receptors (i.e. IL-2R, IL-12R, TNF- α R) and by the fact that activated NK cells produce a variety of soluble factors, including IFN- γ , TNF- α , colony-stimulating factor (CSF), granulocyte-macrophage-CSF (GM-CSF), IL-3 and IL-5^{47, 61}. Many of these soluble factors are released by NK cells in response to other cytokines or upon contact with tumor targets. These results are suggestive of an active ongoing cross-talk of NK cells with other cells of the immune system and propose that NK activity can influence, and be influenced by, the surrounding innate or adaptive immune responses. Much evidence has indeed provided support to this hypothesis. For instance, NK cells have been shown to be attracted by CC chemokines, previously described as chemoattractants for monocytes, eosinophils, basophils and lymphocytes^{38, 50}, and the activity of NK cells was elicited by IFN- γ produced by α -galactosylceramide-activated NKT cells¹⁰. Moreover, NK cells were required for the *in vitro* generation of human alloantigen-specific cytotoxic T cells³¹ and were shown to play an important role in the down-regulation of T helper 1 (Th1)-mediated colitis by controlling the responses of effector T cells to gut bacteria¹⁷. Finally, a regulatory role for NK cells in experimental autoimmune encephalomyelitis⁶⁵ and in the development of allergen-induced eosinophilic airway inflammation^{30, 61} have been reported.

This article reviews the most recent data supporting the idea that the activity of NK cells can influence and be influenced by other cells of the adaptive and innate immune systems not only through soluble mediators³, but also through cell-to-cell contact.

Activating and Inhibiting NK Cells

The fact that NK cell activation results from a balance between positive and negative signals has been long established³³. It has become increasingly clearer that this is not exclusive of NK cells, but that this could also apply to other components of the immune system^{12, 25, 56}.

NK cells express surface receptors that bind to MHC-I molecules on target cells and, as a consequence, inhibit NK cell-mediated cytotoxicity. Several of these molecules with inhibitory function have been identified and cloned in humans and mice. Thus, human NK cells express different members of the Ig-like killer-inhibitory receptor (KIR) family^{33, 43}, which are clonally distributed and represented by circulating NK cells in healthy individuals. In addition, NK cells ex-

press the lectin-like receptor CD94, which forms disulfide-linked heterodimers with the NKG2 molecule⁷. In mice, the best characterized receptors with inhibitory function are those belonging to the Ly-49 family⁵⁸. In addition, the heterodimer CD94/NKG2 has also been found expressed by murine cells⁵⁹.

On the other hand, it has also been demonstrated that NK cells can be triggered by non-MHC-specific activating receptors. Indeed, several cell surface molecules, including NKp44, NKp46, CD16, CD27, NKR-P1, 2B4, LAG-3, CD2, CD69, and Ly-6^{48, 57, 60, 64} have been shown to activate both human and murine NK cells upon triggering. Finally, NK cells also express receptors that bind to MHC-I molecules on target cells that, instead of preventing NK cell activation, activate NK cells^{34, 41, 44}. The significance of these receptors remains, however, to be defined.

NK Cell Activation by Costimulatory Molecules

Even though mature NK cells clearly represent a distinct population of cells that play a unique role in the course of an immune response, many similarities are shared between NK cells and CD8⁺ T lymphocytes. Indeed, both cell types share the expression of several cell surface markers⁵⁵ and the ability to secrete certain cytokines⁴⁷, and both cell types use similar mechanisms to kill cellular targets⁵. In addition, it has been demonstrated that NK cells and T cells share a common thymic precursor⁴². Because of these striking similarities, it is reasonable to imagine that evolution would have selected similar molecular mechanisms to regulate the activation of these cell types. In particular, it would not be surprising to identify a role for costimulatory ligand/receptors during NK cell activation.

