The Murine Nonclassical Class I Major Histocompatibility Complex–like CD1.1 Molecule Protects Target Cells from Lymphokine-activated Killer Cell Cytolysis

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Summary

Classical class I major histocompatibility complex (MHC) molecules, as well as the nonclassical class I histocompatibility leukocyte antigen (HLA)-E molecule, can negatively regulate natural killer (NK) cell cytotoxicity through engagement of NK inhibitory receptors. We show that expression of murine (m)CD1.1, a nonpolymorphic nonclassical MHC class I–like molecule encoded outside the MHC, protects NK-sensitive RMA/S target cells from adherent lymphokine-activated killer cell (A-LAK) cytotoxicity. Passage of effector cells in recombinant interleukin (rIL)-2 enhanced protection by mCD1.1, suggesting an expansion of relevant A-LAK population(s) or modulation of A-LAK receptor expression. Murine CD1.1 conferred protection from lysis by rIL-2–activated spleen cells of recombination activating gene (Rag)-$1^{-/-}$ mice, which lack B and T cells, demonstrating that mCD1.1 can protect RMA/S cells from lysis by NK cells. An antibody specific for mCD1.1 partially restored A-LAK lysis of RMA/S.CD1.1 transfectants, indicating that cell surface mCD1.1 can confer protection from lysis; therefore, mCD1.1 possibly acts through interaction with an NK inhibitory receptor. CD1.1 is by far the most divergent class I molecule capable of regulating NK cell activity. Finally, mCD1.1 expression rendered RMA/S cells resistant to lysis by A-LAK of multiple mouse strains. The conserved structure of mCD1.1 and pattern of mCD1.1 resistance from A-LAK lysis suggest that mCD1.1 may be a ligand for a conserved NK inhibitory receptor.

Key words: CD1 • lymphokine-activated killer cell • natural killer cells • lysis • resistance

Natural killer (NK) cells can mediate innate resistance against microbes and tumor challenges (1, 2). They can carry out these functions by secreting cytokines, particularly IFN-γ, and by lysing virally infected or transformed cells, without prior sensitization (3, 4). NK cells do not express Igs or TCRs, yet they are able to discriminate self from non-self. According to the "missing self" hypothesis of Kärre and colleagues (5, 6), NK cell cytotoxic activity is regulated by self–MHC class I molecule expression, such that a tumor with downregulated MHC expression can be highly susceptible to NK cell lysis (7, 8). This model is supported by the identification of various class I MHC–specific inhibitory receptors expressed by NK cells of both humans and mice (9–11). For example, Ly-49A, a lectin-like NK inhibitory receptor expressed in mice, recognizes H-$2D^d$ and D^k, and downregulates Ly-$49A^+$ NK cell cytotoxic activity (12–14). The human p58 and p70 killer cell inhibitory receptors (KIRs)[1] are known to interact with various alleles of HLA-B and HLA-C, with a similar functional outcome (15, 16). Therefore, it is well established that classical class I MHC molecules can regulate NK cell activity.

Unlike classical class I MHC genes, the nonclassical class I MHC genes, such as those encoded by the murine *T*, *Q*, and *M* regions, exhibit little or no polymorphism (17). In general, they also have a more restricted expression pattern than classical class I MHC products. Although the function of some of these nonclassical class I molecules is unknown, there is evidence suggesting that some of them have spe-

[1]*Abbreviations used in this paper:* A-LAK, adherent lymphokine-activated killer cell; KIR, killer cell inhibitory receptor; m, murine; NA-LAK, nonadherent lymphokine-activated killer cell; NKT cells, natural T cells; NP, nucleoprotein; Rag, recombination activating gene; TAP, transporter of antigen presentation.

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cialized antigen-presenting functions (17, 18). CD1, a nonclassical MHC class I–like molecule, is encoded outside the MHC region (17, 18). Although classified as a class I molecule, CD1 molecules share only 5–15% sequence identity with classical class I molecules in the α1 and α2 domains. In the more conserved α3 extracellular domain, the sequence identity is $\sim$30%. In fact, CD1 molecules are about as closely related to MHC class II as they are to MHC class I molecules (19), suggesting that they diverged relatively early in evolution from the MHC-encoded antigen-presenting molecules. The CD1 family is itself quite divergent, and based on amino acid sequence homology, human CD1 molecules can be classified into groups 1 and 2 (20). The former group includes CD1a–c and e, whereas CD1d belongs to the latter group (17, 18, 20). In mice, CD1 is encoded by two closely related genes, murine (m)CD1.1 and mCD1.2, that are most homologous to human CD1d (17, 18, 20). Like classical MHC class I molecules, CD1 expression is mostly β_2-microglobulin dependent (21, 22). The available x-ray crystallographic data show that mCD1.1 cocrystalizes with β_2-microglobulin and adopts a structure generally resembling classical class I and class II antigen-presenting molecules, although the antigen binding groove is more narrow and hydrophobic (23). The CD1 molecule is a ligand for the TCR expressed by a subset of T cells expressing NK markers, termed natural T (NKT) cells (24). These T cells secrete large amounts of IL-4 and IFN-γ upon activation by anti-CD3 mAbs (25). Unlike classical MHC class I molecules, CD1 antigen presentation is transporter of antigen presentation (TAP) independent (21, 22). Although mCD1.1 can present peptides with a hydrophobic binding motif (26), this unusual TAP-independent behavior is explained in part by the observation that CD1 is also capable of presenting glycosylceramides and microbial lipoglycan antigens to NKT cells (27–30).

