Natural Killer Cells and Mast Cells from gp49B Null Mutant Mice Are Functional

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Immune responses are controlled by a combination of positive and negative cellular signals. Effector cells in the immune system express inhibitory receptors that serve to limit effector cell expansion and to protect the host from autoreactivity. gp49B is a receptor of unknown function that is expressed on activated mast cells and natural killer (NK) cells and whose cytoplasmic tail endows it with inhibitory potential. To gain insight into the function of gp49B in mice, we disrupted the gp49B gene by homologous recombination. gp49B^0 mice were born at expected ratios, were healthy and fertile, and displayed normal long-term survival rates. gp49B^0 mice showed no defect in NK or mast cell development. Furthermore, NK and mast cells from the gp49B^0 mice showed activation properties in vitro similar to those of cells isolated from wild-type mice. Therefore, gp49B is not critical for the development, expansion, and maturation of mast cells and NK cells in vivo. The healthy status of gp49B^0 mice makes them suitable for testing the role of gp49B in immune responses to infectious agents.

The regulation of target cell lysis by natural killer (NK) cells is provided in part by the expression of inhibitory receptors capable of blocking activation signals (9). In the mouse, NK cell activation is inhibited by the lectin-like receptors Ly49 and the CD94-NKG2 complex, which recognize class I molecules of the major histocompatibility complex (MHC), thereby protecting normal cells from NK lysis. In contrast, human NK cells use two types of MHC class I-specific inhibitory receptors, the lectin-like CD94-NKG2 and the killer cell immunoglobulin-like receptors (KIR). The closest relative of KIR in the mouse is a molecule called gp49 with two immunoglobulin (Ig) domains that share 33% sequence identity with those of KIR (1, 15). gp49 is expressed on a variety of cell types, including mast cells, NK cells, and 60% of bone marrow cells that also express the myeloid markers Mac1 and Gr-1 (1, 7, 13, 16, 17). Using a new monoclonal antibody (MAb) specific for gp49, Wang et al. showed that gp49 expression is induced on NK cells upon activation (16).

Two closely linked genes on mouse chromosome 10 encode the related proteins gp49A and gp49B (reference 8 and our unpublished results). These two receptors are almost identical in their extracellular regions but differ in their cytoplasmic domains (1). The cytoplasmic tail of gp49B contains two immunoreceptor tyrosine-based inhibition motifs that can deliver an inhibitory signal in mast and NK cells (7, 11, 13, 16). gp49A has a short cytoplasmic tail without any immunoreceptor tyrosine-based inhibition motifs and lacks the characteristic charged amino acid in the transmembrane region which is present in the activating forms of the KIR, CD94-NKG2, and Ly49 molecules (9). The ligand(s) for gp49A and gp49B remains unknown.

In an effort to determine the role of the potentially inhibitory gp49B receptor, we generated mice deficient in gp49B expression (gp49B^0 mice).

MATERIALS AND METHODS

Cell lines and reagents. Mouse NK cell populations and bone marrow-derived mast cells (BMMC) were generated as described previously (2, 3, 6). The human B-cell line 721.221 (14) was maintained as described previously (15). The anti-HLA-DR MAb L243 (American Type Culture Collection) used in cytotoxicity assays was used as purified IgG.

Generation of gp49B^0 mice. The strategy depicted in Fig. 1 was used to generate the targeting construct. A fragment of 220 bp containing the first exon of gp49B was replaced by the *neo* gene flanked by two loxP sites. The fragments between the *Eco*RI and *Ppu*MI sites (1.5 kb) and between the *Bam*HI and *Pac*I sites (5.2 kb) were extracted from a bacterial artificial chromosome (BAC) genomic clone containing the gp49A and gp49B genes. These fragments were independently blunt-end cloned into the pSP72 vector. The *neo* gene from the pL2neo2 plasmid (a kind gift from Hua Gu, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health) was cloned 3' to the short fragment of gp49B, and the *tk* gene from the pHSV-tk plasmid (12) was cloned 3' to the long fragment of gp49B. Finally, the short fragment of gp49B and the *neo* gene were cloned 5' to the long gp49B fragment. Thirty micrograms of the targeting vector was linearized with *Sal*I and electroporated into E14.1 embryonic stem (ES) cells. Clones resistant to G418 and ganciclovir were screened for homologous recombination by Southern blot hybridization after digestion of the DNA with *Hin*dIII and *Bam*HI. The probe in this case was outside the targeting construct just 3' of the first *Hin*dIII site, and in homologous recombination-positive ES cells a 2.5-kb band shifted to 9 kb, as expected. To exclude the possibility that the targeting construct inactivated the gp49A gene in addition to the gp49B gene, positive clones were further screened by Southern blotting after digestion with *Pac*I and *Rsr*II. Targeting of the gp49A gene would have resulted in a shift of a 23-kb band to 18 kb; no ES cells containing this targeting of gp49A were detected. Selected ES clones were injected into blastocysts to obtain chimeric mice. gp49B^0 mice were identified by Southern blot hybridization after digestion of tail DNA with *Eco*RI. Mice used in these experiments were (C57BL/6 × O1a)F$_1$.