The function of costimulation has mostly been characterized during antigen-bearing APC-mediated CD4 and CD8 T lymphocyte activation. Thus, the binding of B7-1 and B7-2 molecules expressed by professional APC to the CD28 and CTLA-4 receptors expressed on the surface of T cells has been shown to determine the fate of T lymphocyte activation, cytokine production and proliferation³⁵. In addition to B7 ligands, CD40 expression by APC, which is induced upon T cell-APC interaction, is also important for T cell priming, for T cell-mediated effector functions^{4, 49, 52}, and during T cell-mediated antitumor responses^{18, 39}. Homologous molecules to B7^{14, 36} and CD28²⁷ with costimulatory function have been recently reported.

The first indication that costimulation was actively

involved in NK cell activation was obtained in the early nineties, when the human NK leukemia cell line YT¹ was found to kill mouse cells expressing human B7-1 and not the parental B7-negative cells. Blocking experiments performed with mAb inhibited B7-mediated cytotoxicity, indicating that CD28 expressed on the surface of the YT cells was directly involved. Recently, an increasing amount of experimental evidence has demonstrated the triggering of human and murine NK cells by B7-1^{13, 21, 62}, B7-2^{20, 40, 62} and CD40^{8, 40}. B7-mediated costimulation, not only augmented NK cytotoxicity, but other NK cell-mediated effector functions as well. Indeed, HUNTER et al.²⁶ showed that B7-transfected cells enhanced IL-12-induced IFN- γ production by activated CD28-expressing NK cells. Interestingly, this B7-mediated effect was dependent on ICAM-1/LFA-1 interaction and could be inhibited by TGF- β , but not by IL-10. In addition to cytokine production, B7/CD28-mediated costimulatory signals have also been shown to be required for optimal proliferation of murine NK cells⁴⁵.

Different lines of evidence suggest that costimulatory molecules might be involved in NK cell activation also *in vivo*. Infection of SCID mice with *Toxoplasma gondii* resulted in the appearance of CD28⁺ NK cells and a parallel increase of NK-derived IFN- γ production and NK cytolytic activity²⁶. This NK-mediated increased activity was dependent on CD28 triggering, since administration of CTLA-4-Ig (which prevents B7-CD28 interaction) to the infected mice inhibited IFN- γ secretion and resulted in a significant increase in parasite burden. In a different experimental model we obtained similar results. Indeed, the antitumor response induced by a B7-1-expressing adenocarcinoma TS/A cell line was diminished whenever NK cells were absent, suggesting that NK cells were playing an important role in the antitumor response¹¹. In parallel experiments, the role of B7-2 and CD40 was similarly established. In this setting, tumor cell lines expressing CD40 and B7-2 were labeled with⁵¹Cr, injected *in vivo* into naive syngeneic mice⁴⁰, and the radioactivity was measured in the lungs of inoculated mice 24 h after injection as an indication of NK activity. The amount of residual radioactivity was lower in those mice with respect to the levels detected in mice injected with B7/CD40-negative control cells, suggesting that B7 and CD40 expression was mediating cell clearance *in vivo*. This effect was abolished when the mice were pre-treated with anti-NK-depleting mAb prior to the tumor inoculation, suggesting that NK cells were indeed involved in the B7-mediated tumor cell elimination.

The NK Receptors for Costimulatory Molecules

Even though a role for costimulation has been identified in several NK-mediated processes *in vitro* and *in vivo*, it remains to be established whether NK cells express the same natural ligands for B7 (CD28/CTLA-4) and CD40 (CD40L) which have been characterized on T cells. Expression of CD28, CTLA-4 and CD40L on NK cells has been shown to be dependent on the maturation state of the cells, on the stimulus used to activate the cells, and on the origin of the NK cells. Indeed, although CD28 was found to be expressed on human fetal NK cells⁵¹, it was reported to be lost during maturation and was absent on cells obtained from adult peripheral blood. Moreover, FACS analysis and redirected lysis assays failed to detect CD28 and CTLA-4 expression on human NK cell lines generated from peripheral blood⁶². In mice, data regarding the expression of CD28 are controversial. Its expression has been reported to be inducible upon IL-2 stimulation on bone marrow-derived²⁶, but not on spleen-derived NK cells¹³. CD40L, on the other hand, has been reported to be expressed upon short-term culture of human NK cells⁸, but it was absent on resting or IL-2-activated murine NK cells⁴⁰.