Recently, the human nonclassical MHC class I molecule HLA-E has been demonstrated to inhibit NK cell cytotoxic activity through interaction with the CD94/NKG2A NK inhibitory receptor (31, 32). In addition, HLA-G is able to interact with an Ig-like inhibitory receptor, ILT4 (33). These results raise the possibility that other nonclassical class I MHC products might regulate NK cell activity. In this report, we present evidence that mCD1.1 negatively regulates murine NK cell cytotoxic activity. This is the first report to show that a class I molecule encoded outside the MHC region, can regulate NK cell function. mCD1.1 is now the most divergent antigen-presenting molecule known to regulate NK cell function. Our data further suggest the possible existence of a conserved receptor(s) which recognizes mCD1.1.

Materials and Methods

Mice. Female C57BL/6 (B6, H-2^b) mice were obtained from The Jackson Laboratory and Charles River Laboratories. Female CBA/J (H-2^k), NZB/BinJ (H-2^d), and 129 $\times$ B6 recombination activating gene (Rag)-$1^{-/-}$ (H-2^b) mice were purchased from The Jackson Laboratory. All mice were 8–12 wk of age, except the NZB/BinJ mice, which were 9 mo of age.

mAbs. The 1B1 (rat IgG_{2b}) mAb, anti-mCD1, has been described previously (34). M1/89.18.7.HK (rat IgG_{2b}), the anti-CD45 hybridoma, was obtained from American Type Culture Collection. Tissue culture supernatants were prepared from the 1B1 and M1/89.18.7.HK hybridomas incubated in protein-free hybridoma medium. 1B1 was purified using a protein G–sepharose 4B affinity column and dialyzed against PBS. The M1/89.18.7.HK mAb was obtained from the M1/89.18.7.HK hybridoma supernatant by ammonium sulfate precipitation and dialysis against PBS. FITC-conjugated goat anti–rat IgG was purchased from Jackson ImmunoResearch Laboratories.

Tumor Cell Lines. The NK-sensitive target Yac-1 T lymphoma was obtained from American Type Culture Collection. The TAP-2–deficient RMA/S T lymphoma and the RMA/S.CD1.1 transfectant have been described previously (22). The RMA T lymphoma was a gift from Dr. Jefferies (University of British Columbia, Vancouver, Canada). All of the tumor cell lines were maintained in RPMI, with 5% heat-inactivated FCS, 20 mM Hepes, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For RMA/S.CD1.1, 100 μg/ml of G418 was also added to the culture.

Cloned CTL. Clone 3/4 is a C57BL/6-derived CTL clone specific for D^b and the influenza nucleoprotein peptide (NP) (360–380) of the A/PR/8/34 influenza virus (35). Clone 3/4 was maintained as described previously (35).

Generation of Poly I:C–activated Killer Cells. Each C57BL/6 mouse was injected with 200 μg i.p. of poly I:C. After 18 h, spleen cells were harvested from these mice and RBCs were removed by lysing with 0.14 M NH_4Cl. Adherent cells were removed by culturing the splenocytes on tissue culture–treated plates for 1 h at a density of 5×10^6 cells/ml at 37°C. The nonadherent cells were then used as effector cells in ^{51}Cr-release assays.

Generation of Adherent Lymphokine-activated Killer Cells. Adherent lymphokine-activated killer cells (A-LAKs) from different mouse strains were generated as described (36). In brief, adherent cells from the total splenocyte population were removed by culturing them on tissue culture–treated plates for 1 h. Nonadherent cells were then harvested and passaged through a nylon wool column to remove B cells. The nylon wool nonadherent cells, at a density of 10^6 cells/ml, were cultured in RPMI/10% FCS supplemented with sodium pyruvate, nonessential amino acids, 5×10^{-5} M 2-ME (hereafter referred to as complete medium) and with 800 U/ml of recombinant human IL-2 (rIL-2) expressed in and isolated from *Escherichia coli*. After 3 d of culture, the nonadherent (NA)-LAKs were removed, and the A-LAKs were then maintained in complete medium with 800 U/ml of rIL-2 for an additional 4 d. Operationally, these A-LAKs are referred to as day 3 A-LAKs. The NA-LAKs were recultured in the presence of the same concentration of rIL-2, and the A-LAKs generated from this culturing condition on day 4 are referred to as day 4 A-LAKs. After 1 d of culture, the NA-LAKs generated from the day 4 A-LAKs were removed and recultured as before, and are referred to as day 5 A-LAKs. The process was repeated until day 7. Like day 3 A-LAKs, day 4, 5, and 6 A-LAKs were cultured until day 7 in rIL-2 after their selection. In all instances, only the A-LAKs were used as effector cells in ^{51}Cr-release assays. The 129 $\times$ B6 Rag-$1^{-/-}$ A-LAKs were obtained by culturing the RBC-depleted splenocytes with 800 U/ml of rIL-2 for 6 d. The NA-LAKs were then removed, and the A-LAKs were cultured for an additional 6 d in the presence of rIL-2.