Preparation of gp49-specific antisera. An antiserum reactive with the Ig domains of gp49B was generated by immunizing rabbits with a gp49B-Ig fusion protein containing the extracellular portion of gp49B. This antiserum has been shown to react with both gp49A and gp49B (data not shown). Antisera specific for the gp49A or the gp49B cytoplasmic tail were generated by immunization with keyhole limpet hemocyanin-conjugated peptides CPRELSTPR and CIRTQEQNNS, respectively, which correspond to the unique C-terminal regions of gp49A and gp49B, respectively. The specificities of the C-terminal antisera were tested by Western blot analysis of lysates from 721.221 cells infected with recombinant

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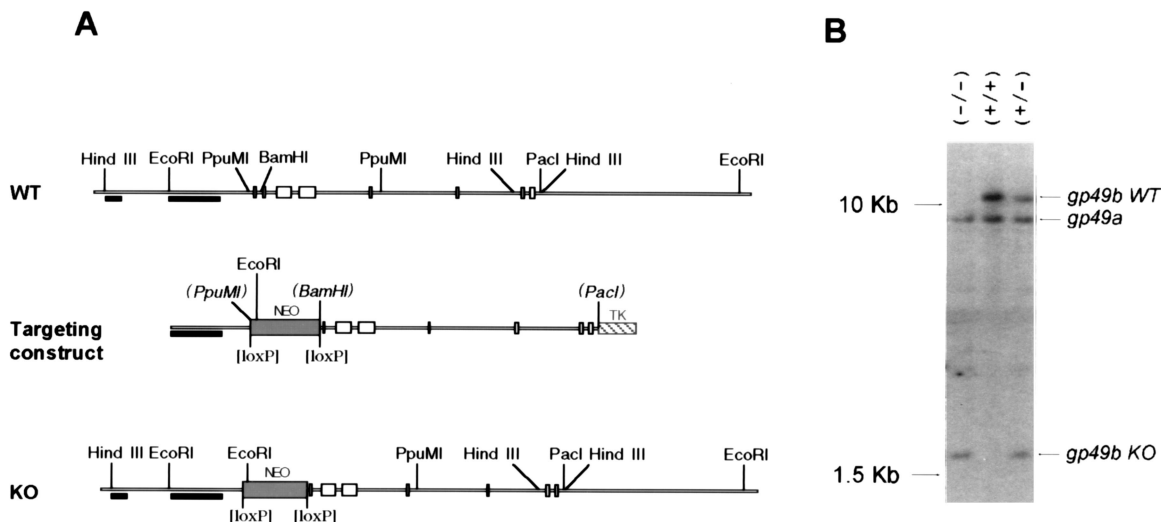† Present address: Novo Nordisk, DK-2880 Bagsvaerd, Denmark.

FIG. 1. Disruption of the gp49B gene. (A) The gp49B targeting construct was made by replacing the first exon between the *Ppu*MI and *Bam*HI sites with a *neo* gene flanked by loxP sites. Sites in parentheses were lost during construction of the targeting vector. The probe outside the targeting construct (short filled bar) was used to screen ES cell transfectants for homologous recombination, and a larger probe inside the construct (long filled bar) was used for screening mouse tail DNA. (B) Southern blot analysis of tail DNA from wild-type (+/+), gp49B$^{+/-}$ (+/−), and gp49B^0 (−/−) mice. Insertion of the *neo* targeting construct into one gp49B allele results in the addition of an *Eco*RI site detected by the appearance of a 1.9-kb band and the loss of the 11-kb band. WT, wild type; KO, knockout.

vaccinia viruses encoding the cDNAs for either gp49B or a chimera of the cytoplasmic tail of gp49A linked to the extracellular portion of gp49B.