Recent data have suggested that expression of CD28-related molecules able to bind B7 family members, but not be detected by the existing reagents, may explain the discrepancy between the function and the expression of B7-binding proteins. Indeed, in a recent publication, GALEA-LAURI et al.¹⁹ have reported that detection of CD28 expression on peripheral blood NK cells was dependent on the mAb used. Thus, 2 out of 4 anti-CD28 mAbs positively stained NK cells, whereas all 4 stained T cells, suggesting that NK cells may express a variant isotype of CD28 not recognized by some of the mAbs.

Finally, the level of expression of B7 ligands and CD28 receptor(s) may also contribute to the costimulation-sensitive NK activity. Indeed, we have observed that only transfectants expressing high levels of B7-2 were able to trigger NK-mediated cell killing⁴⁰, and the level of CD28 was shown to be variable among different individuals and on different human NK lines¹⁹. These observations might help to explain why, in some cases, the contribution of B7-CD28/CTLA-4- and CD40-mediated effect on NK cell function was not revealed^{32, 60}.

The existence of alternative receptors/ligands for B7 and CD40 was suggested by experiments performed with CD28 and CD40L knock-out (k.o.) mice. Thus, NK cells obtained from CD28 and CD40L k.o.

mice^{13, 40} were able to kill murine tumor cell lines expressing B7 and CD40 respectively, and not the untransfected parental cells. Furthermore, tumor cell lines expressing B7-2 and CD40 were eliminated upon inoculation in CD28 and CD40L k.o. mice with efficiency comparable to normal wild-type mice⁴⁰. Moreover, murine tumor cell lines transfected with the human CD40 molecule were specifically killed by NK cells generated from X-linked hyper-IgM patients, known to be defective of CD40L expression (RUGGIERO et al., unpublished observations). Altogether, these experiments do not exclude a role for CD28, CTLA-4 and CD40L on NK cell activity in normal conditions (k.o. animals have evolved in the absence of these molecules and could, therefore, have evolved compensatory mechanisms), but they strongly suggest that costimulation and, therefore, active triggering of NK activity may occur through alternative B7/CD40 receptors/ligands expressed by NK cells.

When NK Cells meet Dendritic Cell

Dendritic cells (DC) are believed to be the orchestrators of the adaptive immune response. These cells in the immature form are able to capture antigens in peripheral tissues, processes and represent them in the context of MHC molecules, migrate into secondary lymphoid organs and present the MHC-antigen complex to naive T cells².

Since DC constitutively express costimulatory molecules and NK cells can be triggered by such molecules, it is tempting to speculate that interaction between NK cells and DC might occur *in vivo* and influence the outcome of an immune response. Indirect data suggesting that NK cells and DC interact come from experiments performed in the mid-eighties by GILBERTSON et al.²². These investigators noticed that DC were unable to stimulate CD8⁺ T cell responses *in vitro* when splenocytes from mice treated with poly I : C, an NK activator, were added to the culture. These experiments suggested that, in the presence of activated NK cells, DC could not exert their function as APC, and that the DC were possibly killed by NK cells. Direct evidence for this possibility has been obtained recently. Indeed, bone marrow-derived macrophages and DC could be killed by murine autologous NK cells in *in vitro* assays^{13, 20}. Similarly, short-term-activated polyclonal human NK cells, as well as human NK lines, efficiently lysed autologous and allogeneic peripheral blood-derived DC^{9, 63}. In both studies, the susceptibility to lysis was a particular feature of immature DC. In-

deed, induction of maturation via different stimuli (anti-CD40 triggering, culture in the presence of TNF- α , LPS- or monocyte-conditioned medium, and infection with influenza virus) rendered the cells less susceptible to lysis. *In vitro* killing was in part mediated by the interaction between CD40 expressed on DC and CD40L expressed on NK cells, in one case⁸, and by triggering of receptors other than CD28/CTLA-4 in other cases^{13, 63}. Although these data need further investigation, they strongly suggest that DC are potential targets of NK cells.