Cytotoxic Assay. Targets cells were labeled with 100 μCi of $Na^{51}CrO_4$ for 1 h. After extensive washing, 10^4 ^{51}Cr-labeled target cells were incubated with poly I:C–activated killer cells or A-LAKs at various E/T ratios for 4–5 h at 37°C in a V-bottomed microtiter plate in triplicate. For killing by CTL clone 3/4, RMA/S and RMA/S.CD1.1 were incubated at 26°C overnight to induce higher expression of D^b. The cells were then labeled with ^{51}Cr and pulsed with 400 μg/ml of NP peptide, YASNENMETM, at 37°C for 1 h before the cytotoxic assay. In all instances, the percentage of lysis was determined as [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Unless stated otherwise, all cytotoxic assays in this report were performed in triplicate.

mAb Blocking Assay. The ^{51}Cr-labeled targets were incubated with 40 μg/ml of M1/89.18.7.HK or 1B1 for 30 min at room temperature. The effector cells were then added to the wells diluting the mAb concentration to 20 μg/ml. The ^{51}Cr-release assays were carried out in the presence of these mAbs for 4–5 h at 37°C.

Results

mCD1.1 Expression Inhibits A-LAK Cytotoxic Activity. We were interested in examining the ability of mCD1.1 to inhibit NK cytotoxic activity. To address this issue, we took advantage of the fact that mCD1.1 expression is TAP independent (21, 22), and thus mCD1.1 can be expressed at the cell surface by the NK-sensitive TAP-2–deficient T lymphoma cell line, RMA/S (22). As detected by the mCD1.1-specific mAb 1B1, RMA/S expresses high levels of mCD1.1 upon transfection compared with that of the untransfected control (22). Since classical class I MHC expression requires the presence of functional TAP, this system enables us to assess the potential protective effect of mCD1.1 without the complication of inhibition also mediated by the endogenous classical class I MHC products D^b and K^b.

We first carried out in vitro killing assays of RMA/S and RMA/S.CD1.1, using poly I:C–activated B6 NK cells. As expected, the class I MHC–deficient cell line, RMA/S, is susceptible to NK cell lysis. However, a modest but reproducible reduction in lysis of the RMA/S.CD1.1 transfectant was detected at higher E/T ratios where cytotoxicity is substantial, but not apparently at plateau, and can be readily compared between the experimental groups (Fig. 1). This suggested that mCD1.1 is able to inhibit NK cell cytotoxic activity, but perhaps only a small fraction of the total poly I:C–activated NK cell population is negatively regulated by mCD1.1. Therefore, we performed cytolytic assays using B6 A-LAKs to test the possibility that mCD1.1-regulated killer cells could be expanded when cultured in the presence of rIL-2. The RMA/S cell line and two independent RMA/S.CD1.1 transfectant clones were compared for their sensitivity to lysis by nylon wool nonadherent B6 spleen cells that become plate adherent in succeeding days of culture in rIL-2. RMA/S and the two RMA/S.CD1.1 transfectants were susceptible to lysis by the day 3 and 4 A-LAKs. Nevertheless, a moderate reduction in lysis by these A-LAKs of both RMA/S.CD1.1 transfectants relative to RMA/S was detected (Fig. 2). Interestingly, day 5 and 6 A-LAK cytotoxic activities against the RMA/S.CD1 transfectants was substantially reduced relative to the highly sensitive RMA/S target cell (Fig. 2). Resistance to lysis of the

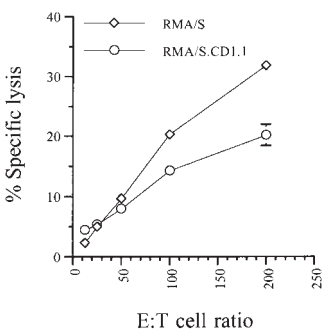

Figure 1. mCD1.1 partially inhibits the cytotoxic activity of poly I:C–activated B6 NK cells. Mice were injected with 200 μg of poly I:C, and 18 h later the spleen cells were harvested and the plastic nonadherent spleen cells were used as effector cells for the lysis of ^{51}Cr-labeled RMA/S and RMA/S.CD1.1. Each E/T ratio was used for triplicate determinations, and the results are expressed as means ± SD. Spontaneous release values were <10.3%. Similar results were obtained in three separate experiments.

