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Murine NK cells express inhibitory receptors belonging to the C-type lectin-like (Ly-49, CD94/NKG2) and Ig superfamily-related (gp49) receptors. The murine gp49B receptor displays structural homology with human killer inhibitory receptors, and was previously identified to be a receptor on mast cells and activated NK cells. The gp49B receptor is highly related to gp49A, a receptor with unknown function. In this study, using a novel mAb produced against soluble gp49B molecules that cross-reacts with gp49A, we examined the cellular distribution and function of these receptors. gp49 is constitutively expressed on cells of the myeloid lineage throughout development, as well as on mature cells. Importantly, gp49 is not expressed on spleen- and liver-derived lymphocytes, including NK cells, but its expression is induced in vitro on NK cells following IL-2 stimulation, or in vivo by infection with murine CMV. Molecular studies revealed that both the immunoreceptor tyrosine-based inhibitory motif-containing gp49B as well as immunoreceptor tyrosine-based inhibitory motif-less gp49A receptors are up-regulated on NK cells following murine CMV infection. When co-cross-linked with NK1.1, gp49B can inhibit NK1.1-mediated cytokine release by NK cells. Taken together, these studies demonstrate that the expression of gp49B on NK cells is regulated, providing the first example of an in vivo activation-induced NK cell inhibitory receptor, in contrast to the constitutively expressed Ly49 family. The Journal of Immunology, 2000, 164: 5215–5220.

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Abbreviations used in this paper: KIR, killer inhibitory receptor; CHO, Chinese hamster ovary; HPRT, hypoxanthine phosphoribosyltransferase; ILT, Ig-like transcript; ITIM, immunoreceptor tyrosine-based inhibitory motif; LAK, IL-2-activated NK cell; LIR, leukocyte Ig-like receptor; MCMV, murine CMV; SHP, src-homology-2 phosphatase-1.
study, we utilize a novel anti-gp49 Ab to further characterize the gp49 receptors. Unexpectedly, we failed to detect gp49 expression on naive splenic and liver-derived NK cells. Instead, only cells of the myeloid lineage constitutively express gp49. Although unstimulated NK cells are devoid of gp49, NK cells stimulated in vitro by IL-2 express gp49, including the inhibitory form, gp49B. Most importantly, gp49 expression is also induced on NK cells upon in vivo murine CMV infection, suggesting an important physiological role for gp49 receptors in regulating NK cell activity following viral infection.

Materials and Methods

Mice and mouse infections

C57BL/6, C57BL/6 (IFN-γ−/−), and C57BL/6 (IL-12−/−) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions at Washington University. Mice were analyzed between the ages of 10 and 16 wk. For mouse infections, under specific pathogen-free conditions at Washington University. Mice purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in St. Louis, MO). P815 and P8151 were grown in complete RPMI. CHO cells (dhfr−) were grown in complete F12 media, and P815 cells were transfected in complete RPMI. CHO + gp49 was obtained using Lipofectamine (Life Technologies, Rockville, MD)-facilitated stable transfection of CHO, with full-length gp49B cDNA in a dhfr-expressing vector pH6p-neo (35). Clones were selected based on ability to grow in α-MEM (no nucleosides), and expression was amplified by selection in 5 μM methotrexate (Sigma, St. Louis, MO). P815 and P815 + gp49A (14) were generously gifts from H. Katz (Harvard Medical School, Boston, MA). For the production of mAb H1.1. Armenian hamsters were immunized i.p. with 100 μg of gp49B-Fc (11) in Ribi adjuvant (RIBI ImmunoChem Research, Hamilton, MT) at 0, 4, 8, and 13 wk and boosted with 50 μg of gp49B-Fc 4 days before fusion. B cell hybridomas were produced by the Washington University Hybridoma Center and screened for anti-gp49B activity by ELISA. Candidate hybridomas were reactive with gp49B-Fc, but not Fc, and were cloned by limiting dilution. Two hybridomas met this criterion, and mAb H1.1 was selected for further analysis. mAb H1.1 was purified from cell-free culture supernatants by protein G-Sepharose chromatography and was labeled with 30% Percoll gradient (Sigma).

