Recognition of CHO cells by inhibitory and activating Ly-49 receptors

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Abstract: Upon ligand recognition, members of the murine Ly-49 receptor family can transmit inhibitory or activating signals that regulate NK cell function. Ly-49A, G, and D have been shown to recognize the murine class I molecule H-2D\textsuperscript{d} as a potential ligand. Recent studies also have demonstrated also that Ly-49D\textsuperscript{+} NK cells can lyse CHO cells, although the ligand responsible for this recognition was not identified. Because allorecognition by NK cells may be important in bone-marrow transplantation and because of the overlapping class I recognition by these receptors, recognition of CHO cells by Ly-49G and A was investigated. Our data suggest that Ly-49G and probably A transmit inhibitory signals in response to CHO cells. Receptor inhibition was assessed by examining NK lytic function, IFN-\(\gamma\) secretion, and DAP12 phosphorylation in response to CHO cells by sorted subsets of Ly-49D vs. G B6 NK cells. Our results suggest that CHO cells may express a common ligand(s) that is capable of engaging Ly-49D, G, and possibly A in C576BL/6 NK cells. In addition to our findings that Ly-49 inhibitory receptors also recognize CHO cells, activating receptors other than Ly-49D are present in B6 mice that can lyse CHO cells. J. Leukoc. Biol. 68: 583–586; 2000.

Key Words: Ly-49 · NK · CHO · IFN-\(\gamma\) · DAP-12

INTRODUCTION

Natural killer (NK) cell killing appears to be regulated by a combination of inhibitory and activating cell-surface receptors. Although structurally distinct, functionally similar families of receptors exist on human (KIR/KAR) [1, 2] and mouse (Ly-49) [3, 4] NK cells. Although the Ly-49 family contains multiple inhibitory receptors (Ly-49A, B, C, E, F, G, and I-L), only two activators, Ly-49D [5] and H, are known to be expressed on NK cells. Ly-49A and G are inhibitory receptors that recognize H-2D\textsuperscript{d}, and the activating Ly-49D receptor also recognizes this ligand [6–8]. Recognition of “self” can be explained readily by the inhibitory Ly-49s in the context of the “missing self hypothesis,” where these inhibitory receptors function normally to down-regulate NK cell activity against self major histocompatibility complex (MHC). However, recognition of self by activating Ly-49 receptors is more difficult to reconcile.

Ly-49D has been characterized as an activating receptor on murine NK cells because Ly-49D\textsuperscript{+} NK cells: 1) mediate redirected lysis of FcR\textsuperscript{+} target cells in the presence of specific antibody to Ly-49D [5], 2) mediate rejection of H-2\textsuperscript{d} bone marrow cells [6], 3) show enhanced lysis of H-2\textsuperscript{d} concanavalin A (Con A) lymphoblasts [7], and 4) demonstrate enhanced lysis of H-2\textsuperscript{d} transfected target cells [8]. Furthermore, Ly-49D associates with the signaling moiety DAP12 that is phosphorylated on tyrosine residues upon receptor cross-linking [9–11]. Recently, we have shown enhanced secretion of interferon (IFN)-\(\gamma\) and tyrosine phosphorylation of DAP12 upon Ly49D/ H-2D\textsuperscript{d} interaction [12]. Together, this evidence strongly suggests that Ly-49D recognizes H-2D\textsuperscript{d}, resulting in NK cell activation.

Recent data by Idris et al. [13] suggest that Ly-49D\textsuperscript{+} NK cells recognize Chinese hamster ovary (CHO) cells and are responsible for their elimination in vitro. In this study, most but not all lysis of CHO cells could be blocked by monoclonal antibody (mAb) to Ly-49D. We were interested in whether the class I inhibitory receptors Ly-49G and/or A might recognize CHO cells. Our data suggest that Ly-49G and possibly A can recognize CHO cells, because their inhibitory functions can be blocked by the addition of mAb to these receptors. Our results demonstrate that although Ly49D recognizes CHO cells in B6 mice as activating, Ly-49G also recognizes CHO cells as inhibitory. Furthermore, activating receptors other than Ly-49D are capable of recognizing CHO cells, leading to their destruction.

