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Inhibition of Human NK Cell-Mediated Killing by CD1 Molecules

Ennio Carbone,‡ Giuseppe Terrazzano,‡ Augustin Melián,§ Delia Zanzi,‡ Lorenzo Moretta,§ Steven Porcelli,‡ Klas Kärre,§ and Serafino Zappacosta‡

It is now well established that NK cells recognize classical and nonclassical MHC class I molecules and that such recognition typically results in the inhibition of target cell lysis. Given the known structural similarities between MHC class I and non-MHC-encoded CD1 molecules, we investigated the possibility that human CD1a, -b, and -c proteins might also function as specific target structures for NK cell receptors. Here we report that expression of CD1a, -b, or -c can partially inhibit target cell lysis by freshly isolated human NK cells and cultured NK lines. The inhibitory effects of CD1 molecules on NK cell could be shown upon expression of individual CD1 proteins in transfected NK-sensitive target cells, and these effects could be reversed by incubation of the target cells with mAbs specific for the expressed form of CD1. Inhibitory effects of CD1 expression on NK-mediated lysis could also be shown for cultured human dendritic cells, which represent a cell type that prominently expresses the various CD1 proteins in vivo. In addition, the bacterial glycolipid Ags known to be bound and presented by CD1 proteins could significantly augment the observed inhibitory effects on target cell lysis by NK cells. The Journal of Immunology, 2000, 164: 6130–6137.

N atural killer cell killing is not MHC class I restricted in the classical sense, but is influenced by the expression of MHC class I molecules on the target cell surface. NK cells spare target cells expressing self MHC class I molecules while efficiently eliminating MHC class I-deficient normal and tumor cells (1). In mice and humans, there are two groups of MHC class I inhibitory receptors, both characterized by the so called immunoreceptor tyrosine-based inhibition motifs in their cytoplasmic tails. One group contains the Ly49 homodimers expressed in mice and the CD94/NKG2A heterodimers expressed in mice and in humans (2). The other group of inhibitory receptors consists of the killer cell-inhibitory receptors (KIR)3 expressed in humans belonging to the Ig gene superfamily. Activating variants of these receptors (e.g., Ly49D, CD94/NKG2C, and KIR2DS) that lack immunoreceptor tyrosine-based inhibition motif domains have also been described (2). Other activating pathways are also used by NK cells (3), and we and others have found that costimulatory molecules expressed on the APC surface may serve as NK cell-activating elements (4, 5).

Because CD94/NKG2A NK cells have been reported to recognize the nonpolymorphic HLA-E MHC class Ib molecule (or the homologous Qa-1 molecule in mice) (6, 7), these observations led us to ask whether CD1 molecules can also be recognized by NK cells. CD1 molecules are nonpolymorphic MHC class I-like glycoproteins that are encoded by a family of genes outside of the MHC locus. The expression of CD1 proteins does not require TAP-1/2 transporters, although it is dependent on β2 microglobulin (8). The genes and amino acid sequences of CD1 and MHC class I and class II show a limited but significant level of homology, which approaches ~30% identity in the α2 and α3 domains (8). CD1 genes are evolutionarily old and are present in rodents and human, as well as in all other mammalian species so far examined (8). In contrast to HLA-A, -B, -C, and -E Ags, which are broadly expressed in almost all tissues, CD1 proteins have a more restricted and cell type-specific expression. All CD1 proteins are strongly expressed by thymocytes. In extrathymic tissues, CD1a, -b, and -c are expressed primarily on specialized APC (8).

CD1b and CD1c in humans and CD1d1 in mice have the property to present lipid Ags (8, 9). Human T cells belonging to either CD4+CD8− or CD8+ subsets can recognize Mycobacterium tuberculosis (M.tb) lipids presented by CD1d1 (9–12). Although not as extensively studied as for CD1b, it appears that human CD1a and CD1c molecules can also present mycobacterial lipid Ags to specific T cells (12, 13). In addition, CD1d proteins in both mouse and human have recently been demonstrated to present synthetic α-glycosyl ceramides to a subset of T cells that bear highly conserved TCRs and coexpress several NK-associated lectin-type receptor molecules (14, 15).

