CD100 on NK Cells Enhance IFNγ Secretion and Killing of Target Cells Expressing CD72

Sa’ar Mizrahi1, Gal Markel1, Angel Porgador2, Yuri Bushkin3, Ofer Mandelboim1,*

1The Lautenberg Center for General and Tumor Immunology, Hebrew University-Hadassah Medical School, Jerusalem, Israel, 2Department of Microbiology and Immunology, Faculty of Health Sciences, The Cancer Research Center, Ben Gurion University, Beer-Sheva, Israel, 3Public Health Research Institute, NJMS-UMDNJ, Newark, New Jersey, United States of America

Background. NK cells are able to kill tumor and virus-infected cells without the need of prior antigen stimulation. The killing of these target cells is regulated by inhibitory, lysis and co-stimulatory receptors that are expressed on the surface of NK cells. Principal Findings. CD100 (Semaphorin 4D), a 150kD transmembrane protein, is expressed on the surface of activated NK cells as a homodimer, mediates the killing of target cells by binding to CD72. CD100 is not involved directly in the killing process but is rather increases NK cytototoxicity by enhancing the adhesion between NK cells and their targets. This increased adhesion leads to a more efficient killing and enhanced IFNγ secretion. Significance. Since CD72 is expressed on antigen presenting cells (APC) and the CD100-CD72 interaction lead to the shading of CD100, we suggest that NK interacting with APC cells could be the early source of soluble CD100 which is crucial for the formation of antigen specific immune response. CD100-CD72 interaction can be the mechanism by which NK cell communicate with B cells.

INTRODUCTION

NK cells are part of the innate immunity system and are able to kill tumor and virus-infected cells that have lost, in most cases, class I MHC protein expression [1]. The recognition of MHC class I proteins by NK inhibitory receptors leads to inhibition of NK killing and in the absence of MHC class I proteins, these inhibitory constraints are removed and NK cytocytotoxicity is restored[2–9]. In recent years it was realized however that NK cytototoxicity is much more complicated [10] and that the killing of NK cells is also controlled by activating receptors, among these are Natural Cytotoxicity Receptors (NCRs), NKGD2D, CD16 (low affinity FcyRIII), 2B4 and NKp80 [11]. NK cells are also capable of producing cytokines, including TNFα, GM-CSF, and a large quantity of IFNγ. IFNγ affect many cellular responses, including the control of viral replication, up-regulation of MHC class I and class II protein expression and activation of macrophages. It can also direct the adaptive immune responses towards the Th1 type [12].

The semaphorins which are characterized by “Sema” domain (~500 A.A.) in their extracellular region were initially recognized for their role as chemorepellents during neural development [13]. The semaphorin CD100 is the first semaphorin to be found on the surface of immune cells[14–16] and is the best semaphorin characterized so far [15,17–20]. Membrane bound CD100 is a 150-kDa trans-membrane protein, express as a homodimer [14–16] with high levels of expression both in lymphoid organs such as thymus, spleen and lymph node, and on non-lymphoid organs such as brain, kidney and heart [14,15,21]. On hematopoietic cells it can be found on resting T cells, B cell, macrophages, dendritic cells (DC) and its expression is up-regulated significantly after cellular activation [15,16,22,23]. CD100 can be cleaved from the membrane to form a functional soluble homodimer in the size of 240-kDa [24–26]. Two distinct receptors were identified for CD100: plexin-B1, which is the high affinity receptor for CD100, is found on many tissues with high levels of expression in the fetal brain and kidney [27]. The low affinity receptor for CD100 is CD72, the major receptor for CD100 in immune cells [22]. CD72 is expressed during all stages of B cell maturation, except for plasma cells [22], and is also expressed on other antigen presenting cells such as dendritic cells and macrophages [28,29].

CD100 has many biological activities in the immune system. It enhances B cells response to stimulation with CD40 and LPS both in vitro and in vivo [15,16,22,23,30]. B cells derived from CD100-/- knockout mice demonstrate a reduction in B cell activity and antibodies specific to T cell dependent (TD) antigens [31]. In contrast transgenic mice expressing functional soluble CD100 demonstrate the reverse pattern [32].

CD100 has also been found to have an important function in DC. CD100-deficient mice were resistant to autoimmune diseases models such as experimental autoimmune encephalomyelitis (EAE) [28] and immune complex glomerulonephritis (ICG) [33]. This effect was due to the lack of proper mature DC in the CD100 knockout mice. In human monocytes, soluble CD100 inhibits migration and induce the production of pro-inflammatory cytokines [28] and inhibit their migration [26,34]. Here, by screening for novel antibodies that affect NK killing we identified a new mAb that recognizes CD100. Using this mAb we demonstrate a novel role for CD100 in the augmentation of NK killing and cytokine secretion.