MATERIALS AND METHODS

NK isolation, cell sorting, and antibodies utilized

Splenic NK cells were isolated from C57BL/6 mice, as previously described [14]. Fluorescein isothiocyanate (FITC)-4D11 (Ly-49G), FITC-YE1-48 (Ly-49A), and phycoerythrin (PE)-4E5 (Ly-49D) were used to separate Ly-49 A, G, and D subsets on a MoFlo Cell Sorter (Cytomation, Ft. Collins, CO). NK subsets were cultured for 9–10 days in 1000 U/ml interleukin (IL)-2 (Chiron, Emeryville, CA). Ly-49D-G and Ly-49D-A cell subsets consisted of >95% Ly-49D\textsuperscript{+} and <5% Ly-49G/A\textsuperscript{+} cells; the Ly-49D-G subset contained >95% Ly-49D-G\textsuperscript{+} and 27% Ly-49G\textsuperscript{+} cells; and the Ly-49D-D\textsuperscript{+} subset contained >90% Ly-49G\textsuperscript{+} and <3% Ly-49D\textsuperscript{+} cells and 40% Ly-49A\textsuperscript{+} cells. Control antibodies consisted of a rat immunoglobulin G
DAP12 phosphorylation in response to CHO cells. Ly-49D\(^\text{+}\)G\(^\text{+}\) A\(^\text{-}\) and Ly-49D\(^\text{+}\)G\(^\text{+}\) B6 NK cells were combined with CHO cells for the indicated times at 37°C and examined for phosphorylation of Ly-49D-associated DAP12 upon immunoprecipitation with mAb 4E5. These data are representative of at least three such experiments performed.

Fig. 1. DAP12 phosphorylation in response to CHO cells. Ly-49D\(^\text{+}\)G\(^\text{+}\) A\(^\text{-}\) and Ly-49D\(^\text{+}\)G\(^\text{+}\) B6 NK cells were combined with CHO cells for the indicated times at 37°C and examined for phosphorylation of Ly-49D-associated DAP12 upon immunoprecipitation with mAb 4E5. These data are representative of at least three such experiments performed.

Fig. 2. Lysis of CHO cells by Ly-49D vs. G subsets. B6 NK cell subsets were pretreated with the indicated mAb and tested for their ability to lyse CHO cells in a 4-h cytotoxicity assay. This augmentation of lysis by antibody suggest strongly that Ly-49G recognizes a ligand on CHO cells as inhibitory and that blocking this inhibitory receptor/ligand interaction allows lysis to occur. As demonstrated previously, antibodies to Ly-49D blocked lysis of CHO cells by both Ly-49D\(^\text{+}\)G\(^\text{-}\) subsets, whereas PK-136, which binds

RESULTS

Ly-49G inhibits Ly-49D-mediated DAP12 phosphorylation by CHO cells

Recently, our laboratory has described the phosphorylation of Ly-49D-associated DAP12 when Ly-49D\(^\text{+}\)G\(^\text{-}\) A\(^\text{-}\) NK cells are combined with YB20 target cells transfected with H-2D\(^d\) (YB/D\(^d\)) [12]. Phosphorylation of DAP12 could not be detected upon immunoprecipitation with mAb 4E5 and required the use of rabbit antisera prepared against Ly-49D/DAP12 immune complexes. In this study, DAP12 phosphorylation was observed after a 1-min incubation of Ly-49D\(^\text{+}\)G\(^\text{+}\) A\(^\text{-}\) NK cells and YB/D\(^d\) targets, after which it was no longer detectable.

Furthermore, Ly-49D\(^\text{+}\)G\(^\text{+}\) cells did not respond to YB/D\(^d\) targets with Ly-49D-associated DAP12 phosphorylation, consistent with the inhibitory role of Ly-49G for H-2D\(^d\). Here, we examined the ability of CHO cells to mediate phosphorylation of Ly-49D-associated DAP12 using similar NK cell subsets. Figure 1 demonstrates that CHO cells are potent mediators of Ly-49D-associated DAP12 phosphorylation as compared with our previous studies using the YB/H-2D\(^d\)-transfected cell line. The following observations suggest that Ly-49D interacts strongly with CHO cells as compared with our previous studies using H-2D\(^d\)-transfected YB20 target cells: (1) Phosphorylation of DAP12 is prolonged significantly up to 20 min following CHO cell stimulation. (2) Much lower numbers of NK cells can be used to demonstrate DAP12 phosphorylation. (3) DAP12 phosphorylation can be detected using mAb 4E5. (4) DAP12 phosphorylation can be demonstrated in the Ly-49D\(^\text{+}\)G\(^\text{+}\) subset of NK cells, although with a somewhat abbreviated response. However, because phosphorylation of DAP12 in the Ly-49D\(^\text{+}\)G\(^\text{+}\) cells was attenuated when compared with the Ly-49D\(^\text{+}\)G\(^\text{-}\) A\(^\text{-}\) and no longer observed after 20 min of incubation, we examined Ly-49G for its ability to inhibit NK cell lysis and IFN-γ secretion in the presence of CHO cells.