The purpose of this study was to test the role of CD1 proteins as potential ligands for NK cells and to evaluate the effect of CD1-presented bacterial lipid Ags on NK cytolysis. In the current study, we have directly examined the effects of CD1 expression on the lysis of two different NK-sensitive target cells (T2 and HeLa). Our results demonstrated a clear effect of each of the human CD1 proteins on the inhibition of target cell lysis by NK cells. Moreover, the preincubation of CD1b− target cells with defined

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[3] Abbreviations used in this paper: KIR, killer cell-inhibitory receptor; M.tb, Mycobacterium tuberculosis; DC, dendritic cell.
bacterial lipid Ags that are known to bind to CD1b led to a significant increase in the inhibitory effect on NK lysis.

Materials and Methods

Cell lines and NK cell culture

T-B lymphoblastoid hybrid wild-type cell line T1 and the TAP-1/2-defective mutant T2 were generated as described (16). The stably MHC class I-reconstituted T3 cell line was produced by transfection of human TAP-1/2 cDNA constructs into T2 cells, as described (17). HeLa cells were obtained from American Type Culture Collection (Manassas, VA). T2 and HeLa transfected with plasmid constructs of the expression vector pSR-Neo encoding either CD1a, CD1b, or CD1c were generated and maintained as described elsewhere (13, 15). Dendritic cell (DC) line cultures were generated by treatment of adherent mononuclear cells with GM-CSF (50 ng/ml) and IL-4 (1000 U/ml) for 48 h before use in cytotoxicity assays. For generation of NK cell lines, freshly isolated nonadherent lymphocytes were washed and resuspended in IMDM medium (BioWhittaker, Berlin, Germany) at a final concentration of 2.5 × 10^6/ml and cultured together with irradiated T2 cells (0.5 × 10^6 cells in a final volume of 500 μl of culture medium and incubating such preparation overnight at 37°C.

To obtain fresh NK cells, PBMC were isolated by centrifugation on Lymphoprep (Nycomed Pharma, Oslo, Norway) gradients from healthy donor buffy coats obtained from the Blood Bank of the Medical School of the Federico II University of Naples. Adherent cells were removed by incubation for 2 h at 37°C in tissue culture flasks, and fresh PBL were stored overnight at room temperature before use as effectors in cytotoxicity assays. IL-2-activated NK cells were generated from PBL cultured in medium supplemented with IL-2 (Cetus, Milan, Italy) (1000 U/ml) for 48 h before use in cytotoxicity assays. For generation of NK cell lines, freshly isolated nonadherent lymphocytes were washed and resuspended in IMDM medium (BioWhittaker, Berlin, Germany) at a final concentration of 2.5 × 10^6/ml and cultured together with irradiated T2 cells (0.5 × 10^6/ml) for 5–7 days of culture. All effectors were depleted of CD3-positive cells by immunomagnetic separation using magnetic beads (Dynal, Oslo, Norway) coated with anti-CD3 mAb. The depletion procedure was repeated twice, and 98% of the remaining cells were CD56^−. To test the effects of expression of human CD1 proteins on NK cell lysis, NK clones were generated as previously reported (4). The stable NK effector line NKL was a gift of Dr. M. J. Robertson (Bone Marrow Transplantation Program, Indiana University, Indianapolis, IN). Generation of NK clones and measurement of NK cytotoxicity by 51Cr release assay were performed according to previously published methods (4).

mAbs, F(ab')2 production, and flow cytometry

Anti-CD1 mAbs used in this study included OKT6 (IgG1, anti-CD1a; obtained from American Type Culture Collection), BCDIb3.1 (IgG1, anti-CD1b; Ref. 13), F10/21A3 (IgG1, anti-CD1c; S. Porcelli, unpublished observations), and 7C6 (IgM, anti-CD1c; Ref. 9). All IgG1 mAbs were purified by protein G column affinity chromatography from ascites fluid (OKT6) or culture supernatants (BCDIb3.1 and F10/21A3). IgM mAbs (7C6) were used as asces fluid dialyzed extensively against RPMI 1640. F(ab')2 were prepared from IgG1 anti-CD1 mAbs by enzymatic digestion of purified Ig using immobilized ficin columns (Pierce, Rockford, IL) and purified by passage over a protein A Sepharose column followed by dialysis against PBS. T2 CD1 transfectant cell lines were incubated with 4 μg/ml of specific anti-CD1 F(ab')2 for 30 min at room temperature, washed twice, and used in the cytotoxicity assay. Phenotypic characterization of monocyte-derived DCs was performed using FITC- and PE-labeled mAbs against CD40, CD80, CD86 (PharMingen, San Diego, CA), CD83 (Immunotech, Marseille, France), HLA class I (Sigma), CD3, CD14, HLA-DR, and isotype-matched control mAbs (Becton Dickinson, Mountain View, CA). Immunofluorescence staining, flow cytometry, and data analysis were performed as described (4).

