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**NKG2D and DNAM-1 activating receptors and their ligands in NK–T cell interactions: role in the NK cell-mediated negative regulation of T cell responses**

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The negative regulation of adaptive immunity is relevant to maintain lymphocyte homeostasis. Several studies on natural killer (NK) cells have shown a previously unappreciated immunomodulatory role, as they can negatively regulate T cell-mediated immune responses by direct killing and by secretion of inhibitory cytokines. The molecular mechanisms of T cell suppression by NK cells, however, remained elusive. Only in the last few years it has become evident that, upon activation, human T cells express MICA–B, ULBP1–3, and PVR, ligands of the activating receptors NKG2D and DNAM-1, respectively. Their expression renders T cells targets of NK cell lysis, representing a new mechanism taking part to the negative regulation of T cell responses. Studies on the expression of NKG2D and DNAM-1 ligands on T cells have also contributed in understanding that the activation of ATM (ataxia-telangiectasia, mutated)/ATR (ATM/Rad3-related) kinases and the DNA damage response is a common pathway regulating the expression of activating ligands in different types of cells and under different conditions. The functional consequences of NKG2D and DNAM-1 ligand expression on activated T cells are discussed in the context of physiologic and pathologic processes such as infections, autoimmunity, and graft versus host disease.

Keywords: NKG2D ligands, DNAM-1 ligands, NK–T cell cross-talk, DNA damage response, cell proliferation

Natural killer (NK) cells contribute to the suppression of T cell responses and to the maintenance of T lymphocyte homeostasis through the release of inhibitory cytokines, such as TGF-β and IL-10, which can inhibit dendritic cell (DC) maturation or T cell activation and functions, and/or through the direct elimination of antigen-presenting cells and activated T cells (Andoniou et al., 2005; Schrama et al., 2006; Galazka et al., 2007; Qiao et al., 2008), monocytes and macrophages (Hamerman et al., 2004; Nowbakht et al., 2005; Nedvetzki et al., 2007; Kloss et al., 2008; Schulz et al., 2010), B cells (Nowbakht et al., 2005), and T cells. In general, NKG2DLs are not expressed by resting T lymphocytes, but their expression can be induced by different stimuli (Table 1). The first evidence of NKG2DL expression on T cells came from a study by Molinero et al. (2002) indicating that human T cells can express MICA in response to alloantigen and to CD3/CD28 cross-linking. Furthermore, also other NKG2DLs namely MICA, MICB, and ULBP1–3, but not ULBP4, are detected on both CD4+ and CD8+ T lymphocytes following stimulation with alloantigens, SEB superantigen, a specific antigenic peptide or upon PMA/ionomycin treatment (Corboni et al., 2007a, 2009). As a consequence, activated T cells become susceptible to autologous NK cell lysis, with an NKG2D/NKG2DL-dependent mechanism (Corboni et al., 2007a). Nielsen et al. (2012) further demonstrated that NKG2D, LFA-1, and Nkp46 are involved in NK cell degranulation triggered by activated autologous CD4+ T cells, with both subsets of human NK cells (CD56bright and CD56dim) equally cytotoxic. Expression of NKG2DLs was described also on regulatory T cells (Treg) in response to Mycobacterium tuberculosis and NK-cell mediated lysis of Treg involves both NKG2D and Nkp46 (Roy et al., 2008). Expression of NKG2DLs was also reported on activated murine T cells. H60 is up-regulated on T cells upon in vitro stimulation with ovalbumin and T cell blasts become susceptible...
to syngeneic NK cell killing (Rabinovich et al., 2003). Of note, an in vivo study showed that chronic antigenic stimulation of CD4+ T lymphocytes determined up-regulation of H60 and MULT1 ligands (Noval Rivas et al., 2010). NKG2DL expression was observed on thymocytes of BALB/c mice and was modulated during thymocyte development, suggesting a possible but yet undefined function in this process (Li et al., 2005). In support of these findings, a role for NK cell-mediated cytotoxicity during thymocyte development was demonstrated (Schott et al., 2003).