Flow cytometry. All antibodies were obtained from PharMingen except the anti-gp49 antiserum described above and the fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit MAb (Jackson Immunoresearch). Samples were analyzed on a Becton Dickinson FACScan.

Immunoprecipitations, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting. For the analysis of both NK cells and BMMC, 30×10^6 cells were surface biotinylated using ImmunoPure NHS-SS-Biotin (Pierce) and lysed in ice-cold lysis buffer (1% Brij 96, 20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) for 20 min on ice. Lysates were cleared by centrifugation ($21,000 \times g$, 4°C, 15 min). Cleared lysates were incubated with streptavidin-agarose beads (Upstate Biotechnology) for 2 h at 4°C. Beads were washed three times with ice-cold lysis buffer and boiled in reducing sodium dodecyl sulfate (SDS) sample buffer. Samples were then separated on a 10% polyacrylamide–Tris-glycine gel (Novex) and transferred to a polyvinylidene difluoride membrane (Immobilon P; Millipore). The membranes were blocked with 5% bovine serum albumin in TPBS (0.05% Tween 20 in phosphate-buffered saline) for 1 h at room temperature and then incubated for a second hour with rabbit antiserum specific for either gp49A or gp49B. Membranes were washed and incubated with peroxidase-coupled goat anti-rabbit antibody (Amersham) and, after a final washing, developed using SuperSignal West Pico (Pierce).

NK cell killing assays. Target cells were grown to mid-log phase and 5×10^5 cells were labeled in 100 µl of CTL medium (Iscove's medium supplemented with 10% fetal calf serum, L-glutamine, and penicillin-streptomycin) with 100 µCi of ^{51}Cr for 1 h at 37°C. Cells were washed twice in CTL medium and resuspended at 5×10^4 cells/ml in CTL medium. A total of 2,500 target cells were used in each sample well. Effector cells were resuspended in CTL medium supplemented with 100 U of recombinant interleukin-2 (IL-2) per ml, and in the case of the antibody-dependent cellular cytotoxicity experiment, 721.221 cells were preincubated with the anti-HLA-DR MAb L243 for 15 min at 25°C. Effector cells were mixed with labeled target cells in a V-bottom 96-well plate. Maximum release was determined by incubation in 1% Triton X-100. For spontaneous release, targets were incubated without effectors in CTL medium alone. All samples were done in triplicate. After a 1-min centrifugation at $200 \times g$, plates were incubated for 3 h at 37°C. Supernatant was harvested and ^{51}Cr release was measured in a gamma counter. The percentage of specific release was calculated as follows: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

Mast cell analysis. Flow cytometric analysis of mast cells was performed on 3×10^5 BMMC as follows. All incubation steps were done on ice for 30 min in 100 µl of phosphate-buffered saline with 0.1% bovine serum albumin and 0.05% sodium azide. Unless specified, all antibody final concentrations were 10 µg/ml. FcεRI expression was determined with biotinylated rat IgE (IR162). After washing, biotinylated rat IgE was revealed with streptavidin-phycoerythrin (diluted 1:2,500). Mast cell identity was confirmed using FITC-labeled anti-c-kit (PharMingen). Cellular degranulation was measured by the release of β-hexoseam-

inidase as described before (5), with minor modifications. Cells were incubated overnight with 500 ng of mouse anti-2,4-dinitrophenol (anti-DNP) IgE (Sigma) per ml per 10^6 cells, washed twice, and stimulated with DNP-albumin (Sigma) at various doses for 20 min.