Cytotoxicity assay

Cytotoxicity was measured using a conventional 4-h 51Cr release assay. Target cells were labeled with Na2 51CrO4 (100 μCi/2 × 10^6 cells), and the percent of specific lysis was calculated as 100 × [(cpm in experimental wells) − (cpm in wells with target cells alone)]/(total cpm incorporated into target cells). The spontaneous 51Cr release from target cells cultured alone was consistently <20% of the total cpm. In mAb blocking experiments, 5 μg/ml of the indicated anti-KIR Abs were added at the beginning of the cytotoxicity test.

Results

Expression of CD1a, CD1b, and CD1c in MHC class I-deficient target cells conferred protection from killing by fresh NK cells and cultured NK cell lines

Because previous studies have shown that CD1 proteins are not dependent on TAP-1/2 for their assembly and can be expressed efficiently in T2 cells, we used this cell line as a recipient in which to test the effects of expression of human CD1 proteins on NK cell lysis. CD1 transfectants were then tested in cytotoxicity experiments for sensitivity to lysis by various NK cell effector populations. These included freshly isolated CD3-depleted PBL (Fig. 1, A–C), the NKL cell line (Fig. 1D), a freshly established polyclonal NK cell line (Fig. 1E), and short-term IL-2-activated NK cells (Fig. 1, F and G). A clear, albeit not complete, reduction of NK susceptibility was found for transfected T2 cell targets expressing each of the different CD1 proteins. This was observed with all of the different NK cell effector populations tested. Note that in some donors no protection or even a slight increase in NK cytotoxicity was observed with some of the CD1 molecule isoforms. However, NK cells from all donors were inhibited by at least one of the CD1 molecules.

To confirm the effect of CD1 molecules in the modulation of NK activity using a second target cell type, CD1 genes were transfected into the epithelial tumor cell line HeLa. Recognition by polyclonal NK cells was reduced for all of the CD1 transfectants. Fig. 1H shows a representative experiment with polyclonal NK cells generated from a donor recognizing all three CD1 isoforms. Pretreatment of CD1-transfected HeLa cells with the specific anti-CD1 F(ab')2 reduced the protection substantially (Table I). These data confirmed that the protection from NK lysis observed for cells transfected with CD1 was specifically due to the expression of CD1 glycoproteins.

Interestingly, in parallel experiments in which a different MHC class I-deficient target cell, C1R, was used, no effect of CD1 expression on NK cell lysis by IL-2-activated NK cells was observed (Fig. 1G), consistent with previously reported results (19).

This may be due to the expression of other dominant inhibitory ligands by C1R or possibly to the lower CD1 surface expression on C1R transfectants (see Discussion). Therefore, the expression of MHC class I and CD1 molecules were measured on the T2, HeLa, and C1R cell lines and their related CD1 transfectants (Fig. 2). The MHC class I expression was not affected by CD1 transfection in all transfectants cell systems studied; on C1R cells, an appreciable level of MHC class I molecules was detected (Fig. 2A). In contrast, the highest CD1 levels were detected on T2 and HeLa, while C1R CD1 expression was approximately half of that observed for T2 and HeLa transfectants (Fig. 2B). A bright staining for CD1 group 1 molecules was observed as well on DC (Fig. 2C).