**DNAM-1 AND ITS LIGANDS**

DNAM-1/CD226 is an activating receptor belonging to the Ig superfamily and is constitutively expressed by most NK cells, T cells, macrophages, and DCs. DNAM-1 interacts with LFA-1, required for its functional activity on both NK and cytotoxic T cells (Shibuya et al., 1996). Ligands for DNAM-1 (DNAM1Ls) include Nectin-2/CD112 and PVR/CD155 belonging to the Nectin/Nectin-like family of adhesion molecules (Bottino et al., 2003; Pende et al., 2006). We have shown that PVR and Nectin-2 are induced on T lymphocytes in response to SEB stimulation at both the mRNA and protein levels, but only PVR can reach the cell surface (Ardolino et al., 2011). PVR expression on CD4+ T lymphocytes was also observed upon phorbolesters stimulation or plating on CD3/CD28 molecules (Cella et al., 2010; Nielsen et al., 2012; Table 1). DNAM-1/PVR axis is involved in the NK cell-mediated lysis of allogeneic activated T cells (Ardolino et al., 2011), while in an autologous setting, NKG2D emerges as the dominant receptor (Rabinovich et al., 2003; Cerboni et al., 2007a; Nielsen et al., 2012).

**ROLE OF THE DDR IN THE REGULATION OF NKG2D AND DNAM-1 LIGANDS ON ACTIVATED T LYMPHOCYTES**

NKG2DL expression is tightly regulated at various levels. During malignant transformation, cells undergo genotoxic or other forms of cellular stress with de novo expression of NKG2DLs. Gasser et al. (2005) demonstrated that murine and human NKG2DLs are up-regulated in fibroblasts by genotoxic stress and stalled DNA replication, conditions known to activate the DNA damage response (DDR) initiated by ATM (ataxia-telangiectasia, mutated) or ATR (ATM/Rad3-related) kinases. Studies aimed at investigating the signaling pathways leading to NKG2DL expression on antigen-activated T cells highlighted a role for the DDR as well. Treatment with ATM/ATR inhibitors blocked MICA induction on T cells with a mechanism involving NF-κB (Cerboni et al., 2007a), which regulates MICA expression on activated T lymphocytes by binding a specific sequence in the long intron 1 of the MICA gene (Molinero et al., 2004). NKG2DLs were found also on HIV-infected CD4+ T cells and their expression requires the activation of the DDR and stress pathways, via the HIV-1-encoded molecule Vpr (Ward et al., 2009; Richard et al., 2010; Pham et al., 2011), a potent activator of ATR and of a cell cycle arrest in G2 (He et al., 1995; Jowett et al., 1995; Re et al., 1995; Roshal et al., 2003). Similarly, oxidative stress and DDR strongly contribute to induce PVR expression on activated T cells (Ardolino et al., 2011). Thus, activation of ATM/ATR kinases and DDR could be a common pathway regulating the expression of different ligands on activated T lymphocytes.

**Table 1** | Different stimuli implicated in the induction or up-regulation of NKG2D and DNAM-1 ligands on activated T cells.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>NKG2DLs</th>
<th>DNAM1Ls</th>
<th>T cell type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>anti-CD3 plus anti-CD28</td>
<td>MICA/B, ULBP1-3</td>
<td>PVR</td>
<td>CD4+ T cells</td>
</tr>
<tr>
<td>Superantigen, allosantigen, PMA/ ionomycine, antigenic peptide</td>
<td>MICA/B, ULBP1-3</td>
<td>CD8+ T cells</td>
<td>Cerboni et al. (2007a)</td>
<td></td>
</tr>
<tr>
<td>Anti-CD3 plus IL-2</td>
<td>MICA</td>
<td>CD8+ T cells</td>
<td>Cerboni et al. (2009)</td>
<td></td>
</tr>
<tr>
<td>Superantigen</td>
<td>PVR, Nectin-2</td>
<td>Jurkat and activated</td>
<td>Cella et al. (2010)</td>
<td></td>
</tr>
<tr>
<td>PHA</td>
<td>PVR</td>
<td>Jurkat and activated</td>
<td>CD4+ T cells</td>
<td>Cella et al. (2010)</td>
</tr>
<tr>
<td>Prophetic acid</td>
<td>MICA/B</td>
<td>Jurkat and activated</td>
<td>T cells</td>
<td>Andresen et al. (2009)</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>ULBR-1</td>
<td>Treg</td>
<td>Roy et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>HIV</td>
<td>MICA, ULBR-1, 2, 3</td>
<td>Jurkat and activated</td>
<td>CD4+ T cells</td>
<td>Cerboni et al. (2007a), Ward et al. (2007)</td>
</tr>
<tr>
<td>HIV</td>
<td>PVR</td>
<td>Activated CD4+ T cells</td>
<td>Matsukai et al. (2012)</td>
<td></td>
</tr>
<tr>
<td>Mouse mHA antigen</td>
<td>H60, MULT1</td>
<td>Jurkat and activated</td>
<td>CD4+ T cells</td>
<td>Noval Rivas et al. (2010)</td>
</tr>
<tr>
<td>ConA, PMA/ionomycine, ovalbumin</td>
<td>H60</td>
<td>Jurkat and activated</td>
<td>T cells</td>
<td>Rabinovich et al. (2003)</td>
</tr>
</tbody>
</table>
activating ligands on T lymphocytes. Of note, we also found that genotoxic stress triggered ATM/ATR-dependent up-regulation of both DNAM1Ls and NKG2DLs on multiple myeloma cells (Soriano et al., 2009).