Cell purification

LAK cells were prepared as previously described (17). For RT-PCR analysis, NK1.1+ cells were isolated from the spleens of unprimed mice or from MCMV-infected mice on day 2 postinfection by cell sorting. Sorted cells were greater than 90% NK1.1+. Liver-derived lymphocytes were isolated by teasing cells into suspension and harvested from the interface of a 30% Percoll gradient (Sigma).

Flow cytometry

Cells were incubated with saturating concentrations of primary Ab(s) for 30 min. All Abs used were directly labeled with fluorochrome, except mAb B23.1. After two washes, cells treated with B23.1 were incubated with goat F(ab′)2, anti-mouse IgG FITC (Cappel) for 30 min. Cells were analyzed on a FACScan (Becton Dickinson, Sunnyvale, CA), and dead cells were excluded by propidium iodide staining. Depicted is the log of the mean fluorescence intensity staining by the indicated Ab.

mAb-mediated co-cross-linking and cytokine assays

A total of 20 μg/ml of streptavidin (Sigma) was immobilized on an Immulon 2 96-well plate (Dynex Technologies, Chantilly, VA). Wells were blocked with 2% BSA/PBS, followed by addition of the activating anti-NK1.1 Ab PK136 biotin (PharMingen). Where indicated, mAb H1.1 biotin or isotype control mAb 2C11 biotin (PharMingen) were also added. After three washes, individual wells were incubated with 2 × 105 KY-2 cells in complete RPMI (no IL-2) for 5 h, followed by harvest of cell-free supernatants. GM-CSF and IFN-γ levels were assessed by ELISA with cytokine minikits (Endogen, Woburn, MA) and extrapolated to a standard curve constructed with known cytokine concentrations.

RT-PCR analysis

Cells from the indicated tissue were homogenized, followed by RBC lysis in hypotonic lysis buffer. Total RNA was prepared using Ultraspec (Bio- tec, Houston, TX), from which first strand cDNA was synthesized using Superscript II (Life Technologies). PCR was performed in a Perkin-Elmer 9600 (Norwalk, CT) for 35 cycles (22 s at 94°C, 22 s at 56°C, 30 s at 72°C) using the following primers: HPRT, 5’ (404–426)-GTAATGATCGT CAACGGGGGC; HPRT, 3’ (580–557)-CGCAAGCTTGAATCCTAAAC CAACGGGGGC; HPRT, 3’ (1110–1096)-AGCCCAAGTTCAAAATTCGATTT; gp49A, 5’ (383–583)-GCAATAGCGCTATTATTTTGTGACGC; gp49B, 5’ (260–282)-AGCCCAAGTTCAAAATTCGATTT; and gp49B, 3’ (383–812)-GCATTAGCCCTCTTTTGTGCCCCAT.

Specificity of the gp49A and gp49B primers was established using an alternatively spliced gp49A cDNA, missing nucleotides 397–496, and full-length gp49B cDNA. PCR products were analyzed on a 1.2% agarose gel.

Results

Production of an anti-gp49B mAb

The gp49 receptor was originally defined by its reactivity with the B23.1 rat IgM mAb, which was, in our hands, produced by an unstable hybridoma (data not shown). To produce a stable hybridoma producing a high affinity anti-gp49B IgG mAb, we immunized Armenian hamsters with a gp49B-Fc fusion protein (11) that is reactive with mAb B23.1 (data not shown). Clone H1.1 was selected based on its specific reactivity with cells expressing transfected gp49B (Fig. 1A). Further demonstrating its specificity, H1.1 immunoprecipitated a ~55-kDa protein that is recognized in Western blot by a gp49B-specific anti-peptide Ab (data not shown) (11). In addition to its recognition of gp49B, mAb H1.1 cross-reacts with gp49A (Fig. 1A), as determined by flow-cytometric data of gp49A-transfected P815 cells. This was not unexpected given the high level of similarity between the extracellular domains of gp49A and B. Surprisingly, however, in contrast to previous published data (14), the B23.1 mAb also recognizes gp49A (Fig. 1A).

Finally, mAb H1.1 stains untransfected LAK cells and the NK cell clone, KY-2 (15) (Fig. 1B), both of which express gp49B transcripts (8). Thus, mAb H1.1 recognizes native gp49 molecules, permitting further analysis of the expression and function of the gp49 receptors.