Reversal of the inhibitory effects of CD1 expression on NK cell killing by mAbs against CD1 proteins

The NK cell killing against each of the CD1 transfectants was significantly augmented by pretreatment of the target cells with F(ab')2 specific for the form of CD1 expressed by the target (Fig. 3). This indicated that the expression of the intact, native form of each of the different CD1 proteins on the surface of the target cells was responsible for the observed inhibition of NK cell-mediated lysis of the T2 transfectants. Similar results were seen with anti-CD1c IgM (mAb 7C6) pretreatment of the CD1c transfectants (data not shown).

We also examined the NK cell-mediated killing of CD1+ monocyte-derived DC. This represents a potentially more physiological...
reaction, because DCs represent one of the major sites of CD1 protein expression in vivo. DC lines generated from autologous or allogeneic monocytes were pretreated separately with F(ab')2 against a single form of CD1, with a F(ab')2 against MHC class I, with a mixture of F(ab')2 against MHC class I and single CD1 molecules, or with a mixture of F(ab')2 against all three CD1 proteins. The DC pretreatment with the mixture of all three CD1 Abs gave rise to strong NK cell-mediated killing against autologous DC (Fig. 4A), whereas F(ab’)2 against CD1c alone (Fig. 4, A–C) or CD1b or CD1a alone (data not shown) had no effect. The pretreatment with anti-MHC class I Ab also increased the DC recognition by polyclonal NK cells. The combination of anti-MHC class I and single anti-CD1 Abs induced a strong synergistic effect on NK recognition (Fig. 4, B and C). This synergistic effect between MHC class I and CD1 blockade was donor dependent (Fig. 4, B and C).

**FIGURE 1.** NK recognition of CD1-transfected T2 cells. Three types of NK effectors were used. A–C, Fresh PBL. D, NKL. E and H, A fresh polyclonal NK line generated by T2 coculture (one representative of five experiments). F and G, CD3 depleted PBL cultured in IL-2 for 48 h. Radiolabeled targets were as follows: (A–F) T2-mock transfectant (○), T2-CD1a (●), T2-CD1b (▲), T2-CD1c (□), and T3 (●); (G) C1R-mock transfectant (○), C1R-CD1a (●), C1R-CD1b (▲), C1R-CD1c (■); (H) HeLa CD1a (▲), CD1b (■), CD1c (●), and HeLa mock (○).
of CD1 as well as MHC class I has some capacity to independently inhibit the NK cell effector population.

Assessment of the CD1 recognition by NK clones

Because differences between donors with respect to CD1 recognition were noticed (Figs. 1 and 4), we assessed the variation in CD1 recognition in a panel of nine established NK clones from four different donors. This revealed a complex and heterogeneous recognition pattern (Fig. 5). For the majority (seven of nine) of clones, target cell lysis appeared to be inhibited by at least one CD1 protein. The exceptions were NK clones 28 and 48, which were not inhibited by expression of any of the CD1 proteins studied. NK clones 1 and 3 appeared to be inhibited by all three CD1 Ags, although with varying degrees of efficiency. Notably, the pattern varied between clones, although the clones derived from an individual donor appeared to show a trend toward inhibition by the same CD1 protein (e.g., clones from donor A were inhibited most strongly by CD1b, whereas three of five clones from donor D were inhibited by CD1a).

Modulation of CD1b-dependent NK inhibition by bacterial lipid Ags

CD1b was shown in previous studies to present M. tb-derived lipid and glycolipid Ags to T cells (9–12). Upon this recognition, T cells were triggered to kill infected presenting cells and to produce IFN-γ. Here, we investigated how NK cells react to the CD1b presentation of a M. tb whole lipid extract or two of its purified constituents: mycolic acid and glucose monomycolate. T2 CD1b and T2 mock transfected were incubated overnight at 37°C with M. tb-derived lipid Ags at a final concentration of 10 μg/ml. Fig. 6, A and B shows one representative experiment on the NK recognition of T2 CD1b and T2 mock cell lines after preincubation with M. tb lipid Ags. The preincubation of T2-CD1b cells with whole extract and the purified glucose monomycolate led to a moderately increased inhibitory effect, while purified mycolic acid caused a marked increase in the inhibition of NK killing. No significant effects were found when the T2-mock cells were exposed to the same lipid preparations (Fig. 6B).