Citation: Mizrahi S, Markel G, Porgador A, Bushkin Y, Mandelboim O (2007) CD100 on NK Cells Enhance IFNγ Secretion and Killing of Target Cells Expressing CD72. PLoS ONE 2(9): e818. doi:10.1371/journal.pone.0000818

Academic Editor: Derya Unutmaz, New York University School of Medicine, United States of America

Received April 26, 2007; Accepted August 5, 2007; Published September 5, 2007

Copyright: © 2007 Mizrahi et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was supported by grants from the US-Israel Bi-national Science Foundation, by a grant from The Israeli Cancer Research Foundation, by a grant from the Israel Science Foundation, by a grant from the AICR, by grants from the European consortium MRTN-CT-2005 and LSCH-CT-2005-518178, and by NIH AI 045761 for Yuri Bushkin.

Competing Interests: The authors have declared that no competing interests exist.

* To whom correspondence should be addressed. E-mail: oferm@ekmd.huji.ac.il
RESULTS

Identification of 172.4 mAb which recognize ligand that is up-regulated after activation of NK cells

Several NK receptors such as NKp44 on NK [33] and NKG2D on T cells [36] are upregulated after activation. Based on that fact, our assumption was that the level of other yet unknown NK activating receptors is also up-regulated after NK cell activation. In order to identify such receptors we immunized mice with the NK cell line-YTS. 2000 hybridomas were obtained and the supernatants of these hybridomas were tested for the binding to IL-2 activated and non-activated peripheral blood lymphocytes (PBL) by flow cytometry. Increased binding to IL-2 activated PBL compared to fresh PBL was observed with the supernatant of hybridoma 172 (data not shown). Hybridoma 172 was re-cloned and a mAb was purified from the hybridoma sub line named 172.4. Supernatants from 172.4 were tested in binding to different sub-population of activated and non-activated PBL. As shown in Figure 1, 172.4 mAb recognizes a sub-population of resting NK, B and T cells (figure 1 A, C, E). A dramatic increase in the binding of 172.4 to all tested populations was observed after 72 hr of activation with IL-2 (figure 1 B, D and F). Almost all activated NK cells express high levels of the ligand for 172.4 (93% in figure 1B).

To further characterize the 172.4 expression on NK cells, we activated the NK cells with different stimuli. The two main NK populations in the blood are CD16\(^+\), CD56\(^{bright}\) and the CD16\(^-\), CD56\(^{dim}\). They differ both in function and in expression of different molecules on their surface [37,38]. Fresh PBMC were incubated with different stimulates (50 ng/ml) for 72 hr and the expression of 172.4-ligand on NK cells was analyze by flow cytometry. As can be seen in figure 1G enhancement of 172.4-ligand expression was observed only after incubation with IL-12 and more pronouncedly after incubation with IL-15. Both cytokines are well known activators of NK cells [39,40]. In contrast, incubation with IL-8, IL-6 and TNF\(\alpha\) had no effect. IL-12 stimulation of NK cells resulted in modest elevation of the 172.4-ligand on CD16\(^-\) NK cells (median fluorescence intensity (MFI) 90.2 verses 60.4 for the negative control) but not on CD16\(^-\) cells (MFI of 59.9 verses 53.3 for the negative control). IL-15 treatment resulted in marked 172.4-ligand expression on both populations (MFI of 235 and 269 in CD16\(^-\) and CD16\(^{+}\), respectively).

Thus, the protein recognize by 172.4 mAb is elevated in a response to a variety of activation stimuli which include IL-12, IL-15 and IL-2 and therefore can serve as a marker of NK activation.

The ligand recognized by 172.4 mAb is not directly involved in the killing of target cells by NK cells

To test whether the protein recognized by 172.4 mAb is involved in the regulation of NK cytotoxicity we performed lysis assays. IL-2-activated NK cells were pre-incubated with various mAbs, indicated in figure 2A, and then subjected to lysis assay with \(^{35}\)S-labeled 721.221 or RPMI 8866 as target cells. No differences in the lysis of 721.221 (figure 2A) or RPMI 8866 (data not shown) were observed regardless of whether the control 12E7 mAb or 172.4 mAb were included in the assay. We concluded that the protein recognized by mAb 172.4 is not directly involved in killing of 721.221 or RPMI 8866 cells. However, it is still possible that the protein is involved in a different way.