Cellular distribution of gp49

Whereas gp49 is expressed on in vitro cultivated bone marrow- or serosal-derived mast cells (18) and LAK cells (8, 9), little is known about gp49 expression on naive cells of hemopoietic origin. We used flow cytometry to more closely examine the cellular distribution of gp49 using fluorochrome-labeled mAb H1.1. Approximately 60% of bone marrow cells from C57BL/6 mice stained with mAb H1.1 (Fig. 2). A similar proportion of cells from BALB/c-derived bone marrow was also mAb H1.1 positive (data not shown), consistent with the observation that gp49 is not polymorphic (8, 10, 13). The majority of the mAb H1.1+ cells also expressed high levels of Mac-1 and Gr-1, placing them in the myeloid lineage. Of the bone marrow lymphoid precursors in C57BL/6, a minor subpopulation of NK1.1+ cells bound to H1.1, while CD19 B cells and CD3+ T cells did not express gp49. Similar results were obtained from day 14 and day 17 fetal liver, in which gp49 expression was limited to cells bearing Mac-1 and Gr-1 (data not shown). These data indicate that gp49 is constitutively expressed on most cells of the myeloid lineage from bone marrow and fetal liver and only on a fraction of bone marrow-resident NK1.1+ cells.
Splenocytes (Fig. 2) and liver-derived lymphocytes (data not shown) were also tested for mAb H1.1 staining. In contrast to bone marrow cells, gp49 was not expressed on NK1.1 cells from spleen or liver. CD19+ B cells and CD3+ T cells also lacked gp49 expression. Instead, similar to the bone marrow, only Mac-1+ and Gr-1+ cells bound to mAb H1.1. These data establish that in contrast to LAK cells, naive mouse NK cells fail to express the gp49 receptor.

**Activation-induced expression of gp49 on NK cells**

Our previous immunofluorescence studies indicated that gp49B is expressed on NK1.1+ cells cultured for 7 days with IL-2 (8). We confirmed this analysis using mAb H1.1 and additionally found that gp49 is inducibly expressed on splenic NK1.1+ cells as early as 2 days after culture in IL-2 (data not shown). Consistent with our previous results, expression of gp49 on NK cells was maintained through day 7 of the IL-2 culture. Inasmuch as gp49 is not expressed on resting splenic NK cells, but is expressed on activated NK cells, these data suggest that NK cell expression of gp49 is induced by cellular activation.

We therefore extended this analysis to in vivo stimulation of NK cells by examining NK cells from mice challenged with MCMV, which is known to elicit an immediate NK cell response in C57BL/6 mice (19, 20). C57BL/6 mice were infected with a sublethal dose of MCMV and, at various time points after infection, cells from the bone marrow, spleen, and liver were examined by flow cytometry (Fig. 3A). In contrast to naive mice, gp49 was induced on splenic NK cells by day 2 postinfection and stayed at high levels on NK cells through day 3 before waning on days 4 and 5 (Fig. 3, A and B). Interestingly, the vast majority of NK1.1+ cells inducibly expressed gp49. Similar results were obtained in the liver, in which NK cell expression of gp49 peaked by days 2 and 3 postinfection. However, the attenuation of gp49 expression, which occurs rapidly on splenic NK cells, is slightly delayed on liver NK1.1+ cells, which express intermediate levels of gp49, 5 days postinfection (Fig. 3B). The expression of gp49 on myeloid cells, T cells, and B cells from spleen, liver, and bone marrow was unchanged following infection (data not shown). Mac-1/Gr-1-positive cells constitutively expressed high levels of gp49, and this remained unchanged through day 5 postinfection. Meanwhile, T and B cells consistently lacked cell surface gp49. These results suggest that gp49 may be an important modulator of NK cell function following activation.

**Cytokine involvement in gp49 induction**

We next addressed the mechanism of gp49 induction. IL-12 and IFN-γ are potent stimuli of NK cells, and both are expressed during MCMV (21). We therefore examined whether the inducible expression of gp49 and CD69 by NK cells is IL-12 or IFN-γ dependent. IL-12-deficient mice were challenged with MCMV, and liver and splenic NK cells were analyzed by flow cytometry (Fig. 5).
NK1.1 cells from infected IL-12-deficient mice expressed comparable levels of gp49 and CD69 as NK cells from MCMV-infected wild-type mice. Similar data were obtained from IFN-γ null mice (Fig. 4), indicating that the inducible expression of gp49 and CD69 on NK cells is not dependent on either IL-12 or IFN-γ, two cytokines whose production is stimulated during MCMV infection.