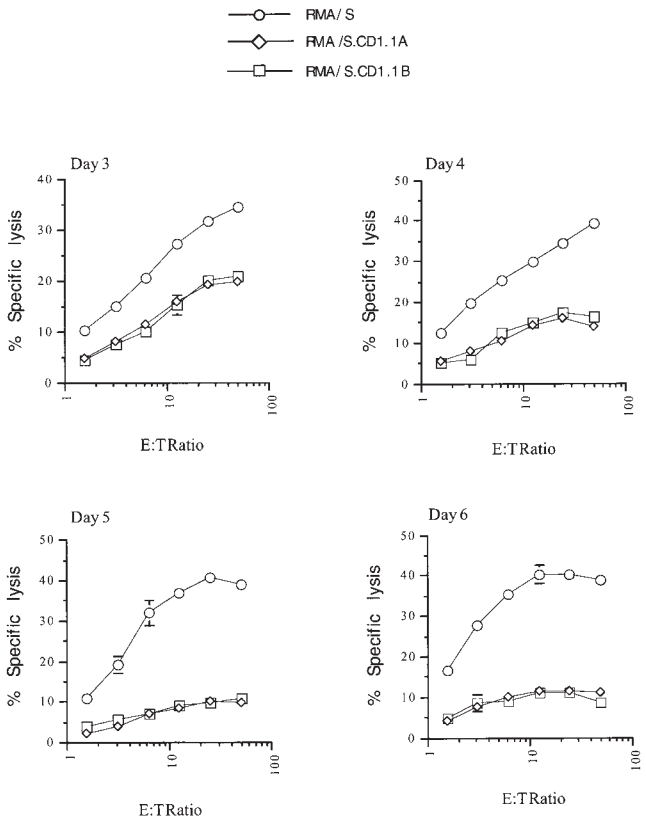

Figure 2. The extent of mCD1.1 inhibition of B6 A-LAK cytotoxic activity is affected by passage of LAKs. B6 A-LAKs were generated by daily transfer after day 3 of the NA-LAKs to new flasks. Lysis of two independent RMA/S.CD1.1 transfectant clones, RMA/S.CD1.1A and RMA/S.CD1.1B, was compared with RMA-S cells. The ^{51}Cr-labeled targets were incubated with the A-LAKs from all groups obtained at day 7 of culture at different E/T ratios for 4 h at 37°C. Each E/T ratio was used for triplicate determinations, and results are shown as the means ± SD. All spontaneous release values were <10.2%. Similar results were obtained in six separate experiments.

RMA/S.CD1.1 transfectants was found to be enhanced despite an increase of lysis observed for the untransfected RMA/S target cell at lower E/T ratios by the day 5 and 6 A-LAKs (Fig. 2). The two CD1 transfectants, RMA/S.CD1.1A and RMA/S.CD1.1B, express comparable densities of CD1.1 (data not shown) and show a similar level of resistance to A-LAK lysis, indicating that the results are reproducible, occurring with more than one CD1.1 transfectant. Subsequent experiments described in this report were carried out with the RMA/S.CD1.1A transfectant, nominally referred to as RMA/S.CD1.1. Taken together, the preceding results obtained with IL-2–activated A-LAKs strongly argue that mCD1.1, like classical class I MHC, may negatively regulate A-LAK cytotoxic activity. In agreement with the poly I:C data, it seems that a small proportion of the initial A-LAK population may be negatively regulated by mCD1.1, which possibly can be expanded by passage and culture in the presence of rIL-2.

The A-LAK–mediated cytotoxicity data demonstrated the protective effect of mCD1.1 expression on RMA/S susceptibility to A-LAK lysis. However, it did not show its target protection/A-LAK inhibitory efficiency compared with that of classical class I MHC. To address this issue, the level of RMA/S.CD1.1 lysis by pooled day 5 and 6 B6 A-LAKs was compared with that of RMA (from which the class I MHC–deficient RMA/S is derived). The class I MHC–positive cell line, RMA, is resistant to day 5 and 6 A-LAK lysis (Fig. 3) and A-LAKs selected at several other time points of culture in rIL-2 (data not shown), whereas RMA/S is lysed to a similar degree as the NK cell–sensitive T lymphoma, Yac-1. In contrast, RMA/S.CD1.1 is as resistant to day 5 and 6 A-LAK cytotoxic activity as RMA (Fig. 3). Thus, these results suggest that mCD1.1 can negatively regulate NK cell cytotoxicity to the same extent as classical class I MHC. Since the day 5 and 6 B6 A-LAKs failed to lyse both RMA and RMA/S.CD1.1, this further suggests that these A-LAKs can be negatively regulated by both classical MHC class I molecules and mCD1.1.

It might be argued that upon mCD1.1 transfection, RMA/S simply becomes resistant to cell-mediated cytotoxicity, independent of killer cell recognition events. However, this interpretation is not supported by the observation that both RMA/S and RMA/S.CD1.1 were lysed efficiently by A-LAKs generated from early time points (Fig. 2). In addition, after induction of higher classical class I MHC expression at 26°C and incubation with peptide antigen, both RMA/S and the RMA/S.CD1.1 transfectant were lysed to a similarly high level by the influenza NP–specific, D^b-restricted CTL clone 3/4 (Fig. 4). For instance, only after pulsing with D^b-specific influenza NP peptide, but not in the absence of peptide or in the presence of the K^b-binding OVA peptide, SIINFEKL (data not shown), can lysis be detected. This indicated that intracellular apoptotic pathways remain intact upon mCD1.1 transfection. Thus, RMA/S.CD1.1 is not intrinsically resistant to cell-mediated lysis.