Increasing evidences show the involvement of DDR in many physiological processes, such as mitosis (Orcchio et al., 2006), insulin response (Yang and Kastan, 2000), V(D)J recombination (Chen et al., 2000) or after lipopolysaccharide stimulation in macrophages (Eissmann et al., 2010). In addition, up-regulation of ATM protein levels was observed in PBMCs (peripheral blood mononuclear cells) in response to mitogenic stimuli (Fukao et al., 1999). Increased phosphorylation of either ATM or one of its substrates, histone H2AX, was described on T cells upon CD3 triggering, PHA or SEB stimulation (Cerboni et al., 2007a; Tanaka et al., 2007; Ardolino et al., 2011). Remarkably, PVR and NKG2DLs expression was mainly observed on T cells that had gone through at least one mitosis (Cerboni et al., 2007a, 2009; Ardolino et al., 2011). This is only one of the numerous examples showing a correlation of either NKG2DL or PVR expression with cell proliferation. In murine bone marrow grafts, Rae-1 was detected on donor proliferating hematopoietic cells in the spleen of the transplant recipients rather than on the long-term hematopoietic stem cells (Ogasawara et al., 2005). The presence of MIC molecules on rheumatoid arthritis synoviocytes was strongly associated with the expression of the nuclear Ki-67 proliferation marker (Gob et al., 2003) and MIC gene promoter contains elements for cell proliferation-associated transcriptional activation (Venkataraman et al., 2007). A preferential expression of PVR on proliferating rat hepatocytes during liver regeneration and acute injury was previously described (Erickson et al., 2006). These authors also reported that PVR expression in epithelial cells was tightly regulated by changes in cell density. NK cells react more efficiently to concanavalin A-stimulated, proliferating MHC class I-deficient target cells than to non-activated cells in vitro and in vivo (Correa et al., 1994) and proliferating T cells become more susceptible to NK cell killing (Ardolino et al., 2011). In line with these results, Davis’s group reported that human NK cells bound to cells in mitosis more effectively than the same cells in other phases of the cell cycle (Nolte-'t Hoen et al., 2007). Thus, we envisage that the expression of PVR and NKG2DLs on proliferating T lymphocytes is a possible mechanism used by NK cells to restrict the expansion of activated/proliferating T cells (Figure 1).

**FIGURE 1** | NKG2D and DNAM-1 ligands are expressed on activated/proliferating T cells. Resting T cells do not express ligands of the activating receptors NKG2D and DNAM-1 and are resistant to NK cell-mediated killing. However, upon T cell activation triggered by different stimuli (listed in Table 1), as well as upon HIV-1 infection, MICA/B, ULBPs, LIRBP, and PVR become detectable on the cell surface, preferentially on T lymphocytes that were undergone at least one cell division. The signaling pathways regulating the expression of NKG2D and DNAM-1 ligands involve the activation of ATM/ATR and of their substrates (e.g., phosphorylation of the histone H2AX). The final outcome is the direct elimination of activated/proliferating T lymphocytes by NK cells.
IN VIVO RELEVANCE FOR NK–T CELL INTERACTION