Inducible expression of the ITIM-bearing receptor gp49B

As stated previously, mAb H1.1 recognizes both known gp49 family members, the ITIM-less gp49A and ITIM-containing gp49B, raising the possibility that either or both are induced following MCMV infection. We used RT-PCR to examine the distribution of the gp49 family members. Primers specific for the A or B form of gp49 were used to amplify cDNA derived from various cell types. cDNA from thymocytes and splenic NK1.1+ (NK) cells contained low to undetectable levels of gp49A and B message (Fig. 5), consistent with our immunofluorescence data. Bone marrow, KY-2, and LAK cells, on the other hand, expressed both forms of gp49 at comparable levels. Although we failed to detect gp49 transcripts in splenic NK1.1+ cells from unchallenged mice, NK1.1+ cells isolated from MCMV-infected mice expressed both gp49A and gp49B.
important role in shaping early immune responses. Because NK cells are a potent source of cytokines in the induction. Because NK cells are a potent source of cytokines in the induction. Because NK cells are a potent source of cytokines in the induction. Because NK cells are a potent source of cytokines in the induction. Because NK cells are a potent source of cytokines in the induction. Because NK cells are a potent source of cytokines in the induction. Because NK cells are a potent source of cytokines in the induction. Because NK cells are a potent source of cytokines in the induction. Because NK cells are a potent source of cytokines in the induction. Because NK cells are a potent source of cytokines in the induction.

NK1.1 bound to immobilized avidin, produce the cytokines GM-CSF (Fig. 6) and IFN-γ.

gp49B. Semiquantitative PCR revealed that both receptors were expressed at comparable levels (data not shown). The significance of gp49A expression is unclear because its function is not known. Nevertheless, our data indicate that in vitro and in vivo expression of an inhibitory receptor, gp49B, on mouse NK cells is regulated by the activation status of the cell.

gp49B-mediated inhibition of cytokine release by NK cells

Because the function of gp49 on NK cells has not been directly tested, we used mAb H1.1 to examine the inhibitory capacity of gp49B on KY-2 cells, known to express high levels of gp49B (11). These cells were preferred for this analysis because of the difficulty in purifying sufficient numbers of NK cells from MCMV-infected spleens. We and others have observed a precipitous drop in the percentage of NK cells in the spleen following infection with MCMV (22) (A. Dokun and W. Yokoyama, manuscript in preparation). KY-2 cells, following stimulation with biotinylated antianti-NK1.1 bound to immobilized avidin, produce the cytokines GM-CSF (Fig. 6) and IFN-γ (data not shown). However, co-cross-linking of the NK1.1 receptor along with gp49B led to dose-dependent diminution of GM-CSF as well as IFN-γ release. In contrast, an isotype control hamster mAb, 2C11, which does not bind to KY-2 cells (15), exerted minimal effects on cytokine production. Because NK cells are a potent source of cytokines in the innate immune response, these data imply that gp49B plays an important role in shaping early immune responses.

Discussion

Mouse gp49 was formerly identified on mast and NK cells. However, in previous studies, only cells cultured in cytokines were examined. In this study, we provide the first comprehensive analysis of the distribution of gp49 on naive, hemopoietic cells. Unexpectedly, we find that resting NK cells lack expression of gp49. Instead, myeloid cells from bone marrow, spleen, and liver, expressing Mac-1 and Gr-1, constitutively express gp49A and B. By contrast, T cells as well as B cells do not express gp49.

Despite that gp49 is not found on naive NK cells, it is induced on NK cells following in vitro and in vivo stimulation. This induced expression is found in NK cell responses to MCMV infection, a notable observation because of the critical role of NK cells in controlling lethality and replication by MCMV (19, 20, 23).

Although the mechanism for induction of gp49 expression was not revealed in our studies, NK cells from MCMV-infected IL-12 or IFN-γ-deficient mice were fully capable of gp49 expression. This does not exclude a role for IL-12 or IFN-γ, but does suggest that neither is required for this process. Although CD69 is also induced on T and B cells, whereas gp49 is not, CD69 is another NK cell activation-induced receptor with kinetics of expression that is strikingly similar to gp49 induction. Therefore, the molecular events underlying gp49 induction are likely to be somewhat distinct from those involved in CD69 up-regulation.