Figure 1. Expression of the ligand of 172.4 on lymphocyte sub-populations and NK line. Biotinylated 172.4 mAb was used in combination with other fluorescently labeled mAbs in a four color staining. 172.4 staining was detected using Cy-5 streptavidin. Cells were analyzed by flow cytometry. T cells were identified as CD3 positive, NK cells as CD56 positive, CD3 negative, and B cells as CD19 positive. Staining was performed on freshly isolated PBL (A, C and E) and on PBL that were cultured for 72 hr in the presence of IL-2 (100 u/ml) and human serum (B, D and F). Each dot blot shows a gated specific sub-population. An isotype matched antibody was used to determent the background staining for each antibody and is represented in the figure as the horizontal line. (H) Freshly isolated PBMC were incubated for 72 hr with 50 ng/ml of the indicated cytokines. Biotinylated 172.4 mAb was detected using Cy-5 streptavidin and used in combination with other fluorescently labeled mAbs in a four color staining. The PBL were analyzed for the expression of CD100 on NK cells and each dot plot represents a gated NK cells (CD3-, CD56\(^{+}\)). Figure shows one representative experiment out of four performed.
Figure 2. Killing of target cells is not influence by mAb 172.4. (A) 35S-labeled 721.221 cells were pre-incubated with the indicated mAbs and tested for lysis by NK cells at different effectors to targets (E:T) ratios as indicated in the figure. (B) 35S-labeled P815 cells were pre-incubated with the indicated mAbs and tested for lysis by NK cells at the indicated E:T ratios. (C) 35S-labeled P815 cells were pre-incubated with the combination of the indicated mAbs and tested for lysis by NK cells. E:T ratio of 10:1 is presented. Figure shows one representative experiment out of four performed. doi:10.1371/journal.pone.0000818.g002

protein recognized by mAb 172.4 is involved in the killing of other cells by NK cells. We, therefore, tested whether this protein is involved in the regulation of NK cell-mediated cytolysis by performing re-directed lysis experiments (figure 2B and 2C). 35S-labeled P815 cells were incubated with different mAbs, as indicated in figure 2B, and then incubated with NK cells. As above, no differences were observed when the control mAb HC10 or mAb 172.4 were included in the assay. Thus, the protein recognized by 172.4 is not directly involved in NK cytolysis. Next, we tested whether the protein recognized by mAb 172.4 may act as co-stimulatory molecule on NK cells. P815 cells were pre-incubated with increasing concentrations of the anti-CD16 mAb B73.1.1 combined either with mAb 172.4 or control mAb 12E7, and then incubated with the NK cells. Efficient lysis of P815 cells was observed even when cells were incubated with 0.02 μg/ml of B73.1.1 mAb (figure 2C). As above, no differences in lysis of P815 cell were observed when either mAb 12E7 or 172.4 mAb were included in the assay together with B73.1.1. Thus, the protein recognizes by mAb 172.4 is unlikely to be directly involved in the regulation of NK cell cytotoxicity.

The protein recognized by mAb 172.4 is CD100
To identify the protein recognized 172.4, we immunoprecipitated the protein with mAb 172.4 from lysate of 125I-labeled YTS cells and analyzed it by two-dimensional (non-reducing/reducing) SDS-PAGE gel. As shown in figure 3A, mAb 172.4 immunoprecipitated a major protein of ~150kDa under reducing conditions. This protein runs as a broad band of about 150–300kDa under non-reducing conditions (data not shown), thus implying that the 150kDa protein recognized by mAb 172.4 could form homodimers (figure 3A). The minor ~120kDa protein band, under reducing condition, that was also precipitated by mAb 172.4 might be a proteolytically cleaved fragment (while the other minor 33kDa protein band is non-specific). A data bank search suggested that the protein recognized by 172.4 might be CD100. CD100 is a 150kDa protein expressed as 300kDa homodimer protein on the surface of T cells and B cells [14,16,25,41]. A soluble form, a homodimer composed of two subunits of 120kDa each has also been described [25,42].

To test whether the 172.4 mAb recognizes CD100 we first performed flow cytometry analysis of various cell types, using both 172.4 and the commercial anti-CD100 mAb A8 and observed identical staining patterns (data not shown). To prove that 172.4 recognize CD100 we used a fusion protein, composed of the extracellular region of human CD100 fused to Fc region of human IgG1 (CD100-Ig). We produced the CD100-Ig protein in COS-7 cells as previously described [43–45] and performed ELISA with increasing concentrations of CD100-Ig and CD99-Ig (used as control) fusion proteins. Figure 3 shows that both the commercial anti-CD100 mAb A8 (figure 3B) and 172.4 (figure 3D) recognized CD100-Ig but not CD99-Ig, whereas 12E7 recognized only the CD99-Ig (figure 3C).

To further verify that mAb 172.4 recognize human CD100 we compare the staining of human NK line by 172.4 mAb to that of the commercial mAb A8 (figure 3E). All activated NK cells were stained positive for both antibodies and demonstrated an identical pattern of staining for both antibodies.

We therefore concluded that mAb 172.4 recognize the protein known as CD100.