NK Cell Cytotoxic Activity Is Inhibited by mCD1.1 Expression. Our results obtained with A-LAKs do not directly assess whether mCD1.1 inhibits the cytotoxic activity of NK cells or some other cell types. Therefore, we directly addressed this issue by using effector cells derived from B6 × 129 Rag-$1^{-/-}$ mice. Since the recombination activating gene, Rag-1, is critical in V(D)J recombination in both B and T cells, Rag-$1^{-/-}$ mutant mice do not produce any mature B and T lymphocytes (37). Therefore, the A-LAKs generated from Rag-$1^{-/-}$ will not contain CTLs or NKT cells, and thus no cell-mediated cytolysis will be contributed by these populations. As expected, upon culturing spleen cells from B6 × 129 Rag-$1^{-/-}$ mice with rIL-2, $CD3^-$, TCR-α/β^-, and $NK1.1^+$ A-LAKs were detected by FACS® and no $CD3^+$ and TCR-α/β^+ were present (data not shown). Our results indicate that RMA/S.CD1.1 but not RMA/S is resistant to lysis by rIL-2–activated Rag-$1^{-/-}$ NK cells (Fig. 5). We conclude that NK cell cytotoxic activity against the NK cell–sensitive RMA/S target cell can be inhibited by mCD1.1 expression.

mCD1.1 Inhibits A-LAK Cytotoxic Activity Generated from Mouse Strains of Different H-2 Haplotypes. Since CD1 is a nonpolymorphic class I MHC molecule, this implies that any NK receptor(s) which may mediate negative regulatory signal(s) upon mCD1.1 engagement could be conserved in mice. Thus, mCD1.1 may be able to inhibit the cytotoxic activities of A-LAKs derived from different inbred mouse strains. To test this hypothesis, we generated A-LAKs from two additional mouse strains, CBA/J ($H\text{-}2^k$) and NZB/BinJ ($H\text{-}2^d$). The results indicated that the A-LAKs from these mouse strains exhibit similar patterns of mCD1.1-

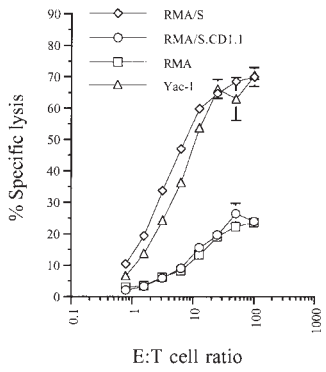

Figure 3. Expression of mCD1.1 renders RMA/S as resistant to day 5 and 6 A-LAKs as the classical class I MHC–expressing RMA cell line. ^{51}Cr-labeled targets were incubated with pooled day 5 and 6 B6 A-LAKs for 4 h. Each data point represents triplicate determinations, and data are shown as means ± SD. All spontaneous release values were <17.9%. This experiment was repeated three times with similar results.

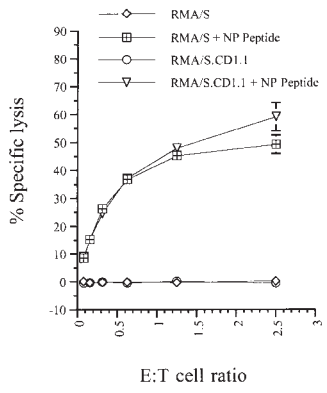

Figure 4. The mCD1.1 transfectant of RMA/S is not intrinsically resistant to cell-mediated lysis. RMA/S and RMA/S.CD1.1 cells were grown overnight at 26°C, then ^{51}Cr-labeled and pulsed with or without 400 μg/ml of NP peptide at 37°C for 1 h. The cells were then incubated with CTL clone 3/4 for 4 h at various E/T ratios in triplicate. Results are shown as means ± SD. In all cases, the spontaneous release values were <9.4%. Identical results were obtained in two additional experiments.

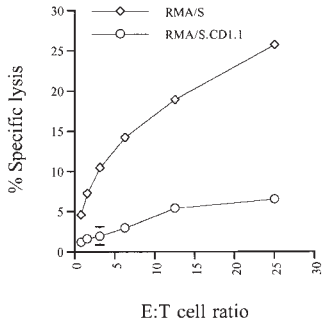

Figure 5. A-LAKs derived from 129 × B6 Rag-$1^{-/-}$ mice lyse RMA/S, but not the RMA/S.CD1.1 transfectant. ^{51}Cr-labeled targets were incubated with Rag-$1^{-/-}$ A-LAKs at different E/T ratios for 4 h. Each E/T ratio was used for triplicate determinations, and the data are expressed as means ± SD. The spontaneous release values were <9.6%. Similar results were obtained in three separate experiments.

mediated inhibition as seen in the B6 A-LAKs (Fig. 6, A and B). For instance, the day 3 A-LAKs lysed RMA/S and RMA/S.CD1.1, whereas a significant reduction in lysis of RMA/S.CD1.1 relative to RMA/S was detected if day 5 and 7 A-LAKs were used as effectors. It is perhaps worth noting that the patterns of inhibition among these mice are not exactly identical. In both B6 and NZB/BinJ mice, RMA/S is highly susceptible to lysis by all A-LAK populations, and the reduction of RMA/S.CD1.1 lysis becomes clear in those A-LAKs obtained at later time points (Fig. 2, and Fig. 6 A). In contrast, A-LAKs derived from CBA/J show a somewhat different kinetic pattern. The day 3 A-LAKs have low cytotoxicity against RMA/S, and it only became strongly observable with day 7 A-LAKs (Fig. 6 B), whereas RMA/S.CD1.1 remained relatively resistant to A-LAKs obtained at all of the various culture periods with rIL-2 (Fig. 6 B). The reasons for the somewhat different kinetic inhibition patterns are not clear. Nevertheless, these results demonstrate that mCD1.1 is able to suppress A-LAK activities of different mouse strains expressing distinct H-2 haplotypes, consistent with the possibility that a conserved NK inhibitory receptor(s) for mCD1.1 may exist that is able to suppress A-LAK cytotoxic activity.