RESULTS AND DISCUSSION

Generation of gp49B^0 mice. The gp49B gene in the ES cell line E14.1 was targeted with a construct in which a 220-bp fragment containing the first exon of gp49B was replaced by a neomycin (*neo*) gene flanked by two loxP sites (Fig. 1A). ES cells carrying this construct were isolated by double drug selection (with G418 and ganciclovir) and screened for homologous recombination by Southern blot analysis (see Materials and Methods). Two different restriction digests were performed to ensure that the construct inserted into the homologous region of the gp49B gene and that the neighboring gp49A gene remained intact (data not shown; see Materials and Methods). Selected ES clones were injected into blastocysts to obtain chimeric mice. gp49B mice were identified by Southern blot hybridization after digestion with *Eco*RI (Fig. 1B).

Surface expression of gp49 in wild-type and gp49B^0 mice. Expression of gp49 at the cell surface was measured by flow cytometry after binding of an antiserum raised against the extracellular domain of gp49B. This antiserum reacts with both gp49A and gp49B (C. Stebbins, unpublished data). BMMC and NK cells from gp49B^0 mice show a reduction in surface expression of gp49 to 26% of the level in wild-type BMMC and 8% of the level in wild-type NK cells (Fig. 2). To test whether residual gp49 expression was due to gp49A, antisera specific for the unique sequences in the cytoplasmic tails of gp49A and gp49B were generated. Cell surface proteins were biotinylated, isolated with streptavidin-agarose, separated by SDS-PAGE, and transferred onto nitrocellulose. Membranes were probed with the specific anti-gp49A or anti-gp49B antisera. Figure 3 shows that BMMC and NK cells from gp49B^0 mice lack gp49B and that, in agreement with cell surface staining (Fig. 2), NK cells express much less gp49A than BMMC do. Inactivation of
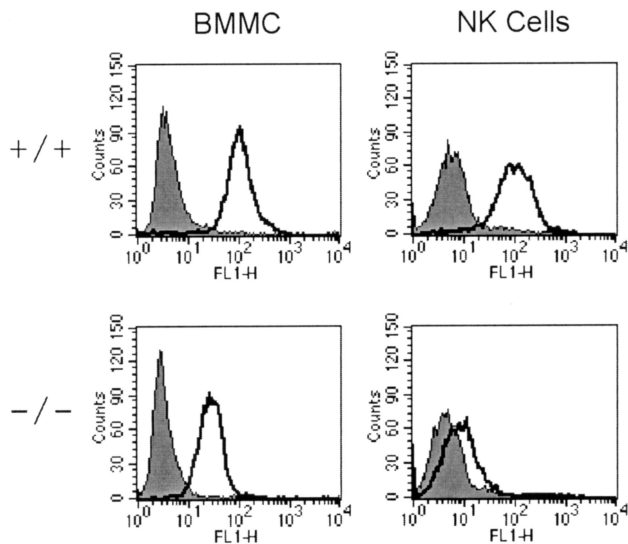
FIG. 2. gp49 surface expression on BMMC and NK cells from wild-type (+/+) and gp49B^0 (−/−) mice. BMMC and NK cells were stained with a rabbit polyclonal anti-gp49 antiserum that reacts with both gp49A and gp49B. FL1-H, green fluorescence.

the gp49B gene did not affect expression of gp49A (Fig. 3). To confirm this observation, gp49A expression was compared in NK cells and BMMC isolated from a C57BL/6 mouse. Direct comparison in Fig. 3 shows that NK cells express only a small fraction of the amount of gp49A expressed by BMMC. These results suggest that NK cells express predominantly gp49B and only a small amount of gp49A. A comparison of gp49A and gp49B expression in NK cells by reverse transcriptase PCR had not revealed this difference (16).

Development of NK and mast cells in gp49B^0 mice appears to be normal. The inhibitory potential of gp49B suggests that this receptor may regulate NK or mast cell activation (7, 13). A phenotypic analysis of NK cells and BMMC was carried out in the gp49B^0 mice to evaluate whether gp49B regulates the development of these two cell types. Splenocytes from gp49B^0 mice were analyzed by double staining with CD3ε and DX5, a MAb specific for a pan-NK cell marker (Fig. 4A). Wild-type
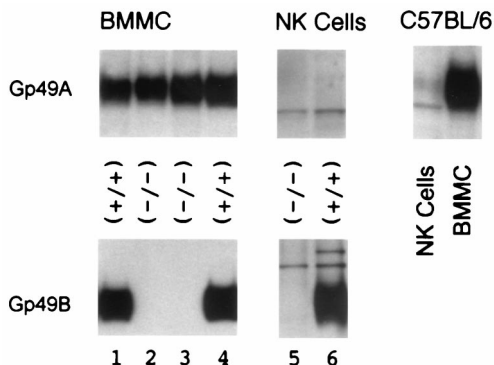