CD100 on NK cells enhance the killing and IFNγ secretion after incubation with targets expressing CD72
Human CD100 interacts with CD72, originally identified as a B cell differentiation antigen [22]. To test whether the CD100-CD72 interaction will affect NK cytotoxicity we generated BW cells stably expressing human CD72 (BW/CD72). BW cells were chosen because their lysis by NK cells is relatively low and this allows small differences in killing to be detected. IL-2 activated NK cells killed more efficiently (up to 64% more at E: T ratio of 5:1) BW cells expressing human CD72 then parental BW (Figure 4A). Blocking of the interaction between CD100 and CD72 by pre-incubating the target cells with CD100-Ig reduced the killing compared with the control fusion protein CD8α-Ig (figure 4B), indicating that the enhanced killing is due to CD100-CD72 interaction. In contrast, pre-incubation of BW cells with either CD100-Ig or CD8α-Ig did not influence the killing of BW cells by NK cells (figure 4C). Thus, CD100 is involved in NK cytotoxicity probably as an adhesion molecule that enhancing the killing of different target cells expressing CD72.

To test whether CD100-CD72 interactions would modify cytokines secretion, freshly isolated NK cell were incubated with
BW or BW/CD72 cells in the absence or presence of NK activating cytokines for 72 hr (figure 4D). Fresh NK cells incubated with BW or BW/CD72 cells did not secrete IFNγ (figure 4D). However, when the (NK) activating cytokines were added to the assay, NK cells that were incubated with BW/CD72 secreted significantly higher amount of IFNγ as compare to NK cells incubated with BW cells.

Figure 3. 172.4 recognize CD100, a 150kDa protein expressed as a homodimer. (A) Surface radiiodinated YTS cell lysate was immunoprecipitated with mAb 172.4. The immunoprecipitate was analyzed first on non-reducing and then on reducing SDS-PAGE. The left lane is an aliquot of immunoprecipitated proteins resolved under reducing conditions. Molecular weight markers are as shown. The two forms of CD100 (150 and 120 kDa respectively) are indicated with arrows. (B, D) ELISA plates were coated with CD100-Ig and assayed for binding to the indicated antibodies as described in "Materials and Methods". (C) ELISA plates were coated with CD99-Ig and assayed for binding to the indicated antibodies as described in "Materials and Methods". The background binding to BSA was subtracted. Figure shows one representative experiment out of four performed. (E) IL-2 activated NK line was stained with either 172.4 or the commercial anti human CD100 antibody A8. FITC conjugated Goat F(ab') anti mouse IgG antibody was used as secondary Ab. Gray histogram is staining with secondary antibody only. Figure show one representative experiment out of 5 performed.

doi:10.1371/journal.pone.0000818.g003
This phenomenon was restricted to IFNγ, because no differences in the secretion of TNFα or GM-CSF were observed when NK cells were incubated either with BW or BW/CD72 (data not shown).

CD100 on NK cells is an adhesion protein

To verify that CD100 function as an adhesion molecule on NK cells we performed an adhesion assay in which BW or BW/CD72 cells were incubated on 96 flat bottom plate for two hours at 37°C. 35S-Met labeled-IL-2-activated NK cells were then added to the plate, washed and radioactivity was measured. As shown in figure 5A, NK cells adhere more strongly to BW cells expressing CD72 compared with BW cells. Blocking CD100-CD72 interaction by pre-incubating BW/CD72 with CD100-Ig reduce the adhesion between the NK cells to the target cells (figure 5B). To further confirm that CD100 is an adhesion molecule on NK cells, we performed signaling experiments. IL-2-activated NK were incubated with BW/CD72 or BW cells for short period of time and then lysed. CD100 was immunoprecipitated with 172.4 mAb. The proteins were eluted under reduce condition and separated on an SDS-PAGE. Western blot analysis with antibody directed against phosphorylated serine residues revealed that cross linking of CD100 by CD72 led to an increase in phosphorylation on serine residues in three unknown proteins that are associated with CD100 (indicated by arrows, figure 5C). In contrast, when NK cells were incubated with BW cells the level of serine phosphorylation did not change or even slightly decreased. To confirm that equal levels of proteins were precipitated in the various treatments, we re-blot the membranes with anti CD100 mAb A8. As shown in figure 5C the level of CD100 is equal in all the lanes. Thus, the changes observed in the levels of serine phosphorylation are genuine. These results were confirmed by the quantification of the level of phosphorylation using densitometrical analysis (figure 5D).

Thus CD100 on activated NK cells strengthens their binding to target cells expressing CD72. The interaction with CD72 leads to increase in serine phosphorylation found in unknown proteins associated with CD100.

DISCUSSION

By immunization of mice with the NK cell line YTS we were able to isolate one mAb named 172.4 that recognized the CD100 protein. The expression of CD100 is up-regulated on the surface of NK cells after their activation with IL-2, IL-12 and IL-15 (figure 1), implicating CD100 as activation marker for NK cells. CD100 can also be used to distinguish between the states of activation of the CD16+ and CD16− NK cell populations. Activation of NK cells with IL-12 elevated the level of CD100 on CD16+ but not on CD16− NK cells compare with IL-15 which elevated the level of CD100 on both populations (figure 1).
We demonstrated that CD100 is not a lysis receptor but could instead enhance the killing and cytokine secretion by interacting with its ligand CD72. The interactions between CD100 and CD72 might be important in killing of transformed cells such as leukemia of B cell origin, which express CD72 on their surface [29,46–48].