Blocking Ly-49G augments lysis of CHO cells

Figure 2 confirms the data of Idris et al. [13] that NK subsets containing Ly-49D\(^-\) cells apparently contain most of the lytic activity against CHO cells. The Ly-49D\(^\text{+}\)G\(^\text{-}\) A\(^\text{-}\) subset and the Ly-49D\(^\text{+}\)G\(^\text{+}\) subset lysed CHO cells, and the Ly-49G\(^\text{-}\)D\(^\text{-}\) subset did not lyse these targets. However, because the lysis of CHO cells by the Ly-49D\(^\text{+}\)G\(^\text{-}\) subset was much less efficient than the Ly-49D\(^\text{+}\)G\(^\text{-}\) A\(^\text{-}\) subset, Ly-49G could recognize a ligand on CHO cells as inhibitory. The addition of antibodies to Ly-49G resulted in a three-fold increase in lysis of CHO cells by the Ly-49D\(^\text{+}\)G\(^\text{+}\) subset and a 20-fold increase in lysis by the Ly-49G\(^\text{-}\)D\(^\text{-}\) subset. This augmentation of lysis by antibody suggest strongly that Ly-49G recognizes a ligand on CHO cells as inhibitory and that blocking this inhibitory receptor/ligand interaction allows lysis to occur. As demonstrated previously, antibodies to Ly-49D blocked lysis of CHO cells by both Ly-49D\(^\text{+}\) subsets, whereas PK-136, which binds

IFN-γ assays

NK cells were pretreated with mAb at concentrations of 5 μg/5 × 10⁵ cells in 0.5 ml RPMI 1640 + 5% fetal bovine serum (FBS) for 15 min at room temperature in 24-well plates. CHO cells (2.5×10⁵) were added to each well, and the plates were incubated for 6 h at 37°C, after which supernatants were collected and analyzed for IFN-γ by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN).

Cytotoxicity assays

NK cell subsets were plated into 96-well plates to yield various E:T ratios, starting at 20:1 (in triplicate), pretreated with mAb at 2 μg/well for 15 min at room temperature, and assayed against 51Cr-labeled CHO cells in a 4-h cytotoxicity assay.

Ly-49D-mediated DAP12 phosphorylation

After each population was chilled on ice for 5 min, NK cells (3×10⁶) were combined with CHO cells (1.5×10⁶) in 1.5 ml microcentrifuge tubes in a total of 100 μl RPMI 1640 + 0.5% bovine serum albumin (BSA). The cells were centrifuged for 5 sec at 3,000 rpm at 4°C. Cell combinations were lysed immediately in ice-cold 1% TX-100 (0°C) or incubated at 37°C for the indicated times and then lysed. Cell lysates were cleared by centrifugation and immunoprecipitated with mAb 4E5 plus protein G sepharose. Proteins were separated by electrophoresis, transferred to immobilon, and blotted with antiphosphotyrosine mAb (Bio 4G10) [9]. A rabbit antiserum prepared against the DAP12 peptide (TB5/20) was used to confirm the presence of DAP12 [11].
to NKR-PIC, had no effect on lysis by any subset. Therefore, Ly-49D and G appear to recognize CHO cells, consistent with their common recognition of H-2Dd.

Although the percentage of Ly-49A+ NK cells in the Ly-49D+G+ and Ly-49G+D− subsets was low (27% and 40%, respectively), addition of antibodies to Ly-49A resulted in some increase in lysis of CHO cells. The augmented lysis observed with antibodies to Ly-49G was increased further when combined with antibodies to Ly-49A (unpublished results). Because the percentage of Ly-49A+D−G− cells in bulk NK cell populations is very low, sorting these cells was not performed to confirm that recognition of CHO cells by Ly-49A occurs. Of particular concern in these assays was the possibility that a small population of Ly-49D+ cells remained in our Ly-49G+D− subset (usually 2–3%), which, potentially, could account for lysis of CHO cells in this population. Our best efforts to remove Ly-49D+ cells from the Ly-49G+ population resulted in a Ly-49G+ subset containing 1% Ly-49D+ cells. As seen in Figure 3, this population of NK cells appeared to have little, if any, innate ability to lyse CHO cells. However, upon addition of antibodies to Ly-49G, CHO cell lysis was enhanced greatly. Furthermore, addition of antibodies to Ly-49G and D resulted in substantial lysis of CHO cells, although almost 50% of this restored ability to lyse CHO cells appeared to be mediated by the Ly-49D+ cells present. Our results suggest strongly that Ly-49G−D− NK cells not only recognize CHO cells as inhibitory but express receptors other than Ly-49D that can recognize these targets and deliver activation signals to NK cells.