VIRAL INFECTIONS

A plethora of studies analyzed the role of NKG2D/NKG2DL axis by looking at infected cells, which very often express one or more ligands. These studies have demonstrated that NKG2D plays an important role in anti-viral immunity, via a direct NK cell-mediated lysis of infected cells. This evidence is also underscored by the countermeasures taken by viruses to avoid NKG2D-mediated triggering (Lanier, 2008; Rossiini et al., 2012). However, NK cell contribution to anti-viral immunity can be seen also from another point of view: NK cells might restrain anti-viral T cell responses, thus promoting the return to T cell homeostasis.

In vivo depletion studies established that NK cells act to negatively regulate CD4+ and CD8+ T cell-dependent IFN-γ production and proliferation during murine cytomegalovirus infection, and they can mediate a similar effect on CD4+ T cell responses during lymphohytic choriomeningitis virus (LCMV) infection in β2-microglobulin deficient mice (Su et al., 2001). Accordingly, a more recent study showed that perforin-deficient mice chronically infected with LCMV contain greater numbers of activated anti-viral T cells compared to control animals. The accumulation of activated CD8+ T cells resulted in mortality within 2–4 weeks, an event which is rarely seen following an i.p. injection with LCMV of normal mice (Matsumi et al., 1999). It was described also a three-way of NK–T cell interaction, where NK cells directly eliminate activated CD4+ T cells (via a perforin-dependent pathway), thereby affecting CD8+ T cell function with beneficial or detrimental effects depending on the viral dose. However, no role for NKG2D/NKG2DLs could be observed (Waggoner et al., 2011). In another study, NK cell depletion promoted LCMV-induced CD8+ T cell immunity with the involvement of both perforin and NKG2D (Lang et al., 2012). Altogether, these studies show that NK cells can be crucial in controlling viral infections not only by a direct elimination of infected cells, but also by altering the number and functions of virus-specific T cells. However, the role of NKG2D and other activating receptors awaits a better elucidation.

Considering HIV-1, a virus replicating (among other cell types) in CD4+ lymphocytes, we face a situation where NK cell targeting of activated T cells via NKG2D means, at the same time, eliminating the infected cell. In fact, expression of several NKG2D ligands was observed on HIV-1 infected CD4+ T cells, with increased susceptibility to NK lysis (Cerboni et al., 2007b; Ward et al., 2007; Fogli et al., 2008; Richard et al., 2010). However, HIV-1 has also evolved its own countermeasures as it can also down-regulate NKG2DLs via Nef and Vif proteins (Cerboni et al., 2007b; Norman et al., 2011). Thus, regulation of NKG2DL expression by Vpu, Nef and possibly other viral proteins might have different impacts on NK cell recognition of infected CD4+ T cells.

The role of DNAM-1 and its ligands in the context of NK–T cell interactions during viral infections has been less investigated. Recently, PVR was detected on HIV-1 infected CD4+ T cells, and when the NKG2D pathway was inhibited, additional blocking of DNAM-1 strongly impaired the capacity of NK cells to kill HIV-1-infected cells, indicating the involvement of both receptors (Matusali et al., 2012). However, expression of PVR on CD4+ T cells might also be responsible for the down-regulation of DNAM-1 on CD8+ T cells observed in chronic HIV-1 infection (Cella et al., 2010).

AUTOIMMUNITY

The mechanisms by which NK cells modulate adaptive immune responses in the course of autoimmune diseases have been addressed by a large number of in vivo and in vitro studies. However, depending on the model system, NK cells might either promote or inhibit the generation and proliferation of autoreactive T cells (French and Yokoyama, 2004; Shi and Van Kaer, 2006; Fodstrom-Tullberg et al., 2009; Lunemann et al., 2009).

Thinking in terms of negative regulation of (autoimmune) T cell responses, NK cells might exert a direct effect on activated, autoantigen-specific T cells. In experimental autoimmune encephalomyelitis (EAE), in vivo depletion of NK cells exacerbated demyelination and the clinical features of EAE; in addition, in vitro studies have shown that direct NK–T cell contact inhibited T cell proliferation and cytokine production triggered by myelin-derived peptides (Zhang et al., 1997; Matsumoto et al., 1998; Stutz et al., 1998; Xu et al., 2003). NK cells might thus ameliorate the course of EAE by limiting the expansion of myelin-reactive T cells in the periphery and in the absence of their suppressive action, central nervous system inflammation became more marked. However, these studies are in conflict with another report showing that NK cell depletion resulted in less severe clinical scores (Shi et al., 2000).