It has long been established that NK cells can interact directly with dendritic cells [49] and macrophages [50]. It is known that NK cells not only influence DC function via cytokine secretion but also via direct killing [51]. Since CD72 is expressed on antigen presenting cells like B cells, macrophages and dendritic cells, the interaction with NK might also influence the function of these cells via the CD100-CD72 interactions. Of a special note is the possible cross-talk between NK cells and B cells. The fact that NK express both CD100 and CD40L and the B cells express their counterpart CD72 and CD40 imply that these cells may interact directly. It was demonstrated that soluble CD100 (sCD100) is crucial for the development of Ag-specific immune responses to T cells-dependent (TD) Ag [28,32,33]. The immune responses can be restore in these mice by applying sCD100 [28,32]. But what could be the early source of sCD100? Both T cell and B cell, express CD100 but they can only release sCD100 after their activation [15,16,22,23] and thus could not be the initial source for sCD100. This raises the possibility that another cell, probably belonging to the innate system is responsible for early release of CD100.

We demonstrate here that NK can produce sCD100 form (~120kDa, figure 3A) and also observed that IL-2-activated NK cells secrete sCD100 (data not shown) and thus we suggest that NK cells are the early source for CD100. We propose the following model. NK cells are activated at the infection site meet APC and secrete sCD100. They interact with APC by several routes among which is the CD100-CD72 interactions. Such interactions lead to IFNγ and sCD100 secretion which in turn activates other cells of the immune system.
MATERIALS AND METHODS

Cells and transfections
The cell lines used were the MHC class I-negative human EBV-transformed B cell line 721.221, simian kidney cells COS-7 and the z/β TCR-negative mouse thymoma BW. Cell lines were grown in complete RPMI-1640 or DMEM (supplemented with 10% FBS, 1 mM L-glutamin, 1 mM penicillin-streptomycin, 1 mM non-essential amino acids and 1 mM sodium pyruvate, Gibco, Minneapolis, MN). NK cells were isolated from peripheral blood mononuclear cells (PBMC) using the human NK cell isolation kit and an autoMACS instrument (Miltenyi Biotec, Bergisch-Gladbach, Germany). NK cells were grown as previously described [43–45]. BW cells were transfected with pcDNA3.1/V5-His-TOPO (Invitrogen, Carlsbad, CA) containing the cDNA of human CD72 (BW/CD72). BW/CD72 cells were grown in complete medium RPMI-1640 supplemented with 5 mg/ml G418 (Gibco). The Human CD72 gene was amplified from 721.221 cells using the 5’ primer (with Kozak and Kpn I site): GGTACCAGGGCGGACCATGCTGAGGCCATCACCTATG and the 3’ primer (with Xba I site): GCTCTAGAGCTTAAATCTGGAAACCTTGAGAAGCTAT. The hCD72 was inserted into the plasmid with Xba I site: GCTCTAGAGCCTAATCTGGAAACCTTGAGAAGCTAT. The 5

Immunoresearch, West Grove, PA).

used as first Ab and then Cy-5 conjugated streptavidin (Jackson Laboratories, Inc.) precoated with irrelevant mouse IgG. Aliquots of precleared lysates were then immunoprecipitated overnight at 4°C with protein A coupled sepharose beads (Zymed Laboratories, Inc.) precoated with irrelevant mouse IgG. Aliquots of precleared lysates were then immunoprecipitated overnight at 4°C with protein A coupled sepharose beads precoated with mAb 172.4. Immunoprecipitated samples were washed four times with lysis buffer and proteins were eluted in the presence of SDS under reducing conditions. The TOPO TA expression Kit (Invitrogen).

Monoclonal antibodies used in this work were mAb B73.1.1, directed against CD16 (IgG1), A8 directed against CD100 (Serotec, Oxford, U.K), 3F3 directed against CD72 (Serotec), 12E7 directed against CD99 (IgG1), HC10 directed against the free heavy chain of MHC class I and mAb 172.4 directed against human CD100 (IgG1). For flow cytometry analysis the following Abs were used: PE-conjugated mouse anti-human CD19 (BD PharMingen, San Diego, CA), Cy-Chrome-conjugated mouse anti-human CD3 (BD Pharmingen), FITC-conjugated mouse anti-human CD56 (Serotec), FITC-conjugated mouse anti-human CD16 (Dako, Glostrup, Denmark), 172.4 mAb bionytinated was used as first Ab and then Cy-5 conjugated streptavidin (Jackson Immunoresearch, West Grove, PA).