The question of whether DC can influence NK cells upon contact has been addressed in recent reports in which the effect of the Flt3 ligand, a hematopoietic cytokine, was studied. Indeed, SHAW et al.⁵⁴ initially demonstrated that Flt3 ligand, in combination with IL-2, augmented the number and activity of murine liver NK cells. Subsequent reports have indicated that, in a model of liver metastases that depends on DC cells for tumor rejection, the antitumor effect of the Flt3 ligand was enhanced by IL-12 and greatly reduced by NK cell depletion⁴⁶. Similarly, FERNÁNDEZ et al.¹⁵ showed that Flt3 ligand-expanded DC adoptively transferred in mice bearing MHC class I-negative tumors promoted antitumor NK cell activity. These investigators also showed that the *in vitro* cell-to-cell contact between DC and NK cells augmented NK cell-mediated cytolytic activity and IFN- γ production by NK cells. Both mature and immature DC were used, but the surface molecules involved in this contact-delivered activity were not defined.

Concluding Remarks

A new aspect in the physiology of NK cells has emerged from recent studies in which the triggering of some NK cell-mediated effector functions occurs upon specific recognition of costimulatory molecules.

NK cell function has so far been regarded as dominated by inhibitory signals delivered to receptors expressed on NK cells by MHC-I molecules expressed by target cells²⁴. MHC-I molecules were, therefore, considered as the key elements able to turn off NK-mediated cell activity. Indeed, lost or aberrant expression of MHC-I alleles was correlated with tumor spread *in vivo*¹⁶ and, therefore, NK cells were thought to be the key players in the immune-surveillance against tumor growth. It was later determined that NK cells were also involved in the clearance of virus and parasite-infected cells^{6, 53}, and recently it has been shown that they are involved in autoimmune diseases⁶⁵.

The present review has presented new insights into the role of costimulatory molecules in NK cell triggering. Expression of costimulatory molecules by either tumor cell lines or by DC was shown to trigger NK-mediated cytotoxicity, cytokine production and NK proliferation *in vitro*, and expression of B7 or CD40 by transfected tumor cells to induce NK cell-mediated clearance *in vivo*. Since expression of the costimulatory molecules is restricted to cells that then determine the outcome of an immune response, it is tempting to speculate that NK cells could, at least in part, control ongoing adaptive immune responses. Some studies have indeed brought together NK cells and DC and have demonstrated that NK cells can kill DC *in vitro*. NK cells may, moreover, receive “positive” signals upon cell-to-cell contact with DC leading, for example, to an elimination of tumor cells *in vivo*, but at the same time they could deliver a “negative” death signal to the interacting DC. Whether this death signal occurs *in vivo* as well remains to be demonstrated.

In conclusion, the outcome of NK-mediated responses upon contact with target cells might depend on the kind of the receptors/ligands expressed by both cell types. Recognition of MHC-I molecules on target cells would prevent NK cell killer activity, while triggering through costimulatory molecules expressed on APC would induce NK lytic activity, cell proliferation and/or cytokine production. In the absence of MHC-I molecule expression, NK cells would be “released” from inhibition and allowed to kill the target. When both class I and costimulatory molecules are expressed on target cells and presented simultaneously to NK cells, the outcome of NK cell activation could depend on the balance between the two. It is equally possible that the inhibitory signals delivered through MHC-I recognition are overcome by the costimulatory signals or that triggering of NK activity via costimulatory molecules occurs independently from the expression of MHC-I on targets.

Where would NK cells “meet” DC *in vivo*? Would such interaction affect the priming of the adaptive immune response? Which would be the receptors/ligands involved? Given the increasing interest in understanding the multiple roles played by NK cells, we hope all these questions will find answers in the very near future.

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