In an in vivo model of colitis, NK cell-depleted animals developed accelerated disease, and it was suggested that NK cells inhibited effector CD4+ T cells in a perforin-dependent manner (Fort et al., 1998; Yamaji et al., 2012). Such a protective effect also occurred in Staphylococcus aureus- and collagen-induced arthritis (CIA; Nils- son et al., 1999; Leavenworth et al., 2011), as well as in NOD mice (Lee et al., 2004).

A number of studies have identified the cytolytic mechanism underlying NK cell-mediated killing of autoreactive T cells, and the NK cell-mediated immunoregulatory activity was shown to be perforin-dependent in animal models of colitis, EAE, and CIA (Fort et al., 1998; Lu et al., 2007; Leavenworth et al., 2011). Thus, the receptor/ligand interactions triggering a perforin-mediated cytotoxicity play a key role in controlling T cell responses, and NKG2D might be part of the picture, since it plays a major role in NK cell lysis of autologous activated T cells (Rubinovich et al., 2003; Cerboni et al., 2007a; Nielsen et al., 2012). Moreover, NK cells can lyse autologous DCs, that under certain circumstances – including EAE – express NKG2DLs (Imashiki et al., 2003a,b; Andonisou et al., 2005; Schrama et al., 2006; Galka et al., 2007; Qiao et al., 2008). These data, together with NKG2DL expression also on activated macrophages and monocytes, bone marrow cells and microglia (Lunemann et al., 2008), indicate that this receptor/ligand pair might play a more general immunoregulatory role besides killing autoreactive T cells, e.g., by eliminating macrophages and other antigen-presenting cells or their precursors under inflammatory conditions, as a feedback mechanism to silence uncontrolled antigen-specific immune responses.

The role of NKG2D/NKG2DLs in autoimmunity has been addressed also from another point of view. In fact, endogenous cells and/or tissues can aberrantly express NKG2DLs (as shown in...
particular for MICA and MICB in humans and Ra-e-1 in mice), promoting activation of autoreactive infiltrating NKG2D+ T cells, leading to tissue destruction. Examples of this condition can be found in human type 1 diabetes and in NOD mice, in patients with rheumatoid arthritis, Crohn’s disease, celiac disease, and in a mouse model of autoimmune vitiligo. These aspects are however reviewed elsewhere (Shi and Van Kaer, 2006; Van Belle and van Herrath, 2009).

Regarding DNAM-1, despite the expression of PVR on activated T cells (Cenci-Bolognetti et al., 2011), its role was not evident in autologous NK–T combinations (Arvidson et al., 2011; Nielsen et al., 2012), while it was relevant in allogeneic settings (Arvidson et al., 2011), suggesting that DNAM-1 might not be involved in autoimmune reactions.

**GRAFT VERSUS HOST DISEASE**

Allogeneic bone marrow transplantation (BMT) was estimated to be an effective treatment for hematologic malignancies and some solid tumors. However, the high incidence of graft versus host disease (GVHD) mediated by the activation and proliferation of alloreactive T cells leads to severe host tissue damage. Previous studies demonstrated that donor NK cells are able to suppress the development of GVHD through the killing of host antigen-presenting cells which are essential for donor T cell activation (Buggeri et al., 2002). More recently, several in vivo studies in the mouse showed a direct effect of donor NK cells on GVHD-inducing T cells. Allogeneic T cells and NK cells trafficking similarly after BMT (Olson et al., 2009) and donor NK cells limited the expansion of syngeneic donor T cells through different mechanisms mediated by perforin and Fas-FasL interaction (Olson et al., 2010). Similarly, in a model of chronic GVHD, the killing of CD8+ cells limited the expansion of syngeneic donor T cells through NKG2D and DNAM-1 regulation of the activating receptor NKG2D ligands released by activated T cells. Blood 113, 2905–2914.

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