Cytotoxicity assays
The cytotoxic activity of NK cells against the various targets was assayed in 3-hr 35S-release assays, as described previously [43–45]. In all presented cytotoxic assays, the spontaneous release was less than 20% of maximal release. In some assays the NK cells were pre-incubated with 0.5 µg/well of each antibody 1 hr on ice prior to the experiment. In the Re-directed lysis experiment target cells were pre-incubated with increasing concentration of anti-CD16 of each fusion protein 2 hours on ice prior to the experiment. In some assays the target cells were pre-incubated with 40 ng/ml than 20% of maximal release. In some assays the NK cells were activated by coincubating 20 x 10⁶ NK cells per sample and 20 x 10⁶ BW/CD72 cells or BW in 500 µl complete RPMI-1640 medium at 37°C. After activation cells were immediately washed four times with ice cold PBS containing 1:100 phosphatase inhibitor cocktail II (Sigma-Aldrich, Inc, St. Louis, MO). Cells were lysed with 500 µl of lysis buffer (150 mM NaCl/50 mM Tris (pH 7.6)/0.5% Nonidet P-40/5 mM EDTA) supplemented with 1:100 aprotinin (Sigma-Aldrich)/1 mM PMSE and 1:100 of phosphatase inhibitor cocktail II (Sigma-Aldrich). Aliquots were preincubated with 0.5 µg/ml of indicated antibodies in 100 µl of blocking buffer/Tween (PBS+1% BSA, Tween 20) and blocked with 200 µl of 1% BSA in PBS for 2 hr on ice. Plates were washed three times with washing buffer (PBS+0.5% Tween 20) and blocked with 200 µl of indicated antibodies in 100 µl of blocking buffer/Tween (PBS+1% BSA, Tween 20). Plates were washed four times with washing buffer and incubated overnight at 4°C with 0.1 µg/well of indicated antibodies in 100 µl of blocking buffer/Tween (PBS+1% BSA, Tween 20). Plates were washed four times with washing buffer and incubated for 1 hr on ice with 100 µl of goat anti-mouse IgG (H+L)-AP conjugated (Bio-Rad, Hercules, CA), diluted 1/2000 in blocking buffer/Tween . Plates were washed six times with washing buffer and incubated for 30–50 min at room temperature with alkaline phosphatase substrate kit (Bio-Rad). Optical density was measured in an ELISA reader at 405 nm.

For the detection of IFNγ secreted by NK cells, 100,000 NK cells per well were incubated with 100,000 BW or BW/CD72 cells in 200 µl of complete RPMI-1640 on flat bottom plate for 72 hours at 37°C with or without 20 ng/ml of the indicated cytokines (Pepro tec). The ELISA for the detection of human IFNγ was performed in accordance with the manufacture protocol and reagents (PharMingen).

Radioiodination, immunoprecipitation, and two-dimensional SDS-PAGE
YT1 cells were radioiodinated with 1 mCi of Na125I (ICN Biomedicals, Inc., Irvine, CA) by the lactoperoxidase method. After extensive washes with cold PBS, labeled cells were solubilized on ice with 0.5% Nonidet P-40/10 mM iodoacetamide/5 mM EDTA/1 mM PMSF/10 µM leupeptin and pepstatin A in PBS, pH 8.3 (lysis buffer). Cell lysates (1 x 10⁶ cells/sample) were preclar on ice with protein A coupled sepharose beads (Zymed Laboratories Inc, S. San Francisco, CA) precoated with rabbit anti-mouse IgG. Aliquots of preclar on ice with protein A coupled sepharose beads precoated with rabbit anti-mouse IgG and mAb 172.4 or control IgG1 mAb. Immunoprecipitated samples were washed with lyso buffer, and labeled proteins were eluted in the presence of SDS under non-reducing conditions. Non-reduced samples were separated on SDS-PAGE. The gels were then cut into strips, each representing an immunoprecipitate, reduced with 2% (V/V) 2-mercaptoethanol at 37°C for 1 hr, and placed on top of the second dimension SDS-PAGE followed by autoradiography. For detection of phosphorylated proteins, NK cells were first activated by coincubating 20 x 10⁶ NK cells per sample and 20 x 10⁶ BW/CD72 cells or BW in 500 µl complete RPMI-1640 medium at 37°C. After activation cells were immediately washed four times with ice cold PBS containing 1:100 phosphatase inhibitor cocktail II (Sigma-Aldrich). Samples were preincubated with 0.5 µg/ml of indicated antibodies in 100 µl of blocking buffer/Tween (PBS+1% BSA, Tween 20). Plates were washed four times with washing buffer and proteins were eluted in the presence of SDS under reducing conditions. The TOPO TA expression Kit (Invitrogen).

ELISA 96 wells MaxiSorp plates (Nunc, Roskilde, Denmark) were pre-coated overnight at 4°C with increasing concentrations of various fusion proteins in 50 µl of PBS per well. Plates were washed three times with washing buffer (PBS+0.5% Tween 20) and blocked with 200 µl of 1% BSA in PBS for 2 hr on ice. Plates were washed three times with washing buffer and incubated overnight at 4°C with 0.1 µg/well of indicated antibodies in 100 µl of blocking buffer/Tween (PBS+1% BSA, Tween 20). Plates were washed four times with washing buffer and incubated for 1 hr on ice with 100 µl of goat anti-mouse IgG (H+L)-AP conjugated (Bio-Rad, Hercules, CA), diluted 1/2000 in blocking buffer/Tween . Plates were washed six times with washing buffer and incubated for 30–50 min at room temperature with alkaline phosphatase substrate kit (Bio-Rad). Optical density was measured in an ELISA reader at 405 nm.