Anti-mCD1.1 mAb, 1B1, Partially Restores the A-LAK Lysis of RMA/S.CD1.1. The preceding in vitro cytotoxic assays suggest that mCD1.1 is capable of negatively regulating A-LAK cytotoxic activity. To confirm this, we tested whether the inhibition of lysis of RMA/S.CD1.1 target could be reversed in the presence of an antibody that recognizes mCD1.1. Consistent with the previous results, reduction of A-LAK–mediated lysis of RMA/S.CD1.1, relative to RMA/S, was observed in the absence of any mAb (Fig. 7). However, with a final concentration of 20 μg/ml of the anti-mCD1.1 mAb, 1B1, lysis of RMA/S.CD1.1 was partially restored (Fig. 7). This restoration of lysis is not mediated by antibody-dependent cell-mediated cytotoxicity, since in the presence of an isotype control mAb, M1/89.18.7.HK specific for CD45, no restoration of lysis was detected (Fig. 7). These data indicate that cell surface expression of mCD1.1 is likely to be critical in inhibiting the A-LAK cytotoxic activity. In all attempts with the 1B1 antibody, we were unable to completely restore the killing of RMA/S.CD1.1 to the same level as that of RMA/S. The reason for this incomplete restoration of lysis by 1B1 is not clear. It is perhaps interesting to note that 1B1 also only partially inhibits peptide-specific and mCD1.1-restricted CTL lysis (38). It is possible that 1B1 recognizes an epitope that is only partially overlapping with the putative receptor(s) that mediates the inhibition of A-LAK lysis.

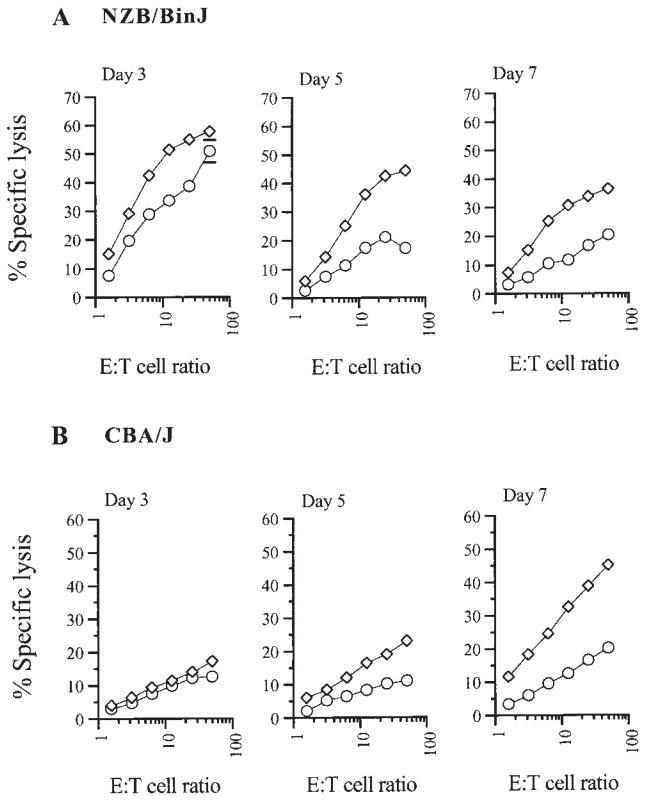

Figure 6. Cytotoxicity by A-LAKs generated from NZB/BinJ (A) and CBA/J mice (B) is inhibited by mCD1.1. The day 3, 5, and 7 A-LAKs from both strains of mice were incubated with ^{51}Cr-labeled RMA/S (◇) or RMA/S.CD1.1 (○) target cells. Each data point is the mean of triplicate wells ± SD. Spontaneous release values were <15%. This experiment was performed twice with effector cells from both mouse strains.

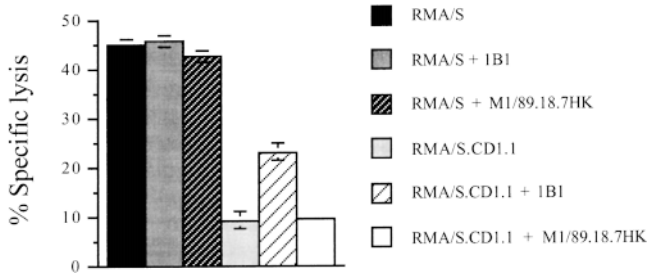

Figure 7. Antibody against mCD1.1 but not CD45 partially restores day 6 A-LAK cytotoxic activity against RMA/S.CD1.1. ^{51}Cr-labeled RMA/S.CD1.1 or RMA/S targets were incubated at a 25:1 E/T ratio with A-LAKs in the presence of 20 μg/ml of 1B1, anti-mCD1.1, or M1/89.18.7.HK, anti-CD45, for 4 h at 37°C. Each mAb treatment was carried out in triplicate, and the percentage of lysis is shown as mean ± SD. Spontaneous release values were <8.6%. This experiment was performed six times with similar results.