**Blocking Ly-49G augments IFN-γ secretion**

To confirm the lytic data, CHO cells were combined with NK cell subsets and examined for secretion of IFN-γ in the presence or absence of mAb to Ly-49D and/or Ly-49G. Figure 4 demonstrates that Ly-49D+G−A− cells secrete large amounts of IFN-γ when cocultured with CHO cells, and the addition of antibodies to Ly-49D inhibited IFN-γ secretion. However, the production of IFN-γ by the Ly-49D+G+ and Ly-49G−D− subsets was much lower in the presence of CHO cells. These data correlated well with our cytotoxicity data, suggesting that Ly-49G may recognize a ligand on CHO cells. Addition of antibodies to Ly-49G resulted in a four-fold increase in IFN-γ secretion by the Ly-49D+G+ subset and a 10-fold increase in IFN-γ secretion by the Ly-49G+D− cells. These results complemented those of our cytotoxicity assays and further support the fact that Ly-49G recognizes a ligand on CHO cells as inhibitory.

**DISCUSSION**

Our findings demonstrate that Ly-49G (and possibly Ly-49A) recognize CHO cells and deliver inhibitory signals to NK cells. These results have been confirmed using three different assays: 1) augmented lysis of CHO cells in the presence of antibodies to Ly-49G; 2) augmented IFN-γ secretion in the presence of antibodies to Ly-49G; and 3) attenuated levels of Ly-49D-associated DAP12 phosphorylation in Ly-49D+G+ NK cells when combined with CHO cells.

It has been shown that Ly-49D can recognize H-2Dα, α, and Dγ2 [8, 9]. The findings of Idris et al. [13] and this study demonstrate that CHO cells are potently lysed by Ly-49D+ NK cells. The ligand recognized by Ly-49D on CHO cells is speculated to be hamster class I protein. Therefore, the multiple target specificity of Ly-49D leaves open the question as to the physiological ligand for this receptor.

The observation that Ly-49D+ NK cells from B6 mice are responsible for recognition and lysis of CHO cells, and therefore represent the Chok locus, was significant in that it identified a unique NK cell-activating receptor that could mediate the rejection of a specific tumor-target cell [13, 15]. Although

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**Fig. 3.** Lysis of CHO cells by purified Ly-49G+D− NK cells. Ly-49G+D− NK cells containing only 1% Ly-49D+ cells were assayed against CHO cells as in Figure 1.

**Fig. 4.** IFN-γ secretion by Ly-49D vs. G subsets in the presence of CHO cells. B6 NK cell subsets pretreated with the indicated mAb were combined with CHO cells, and supernatants were assayed for IFN-γ after 6 h. These data represent one of at least three such experiments performed.
blocking of Ly-49D-mediated lysis of CHO cells with mA b 4E4 greatly reduced the lytic potential of Ly-49D⁺ NK cells in this study, it did not abrogate killing completely. This residual lysis could reflect simply a technical limitation in the ability to totally block lysis but probably represents additional activating receptors that recognize CHO cells (e.g., Ly-49H). The hypothesis that B6 NK cells contain activating receptors that recognize CHO cells other than Ly-49D is supported by our data with the Ly-49G⁺D⁻ subset of NK cells. Ly-49G⁺D⁻ NK cells can lyse CHO cells effectively in the presence of antibodies to Ly-49G and secrete significant amounts of IFN-γ. Furthermore, experiments with BALB/c NK (Ly-49D⁻) cells did not demonstrate lysis of CHO cells upon addition of antibodies to Ly-49G when bulk populations of NK cells were used (unpublished results). Comparable NK cell populations from B6 mice did mediate lysis of CHO cells in the presence of this antibody. These results support the findings of Idris et al. [13] that Ly-49D⁺ NK cells from B6 mice do lyse CHO cells and also demonstrate that other Ly-49 receptors, specifically Ly-49G and A, recognize these cells. In addition, our data suggest that additional receptors, which have the potential to lyse CHO cells, exist on B6 NK cells. Whether Ly-49A, G, and D recognize the same ligand on CHO cells or separate ligands remains to be resolved.

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REFERENCES