For the detection of IFNγ secreted by NK cells, 100,000 NK cells per well were incubated with 100,000 BW or BW/CD72 cells in 200 µl of complete RPMI-1640 on flat bottom plate for 72 hours at 37°C with or without 20 ng/ml of the indicated cytokines (Pepro tec). The ELISA for the detection of human IFNγ was performed in accordance with the manufacture protocol and reagents (PharMingen).

Adhesion assay
NK cells were labeled with 35S-Met as described previously [43–45]. 100,000 BW or BW/CD72 cells per well were incubated in 50 µl complete RPMI-1640 (Gibco) for 2 hr in 37°C on 96 flat bottom plates (Nunc). 50,000 NK cells in 50 µl complete RPMI-1640 medium were added to the plate and incubated for another 20 minutes at 37°C. The medium was aspirated from the plate gently and then the plate was washed once with 200 µl PBS. 100 µl of RPMI-1640 was added to each well and cells were lysed
REFERENCES

1. Ljunggren HG, Karre K (1990) In search of the ‘missing self’: MHC molecules and NK cell recognition. Immunol Today 11: 237–244.

2. Long EO (1999) Regulation of immune responses through inhibitory receptors. Annu Rev Immunol 17: 875–904.

3. Colonna M, Samaridis J (1995) Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. Science 268: 405–406.

4. Watanaga N, Takiguchi S, Winter CG, Peruzzi M, Long EO (1995) Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. Immunity 3: 801–809.

5. D’Andrea A, Chang C, Frang-Bacou K, McClanahan T, Phillips JH, et al. (1995) Molecular cloning of NKB1. A natural killer cell receptor for HLA-B allotypes. J Immunol 155: 2306: 2310.

6. Braud VM, Allan DS, O’Callaghan CA, Soderstrom K, D’Andrea A, et al. (1998) HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nat Immunol 1: 791–797.

7. Braud VM, McMichael AJ (1999) Regulation of NK cell functions through interaction of the CD94/NKG2 receptors with the nonclassical class I molecule HLA-E. Curr Top Microbiol Immunol 244: 85–93.

8. Colonna M, Nakajima H, Navarro F, Lopez-Botet M (1999) A novel family of Ig-like receptors for HLA class I molecules that modulate function of lymphoid and myeloid cells. J Leukoc Biol 66: 371–378.

9. Colonna M, Navarro F, Bellon T, Llano M, Garcia P, et al. (1997) A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. J Exp Med 180: 1899–1918.

10. Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, et al. (2001) Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol 19: 197–223.

11. Lanier LL (2005) NK cell recognition. Annu Rev Immunol 23: 225–274.

12. Boelum U, Klaemp T, Groot M, Howard JC (1997) Cellular responses to interferon-gamma. Annu Rev Immunol 15: 749–795.

13. Tessier-Lavigne M, Goodman CS (1996) The molecular biology of axon guidance. Science 274: 1123–1133.

14. Bougeot C, Mansur IG, Dastot H, Schmid M, Mahouy G, et al. (1992) Increased surface expression of a newly identified 150-kDa dimer early after human T lymphocyte activation. J Immunol 148: 318–323.

15. Delaire S, Elhabazi A, Bensussan A, Boumell L (1998) CD100 is a lymphocyte semaphorin. Cell Mol Life Sci 54: 1265–1276.

16. Hall KT, Boumell L, Schlutte JL, Boussiotis VA, Dorfman DM, et al. (1996) Human CD100, a novel leukocyte semaphorin that promotes B-cell aggregation and differentiation. Proc Natl Acad Sci U S A 93: 11760–11765.

17. Kumanogoh A, Kikutani H (2000) Semaphorins in interactions between T cells and antigen-presenting cells. Nat Rev Immunol 3: 159–167.

18. Kumanogoh A, Kikutani H (2001) The CD100/CD100 interaction: a novel mechanism of immune regulation. Trends Immunol 22: 670–676.

19. Bismuth G, Boumell L (2002) Controlling the immune system through cytokines in complete RPMI-1640 medium (Peprotec, London, U.K.).

20. delaire S, billard C, tordjman R, chelotot A, elhabazi A, et al. (2001) Biological activity of soluble CD100. II. Soluble CD100, similarly to H-SemIII, inhibits immune cell migration. J Immunol 166: 4340–4344.

21. casado M, arriguian S, chen H, ze ming G, et al. (1999) Pexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell 99: 71–80.