Discussion

In this study, we have shown that A-LAK/NK cytotoxic function is negatively regulated by mCD1.1. We first observed that mCD1.1 only partially inhibited the cytotoxicity of poly I:C–activated NK cells. There are at least two possible explanations for this observation: that only a small fraction of the total NK population expresses the KIR(s) for mCD1.1, or that only this population expresses the relevant receptor(s) at high enough density to deliver the inhibitory signal(s). Regardless of the true nature of this population, it or a subset with similar properties can be detected by consecutively transferring NA-LAKs early in culture in rIL-2 to generate A-LAKs later in culture. Whether this protocol actually expands a relevant but small NK subpopulation, or whether it simply induces upregulated expression of an inhibitory receptor specific for mCD1.1, remains to be determined.

The mCD1.1 protein is by far the most divergent class I molecule demonstrated to inhibit NK cell cytotoxic activity. However, it has been reported that human CD1a, b, and c do not protect target cells from NK lysis (39). It is important to note that these group 1 CD1 molecules have rather different amino acid sequences than the group 2 CD1 molecules, which includes human CD1d, mCD1.1, and mCD1.2. For instance, the α1 and α2 domains of human CD1d are more similar to that of mCD1.1 and 1.2 than they are to the other human CD1 molecules (20). These differences might explain why human CD1a, b, and c failed to inhibit NK cell cytotoxic function. Therefore, it will be interesting to determine whether human CD1d also protects target cells from lysis. Another major difference between the earlier human CD1 study and ours is that the NK cells used earlier were isolated from peripheral blood, and in some instances were further activated by rIL-2 for only 18 h (39). It is possible that this short-term culturing condition did not allow the upregulation of an inhibitory receptor(s) specific for CD1, outgrowth of relevant cell subpopulations, or other changes that would result in CD1-mediated protection of target cells.

mCD1 is encoded by two highly related genes resulting from gene duplication, mCD1.1 and mCD1.2, which are $>$95% homologous at the level of amino acid sequences (17, 18). However, based on the presence of mRNAs, their expression levels are rather different. For instance, mCD1.1 is highly expressed in all tissues compared with mCD1.2, except in thymus where both mCD1 are expressed to approximately the same level (40). In addition, mCD1.2 lacks a conserved cysteine residue in its α2 domain, which is presumably important in forming a disulfide bridge in mCD1.1 and other class I MHC molecules (40). Thus, mCD1.2 might not be functional. Whether mCD1.2 can inhibit NK cell cytotoxic activity similar to mCD1.1 remains to be determined. It is reported that B6 mice do not express mCD1.2 due to a frameshift mutation in exon 4 encoding the α3 domain (41). The significance of this is not clear, but it raises the possibility that a deficiency in mCD1.2 expression might influence the expression level of putative inhibitory receptor(s) for mCD1.1, as can occur with NK inhibitory receptors specific for classical class I MHC molecules (42).

The TAP-2–deficient cell line, RMA/S, can present peptides derived from cytosolic proteins on classical class I MHC through a TAP-independent pathway (43, 44). Thus, it can be argued that the protective effect seen in RMA/S.CD1.1 is actually mediated by classical class I MHC and mCD1.1 only indirectly. For instance, mCD1.1-derived peptides might be bound to and stabilize the normally empty and thermally unstable D^b and K^b molecules expressed by RMA/S. This in turn would protect the target cells from A-LAK lysis. However, this argument is not supported by the fact that no increase in cell surface expression of D^b and K^b can be detected in the mCD1.1 transfectant (data not shown). Regulation of NK cell cytotoxicity by human nonclassical class I HLA-E has been described recently (31, 32). It was shown that HLA-E presentation of leader sequences derived from other HLA molecules is critical in this HLA-E–mediated inhibition, through interaction with the CD94/NKG2A heterodimer (31, 32). Since murine nonclassical class I Qa-1^b is the murine homologue of HLA-E, it is possible that the mCD1.1 inhibition of A-LAK cytotoxic activity is actually mediated by Qa-1^b. However, this interpretation is highly unlikely, since the leader sequence of mCD1.1 does not contain the necessary motif to bind Qa-1^b. An additional argument against such interpretations of the data is that an mCD1.1-specific mAb partially restored the susceptibility of RMA/S.CD1.1 to NK lysis. This indicated that the suppression was indeed mediated by intact, cell surface mCD1.1. We have shown previously that untransfected RMA/S cells do express some cell surface mCD1 (34). However, the amount of mCD1 expression by these cells is much less than the expression level in RMA/S.CD1.1 transfectants. In fact, the level of mCD1.1 expression in RMA/S cells supports a response to lipoglycan α-galactosylceramide, but it is not sufficient for stimulating peptide-specific T cells (30), in addition to not inhibiting NK cell activity. However, it should be noted that the level of mCD1.1 expression on the RMA/S transfectant is not unphysiologic, and is similar to the level present on B cells and freshly isolated dendritic cells (41, 45).