Discussion

In this study we provide evidence that CD1 proteins can function as target structures for NK recognition. To our knowledge, this is the first example of human NK cell inhibitory recognition of tissue-specific molecules, as all of the previous ligands of NK cell inhibitory receptors have been widely or ubiquitously expressed MHC class I proteins. Although they differ from MHC class I molecules in their more restricted tissue distribution, a function for CD1 proteins as ligands for inhibitory NK receptors was initially suggested by their known MHC class I-like structure. However, previous investigators did not detect a role for CD1 proteins in the inhibition of NK cell cytolytic responses (19). Based on the results
reported here, this may be related to the exclusive use of C1R B lymphoblastoid cells as the target cell in that study. Also in our hands, the CD1 inhibition was not detectable when the CD1 genes were expressed in C1R cells. This may relate to the relatively low CD1 expression by C1R transfectants, or to the presence of significant levels of residual MHC class I molecules on these cells. Although C1R cells harbor chromosomal deletions that eliminate all expression of HLA-A locus molecules, they express normal levels of HLA-C molecules (Cw04) and detectable amounts of HLA-B35 (Fig. 2B). Partial inhibitory effects mediated by these
molecules may mask the effects of CD1 proteins. Another explanation may relate to the possible HLA-E expression by C1R. HLA-E expression is up-regulated by HLA-Cw04 leader sequence-derived peptides (6) and could thus mediate an inhibition that masks the effect of coexpression of CD1 proteins by these cells.

In contrast to the negative results with C1R cell transfectants, inhibitory effects of CD1a, -b, or -c expression on NK cell-mediated lysis were readily observed in CD1-transfected T2 and HeLa cells. T2 have a deletion of the entire class II region including the TAP-1 and -2 peptide transporter genes. Paradoxically, presentation of signal peptides by HLA-E is TAP dependent, and therefore T2 is not expected to express this CD94/NKG2A ligand (20). HeLa cells were also protected by CD1 transfection, and these express low levels of MHC class I comparable with T2 and remarkably high levels of CD1 on their cell surface.

The specificity of the inhibition of NK cells by CD1 proteins was confirmed using F(ab')2 of mAbs specific for each CD1 protein to reverse the observed inhibition. Because the anti-CD1 mAbs used are known to be reactive with the mature cell-surface form of the CD1 proteins that they recognize, these results indicate that the intact CD1/β2-microglobulin complex must be responsible for the observed inhibition of NK cell-mediated killing of T2 and HeLa cell transfectants and of DCs. Thus, it is unlikely that the inhibitory effect of CD1 expression can be accounted for by indirect mechanisms, such as the presentation of CD1 leader peptides by HLA-E and subsequent interaction with CD94/NKG2A. Instead, we propose that one or more specific receptor molecules on NK cells must interact directly with cell-surface CD1 proteins to generate the putative inhibitory signals.

Using a variety of different NK effector populations, we found that the expression of a single form of CD1 on transfected T2 cells provided significant but incomplete protection from NK cell-mediated killing. In the various experiments performed, inhibition of NK cell lysis of T2 cells by CD1 expression ranged between a 30% to 70% reduction of the level achieved with control target cells. In one set of experiments (see Fig. 1D), the levels of protection achieved on T2 cells transfected with TAP-1/2 to reconstitute normal MHC class I levels was compared with that achieved for cells transfected with the different forms of CD1. Notably, the restoration of MHC class I expression on the target cell membrane almost completely abolished NK killing at all E:T ratios used, whereas the expression of any of the CD1 proteins was able to inhibit the NK killing only partially. The lower potency of CD1 expression compared with MHC class I reconstitution was especially apparent at high E:T cell ratios, at which the protective effects of CD1 but not MHC class I expression were largely overcome (Fig. 1D). However, at low E:T cell ratios, the same level of inhibition was found for either MHC class I-reconstituted or CD1-expressing T2 cells. The lower expression of CD1 on T2 transfectants compared with the levels of MHC class I on the TAP-1/2-reconstituted cells (data not shown), or the fact that TAP-1/2 reconstitution generates multiple inhibitory MHC class I targets on the cell surface, may account for the apparent difference in the potency of inhibition we observed.