FIG. 3. Biochemical analysis of gp49 expression. Cell surface proteins were biotinylated and precipitated with streptavidin-agarose, separated by SDS-PAGE, blotted onto nitrocellulose, and probed with antiserum specific for gp49A or gp49B, as indicated. BMMC and NK cells from wild-type (+/+) and gp49B^0 (−/−) mice were analyzed. Included is a comparison of the level of gp49A expression in BMMC and NK cells from a C57BL/6 mouse.
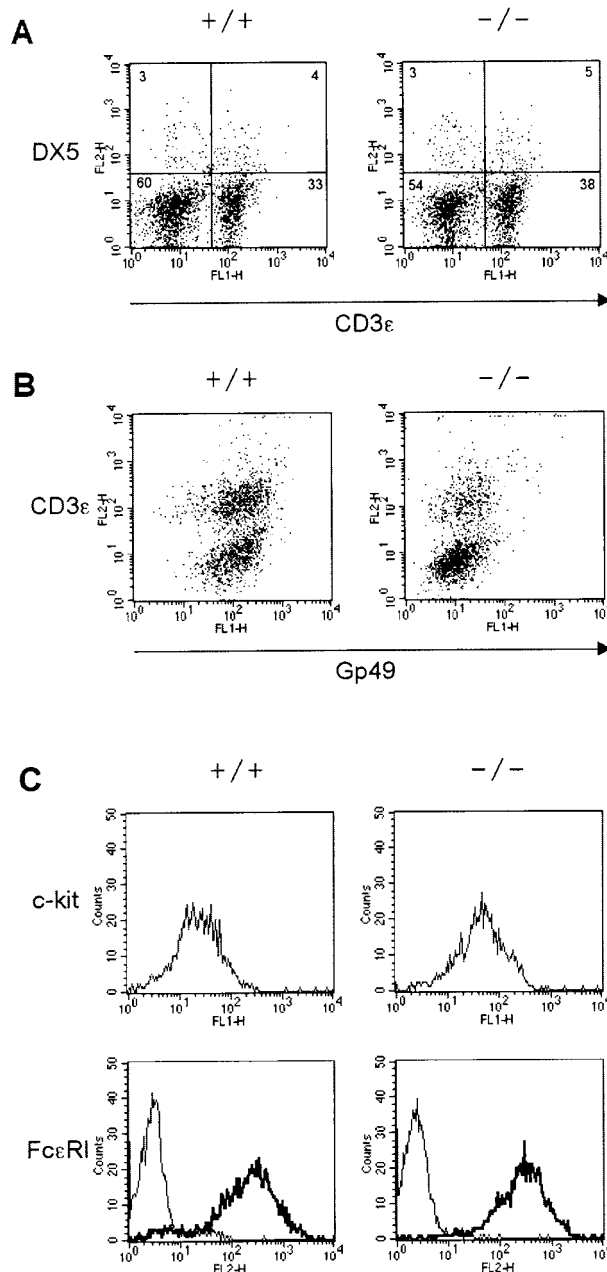

FIG. 4. Analysis of NK and mast cells in wild-type and gp49B^0 mice. (A) Unstimulated splenocytes prepared from wild-type (+/+) and gp49B^0 (−/−) mice double-stained with FITC-conjugated anti-CD3ε and phycoerythrin (PE)-conjugated DX5. (B) IL-2-expanded DX5$^+$ NK1.1$^+$ cell populations from wild-type and gp49B^0 mice double-stained with PE-conjugated anti-CD3ε and anti-gp49 antiserum followed by goat anti-rabbit IgG-FITC. (C) Expression of c-kit and FcεRI in cultured BMMC from wild-type and gp49B^0 mice. FcεRI expression was determined by binding of biotinylated IgE followed by streptavidin-PE (thick line). The control for this experiment was streptavidin-PE alone (thin line). FL1-H, green fluorescence; FL2-H, red fluorescence.