Although both gp49A and gp49B are induced by NK cell activation, the role of these receptors in MCMV infection, particularly that of gp49B, can be best understood in the context of the large amount of data concerning the better characterized inhibitory receptors on NK cells. The inducible expression of gp49B contrasts with the constitutive expression of Ly-49 receptors on overlapping subsets of naive NK cells, LAK cells, and NK cells from MCMV-infected mice. However, certain Ly-49+ NK cell subsets in the spleen appear to be expanded during MCMV infection (22) (A. Dokun and W. Yokoyama, manuscript in preparation), perhaps by redistribution of these subsets or selective outgrowth. Instead, the inducible expression of gp49 appears to be a general response to NK cell activation, because all NK cells early in the course of Listeria monocytogenes infection, another pathogen that involves an NK cell response (24), also inducibly express gp49 (L. Wang and W. Yokoyama, unpublished observations). Additionally, treatment of mice with poly(I:C) stimulates gp49 expression by NK cells (data not shown). Based on our in vitro observation that stimulation of mature NK cells induces gp49, this enhanced expression of gp49 following MCMV infection is very unlikely due to the recruitment of the small number of gp49+ cells in the bone marrow to the periphery or other redistribution phenomena. These data strongly suggest that gp49 expression is induced upon physiological stimuli and that the role of these receptors is to modulate NK cell activity some time after initial activation. This can be differentiated from the role of Ly-49 and other MHC class I inhibitory receptors that are currently conceived to be constitutively and stably expressed on individual NK cells once the mature repertoire is established (25, 26). Perhaps the constitutive and stable expression of such receptors is not surprising because these MHC class I-specific receptors must act immediately to control untoward NK cell activation. Although the ligands for the gp49 receptors are not yet understood, ongoing studies do not clearly implicate MHC class I ligands (L. Wang and W. Yokoyama, unpublished data). Hence, gp49 receptors and their ligands may play critical roles in returning the activated NK cell population back to resting state, thus playing a potentially important role in NK cell homeostasis.

Whereas gp49B is a functional receptor for inhibition, the function of gp49A is not yet understood, even though it is clearly not an inhibitory receptor due to the absence of cytoplasmic ITIMs. On the other hand, it does not contain a charged transmembrane residue required for association with signaling chains expressed by NK cells, such as DAP12, DAP10, FcεRIγ, or CD3ζ. Furthermore,
gp49A does not contain any tyrosine-based cytoplasmic signaling motifs involved in cellular activation. Regardless, the induced expression of gp49A may provide a clue to its physiological function, and this induced expression may be relevant to a large family of related receptors.

Although there are other examples of NK and TCRs that are induced by cytokines (27, 28), such studies were limited to in vitro analysis and did not examine NK cells in virally challenged hosts. In addition to human KIR molecules, mouse gp49 molecules display structural homology with mouse paired Ig-like receptor (PIR) (29, 30) and human ILT/LIR receptors, although only a few of these receptors have been found on NK cells. PIRs are found on cells of the myeloid lineage, but are also expressed on B cells (31), unlike gp49. The recently identified human ILT/LIR receptors (32, 33) may represent functional orthologs of mouse gp49. There are now at least nine known ILT/LIR family members that display heterogeneous tissue distribution. A few members such as ILT1 (LIR-7) and ILT2 (LIR-1) can be found on NK cells, but also display tropism for cells of the myeloid lineage. ILT2 (LIR-2) is also found on B cells and T cell subsets. Like the gp49 family, there exist ITIM-less ILT/LIR molecules. Unlike gp49A, one such receptor, ILT1 (34), through a charged residue in its transmembrane domain, can pair with FcεRy and mediate activation of monocytes. Thus, it is not yet clear how gp49 relates to these otherwise homologous receptors.

Nevertheless, our data also imply that it is worth considering if any member of the growing family of Ig-like receptors may have important functions on not only resting cells, but also on activated cells. A number of these receptors may be induced upon cellular activation on not only NK cells, but also other hematopoietic cells. Such findings would also strongly suggest that the physiological functions of these receptors may be important as regulators of cellular activation, long after the cells are first stimulated, rather than at the time of initial activation, as currently envisioned.

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