22. kumanogoh A, suzuki K, ching E, watanebe C, marukawa S, et al. (2002) Requirement for the lymphocyte semaphorin, CD100, in the induction of antigen-specific T cells and the maturation of dendritic cells. J Immunol 169: 1175–1181.

23. schwarting R, castello R, molenhaeuer G, Pezzutto A, von hoeven I, et al. (1999) Human Lymph-2 hormone, CD100, is a marker for progenitor B-cell leukemias. Am J Hematol 41: 151–156.

24. kumanogoh A, shikina T, watanebe C, takeghara N, suzuki K, et al. (2005) Requirement for CD100/CD102 interactions in fine-tuning of B-cell antigen receptor signaling and homeostatic maintenance of the B-cell compartment. Immunity 17: 1277–1282.

25. shi W, kumanogoh A, watanebe C, uchida J, wang X, et al. (2000) The class IV semaphorin CD100 plays nonredundant roles in the immune system: defective B and T cell activation in CD100-deficient mice. Immunity 13: 635–642.

26. watanebe C, kumanogoh A, shi W, suzuki K, yamada S, et al. (2001) Enhanced immune responses in transgenic mice expressing a truncated form of the lymphocyte semaphorin CD100. J Immunol 167: 4321–4329.

27. Li M, OSullivan KM, Jones LK, Sempfe T, kumanogoh A, et al. (2006) CD100 enhances dendritic cell and CD1+ cell activation leading to pathogenetic humoral responses and immune complex glomerulonephritis. J Immunol 177: 3406–3412.

28. Chabbert-de Poinnat I, Marie-Caroline A, Pasterkamp RJ, sciavon I, tanamone L, et al. (2005) Soluble CD100 functions on human monocytes and immature dendritic cells require plexin C1 and plexin B1, respectively. J Immunol 173: 439–447.

29. vinale M, Bottino C, stevoi S, sanseverino I, castriconi R, et al. (1999) Nkp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. J Exp Med 187: 2063–2072.

30. Dieffenbach A, Tomaselli E, Lucas M, Jamieson AM, Haia JK, et al. (2002) Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. Nat Immunol 3: 1142–1149.

31. cooper MA, fleminger TA, caligiuri MA (2001) The biology of human natural killer-cell subsets. Trends Immunol 22: 633–640.

32. hanna J, bechtel P, zhai Y, vousuff F, mclachlan K, et al. (2004) Novel insights on human NK cells’ immunological modalities revealed by gene expression profiling. J Immunol 173: 6547–6557.

33. carson WE, giri GJ, lindemann MJ, linett MI, Andieh M, et al. (1994) Interleukin IL-15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. J Exp Med 180: 1395–1403.

34. robertson MJ, seiffer RJ, wolf SF, manley TJ, donahue C, et al. (1992) Response of human natural killer (NK) cells to NK cell stimulatory factor (NKSF): cytotoxic activity and proliferation of NK cells are differentially regulated by NKSF. J Exp Med 175: 779–788.

35. billard C, delaire S, raffoux E, bensussan A, boumell L (2000) Switch in the protein tyrosine phosphatase associated with human CD100 semaphorin at terminal B-cell differentiation stage. Blood 95: 965–972.

36. Wang X, kumanogoh A, watanebe C, shi W, yoshida K, et al. (2001) Functional soluble CD100/Sem1a/D1 released from activated lymphocytes: possible role in normal and pathologic immune responses. Blood 97: 3498–3504.

37. mandelboim O, malik P, davis DM, JO CH, boyson JE, et al. (1999) Human CD100 serves as a lysis receptor mediating direct natural killer cell cytotoxicity. Proc Natl Acad Sci U S A 96: 5640–5644.

38. mandelboim O, lieberman N, lev M, paul L, arnon TI, et al. (2001) Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. Nature 409: 1035–1036.

39. arnon TI, lev M, katz G, chernysov Y, porgador A, et al. (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. Eur J Immunol 31: 2680–2689.
46. Pesando JM, Stucki MA (1990) AFTR: a fifth human B-cell-specific surface antigen. Hum Immunol 27: 193–207.
47. Garand R, Robillard N, Bataille R (1994) CD72 is constantly expressed in chronic lymphocytic leukemia and other B-cell lymphoproliferative disorders. Leuk Res 18: 651–652.
48. Myers DE, Uckun FM (1995) An anti-CD72 immunotoxin against therapy-refractory B-lineage acute lymphoblastic leukemia. Leuk Lymphoma 18: 119–122.
49. Shah PD, Gilbertson SM, Rowley DA (1985) Dendritic cells that have interacted with antigen are targets for natural killer cells. J Exp Med 162: 625–636.
50. Hauer WE Jr, Sharma SD, Remington JS (1982) Natural killer cells induced by acute and chronic toxoplasma infection. Cell Immunol 69: 330–346.
51. Ferlazzo G, Tsang ML, Moretta L, Melioli G, Steinman RM, et al. (2002) Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. J Exp Med 195: 343–351.