mCD1.1 is nonpolymorphic (17, 18), and we observed that its expression protected RMA/S from lysis by A-LAKs from a variety of mouse strains. The conserved structure of mCD1.1 and the broad pattern of A-LAK inhibition suggest the existence of a conserved inhibitory receptor(s) specific for mCD1.1 expressed by A-LAK cells of the strains of mice we have tested. It is unlikely that the inhibition is mediated by lectin-like NK inhibitory receptors Ly-49A, G, or C/I, since these molecules are detected on B6 day 3 and 4 A-LAKs as well as A-LAKs obtained later in culture, and RMA/S.CD1.1 is highly susceptible to lysis by these A-LAKs (data not shown). Based on the inhibition pattern of mCD1.1, one would predict that the receptor(s) would be absent or reduced in expression on day 3 and 4 A-LAKs

but present on A-LAKs from later time points. Since no mAbs specific for other potential inhibitory Ly-49 family members such as Ly-49B, E, and F have been described, we do not know the expression pattern of these molecules on A-LAKs generated at different time points. It remains possible that Ly-49 members with divergent amino acid sequences, such as Ly-49B, might recognize mCD1.1. However, it has been demonstrated that inhibition of NK function by class I MHC molecules can be achieved in the absence of Ly-49 receptor expression (46–48). This is especially true for NK cells derived from fetal tissues (46–48). Interestingly, Kumar and colleagues suggest that fetal Ly-49$^-$ NK cells might be regulated by nonclassical class I MHC molecules (47). Nevertheless, the suggestion of a lectin-like inhibitory receptor for mCD1.1 is rather interesting, especially since mCD1.1 possesses five potential N-linked glycosylation sites on its extracellular domains (40). With the recent cloning of murine CD94 and the suggestion of the existence of murine NKG2 (49), CD94 heterodimers can potentially be the receptor(s) for mCD1.1. However, mAbs specific for these receptors have to be developed to assess their involvement in mCD1.1-mediated inhibition. Another candidate for an mCD1.1-specific inhibitory receptor is gp49B1, which belongs to the Ig superfamily (50). However, this inhibitory receptor is expressed on all NK cells, and can be detected on A-LAKs that have been cultured for 3 d (50). Thus, it seems unlikely that gp49B1 is the inhibitory receptor for mCD1.1. At present, the identity of mCD1.1-specific inhibitory receptor(s) remains to be determined.

Mass spectrometry and radiolabeling data have shown that the majority of the material bound to mCD1.1 in normal cells is a glycosylphosphatidylinositol (51), while CD1 molecules can also bind, as well as present, non–self-antigenic glycolipids to NKT cells (29, 30). Therefore, it can be speculated that mCD1.1 may represent a means by which NK cells can differentiate self- and non–self-antigens. For instance, mCD1.1 might only suppress NK cell functions when bound with a self–natural ligand, but not when antigenic microbial lipoglycan antigens are being presented. For the best-characterized Ly-49 family member, Ly-49A, its interaction with class I MHC molecules appears to be peptide dependent, but not peptide specific (52). In contrast, the CD94/NKG2A heterodimer only recognizes HLA-E in the presence of leader peptides derived from certain HLA products (31, 32). Thus, it is possible that mCD1.1 can regulate NK cell function in a ligand-dependent manner, but this remains to be examined.

It is known that the distribution of various inhibitory receptors is not restricted to NK cells. For example, in addition to NK cells, the Ly-49 inhibitory receptors are known to be expressed by subsets of NK1.1$^+$CD3$^+$ NKT cells and NK1.1$^-$CD3$^+$ T cells (53). Upon engagement with class I MHC, these inhibitory receptors can downregulate the cytotoxicity and cytokine production by these T cells (53). Similar results are demonstrated with human CTLs. For instance, cross-linking of the p58 KIR on CTLs can partially inhibit their cytotoxic activities (54). We have provided evidence that mCD1.1 protects target cells from NK cell lysis, and these results suggest that NK cells may express a receptor for mCD1.1. It is possible that the putative mCD1.1-specific inhibitory receptor(s) also has a broader cellular distribution that includes T cells. Since the nature of this receptor(s) is not known, this hypothesis cannot be tested at the present time. However, it is interesting to note that the pool of mCD1.1-autoreactive T cells can be quite large. It is estimated that these T cells range from 0.5 to 2% of the lymphocytes in the spleen, and comprise a much larger percentage of the T cells in the bone marrow and liver (55). These lymphocytes are reported to be mCD1-autoreactive, but how these autoreactive T cells are regulated is not entirely clear. Bendelac and colleagues suggest that the autoreactivity of these T cells is normally suppressed by MHC-specific inhibitory receptor(s) (41). They further suggest that loss of negative regulation through these MHC-specific inhibitor receptor(s) could lead to activation of these autoreactive T cells (41). Consistent with this, the autoreactivity of NKT cells for mCD1 is most striking when hybridomas made from this population are analyzed. The hybridomas lose expression of NK1.1, and as a result of fusion, they also may lose expression of other NK cell receptors, thereby revealing their reactivity for self-mCD1. Thus, it is possible that the mCD1.1-specific inhibitory receptor(s) suggested by our study may also be originally expressed by these mCD1.1-autoreactive T cells. The possibility that this putative receptor(s) recognizes mCD1.1 in a ligand-dependent manner is an attractive model for suppressing mCD1.1-specific autoreactive T cells. Therefore, the identification of inhibitory receptor(s) recognizing mCD1.1 may have relevance in understanding the regulation of both NK and NKT cell activities.

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