**FIGURE 4.** Regulation of NK-DC recognition by CD1 molecules. IL-2-activated PBL (48 h) were used as effector cells against autologous DC subjected to the follow pretreatment: (A) none (○); a mixture containing F(ab')2 anti-CD1a,b,c (●); anti-CD1c F(ab')2 alone (△). The anti-F(ab')2 were used at the final concentration of 4 µg/ml. Pretreatment of DC in B and C: none (○); anti-CD1c fragments (△); anti-HLA class I (□); with a mixture of anti-MHC class I and anti-CD1c fragments (■), anti-HLA class I and anti-CD1a (▲), and anti-CD1b and HLA class I (●).
DC appear to represent a major site of expression of most CD1 proteins in humans, and therefore it is intriguing to note that treatment of monocyte-derived DCs with a mixture of anti-CD1a, -b, and -c mAb increased the sensitivity of these cells to NK lysis in vitro. Abs against a single form of CD1 did not reverse the protection, except when used in combination with anti-MHC class I mAbs, indicating that to overcome the inhibitory effect it is necessary to block NK recognition of most or all expressed CD1 proteins simultaneously or block at the same time MHC class I and CD1.

The observed synergistic effect of the anti-MHC class I and single anti-CD1 F(ab')2 on reversal of protection from NK cells can be interpreted in at least two different ways: 1) one NK receptor may interact with both MHC nonclassical class I and CD1, or 2) two distinct inhibitory receptors recognizing MHC class I and CD1 molecules are involved in NK-DC interactions. This issue is under active investigation in our laboratory. The full DC protection by NK recognition is achieved only when a full inhibitory signal is delivered from the NK receptor(s) for MHC class I and CD1 molecules. Thus the protective effect of CD1 proteins on DCs is apparent despite the presumably normal expression of classical MHC class I and HLA-E molecules on these cells. Although the reason for the failure of these latter proteins to completely protect DCs from NK lysis is not entirely clear, we speculate that this may relate to the extremely prominent expression of costimulatory molecules present on these cells (21). CD1 could also have an important role in virus-infected DC in which the normal TAP-dependent MHC class I pathway is perturbed and fails to protect DC until they can prime T cells. Our results also indicate that the presentation of bacterial lipid Ags by CD1b may be able to significantly augment the protection against NK cell lysis conferred by this CD1 protein. The mechanism for the effect of lipid Ags is currently under investigation. We speculate that this enhanced protection from NK lysis may be important in the setting of bacterial infection. In such circumstances, the NK cytolytic response may be damped by lipid Ag presentation in the context of CD1, while specific double negative and CD8+ T cells may be efficiently activated by such presentation.

**FIGURE 5.** NK clones recognize CD1 Ags. NK clones were generated from three donors as reported on the left of the figure. These clones were tested against T2-mock (□), T2-CD1a (■), T2-CD1b (▲), and T2-CD1c (■) targets as indicated. The E:T ratio was 2:1.

**FIGURE 6.** CD1b-mediated NK protection was increased by preincubation of the target cells with CD1b-presented M.tb lipid Ags. A, T2 CD1b target cells were incubated overnight separately with 10 μg/ml M.tb whole lipid extract (△), purified mycolic acid (gray triangle), or with glucose monomycolate (○). T2 CD1b not pretreated with M.tb lipid Ags (●); T2 mock were used as control (○) in both figures. B, The same treatments with M.tb lipid Ags were performed on T2-mock cells, and no consistent changes were found in NK susceptibility. The effectors used were polyclonal NK cells. These experiments were repeated three times.
While the present study was being conducted, it was reported (22) that cytolysis by mouse lymphokine-activated killer cells is inhibited by target cell expression of the murine CD1 molecule, mCD1d1. The closest human homologue of mCD1d1 is the human CD1d protein, which was not directly assessed for NK cell inhibitory activity in our study. Nevertheless, the inhibitory effect of mCD1d1 on mouse lymphokine-activated killer cells adds further support to our hypothesis that CD1 molecules are ligands of specific inhibitory receptors expressed by NK cells.

Based on the initial results reported here, we envision an important role for negative signaling by CD1 recognition in infections, particularly in the setting of global MHC class I down-regulation by microorganisms.

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