and gp49B^0 splenocytes contained similar proportions of DX5$^+$ cells (3%) and of CD3$^+$ and CD3$^-$ cells. No significant difference was detected in the number of total splenocytes isolated from wild-type and gp49B^0 mice (data not shown). Further analysis by flow cytometry of both fresh and IL-2-expanded NK cells revealed no difference in the expression of
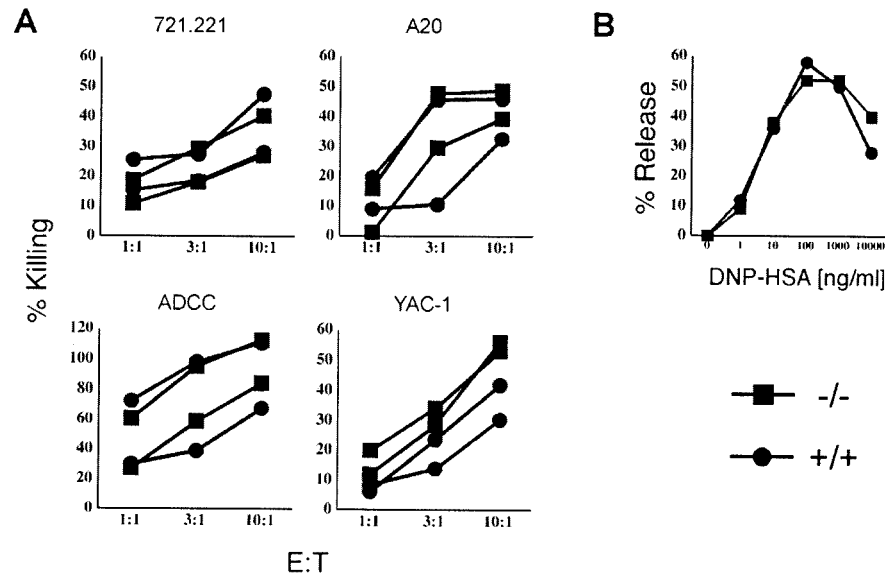
FIG. 5. Functional analysis of NK and mast cells. (A) Killing of different target cells by NK cells. IL-2-expanded NK cells from wild-type (●) and gp49B⁰ (■) mice were used in a standard cytotoxicity assay at the indicated effector-to-target (E:T) ratios. The results are representative of several experiments that included at least four mice of each type. (B) FcεRI-induced degranulation as measured by β-hexoseaminidase release. BMMC from wild-type and gp49B⁰ mice were incubated with 500 ng of DNP-specific mouse IgE per ml per 10^6 cells and stimulated with DNP-human serum albumin (DNP-HSA).

several other cell surface proteins, including Ly49A, Ly49C/I, c-kit, CD16, LFA-1α, and ICAM-1 (data not shown). Of interest, isolation and expansion of DX5⁺ splenocytes in IL-2 often yielded a population of CD3⁺ NK1.1⁺ cells. These results show that gp49 can also be expressed on this T-cell subset (Fig. 4B).

Analysis of BMMC from wild-type and gp49B⁰ mice revealed no difference in expression of the cell surface marker c-kit (Fig. 4C). BMMC from both mice were equally able to bind IgE immune complexes (Fig. 4C). Histological analysis of several tissues rich in mast cells (ear, skin, tongue, and stomach) revealed no difference between the wild-type and gp49B⁰ mice in the number of mast cells resident in these tissues (data not shown).

NK and mast cells from gp49B⁰ mice are functionally normal. We analyzed the ability of NK cells to kill target cells and the ability of BMMC to degranulate upon FcεRI cross-linking. Since gp49B can function as an inhibitory receptor, it was possible that NK cells from the gp49B⁰ mice would exhibit an increase in their ability to kill certain targets. On the other hand, it was also possible that gp49B⁰ mice would compensate for the loss of an inhibitory receptor by downmodulating their activation signals. Such adaptation has been reported for NK cells from mice deficient in MHC class I (4, 10). Two mouse cell lines, A20 and YAC-1, and the human cell line 721.221 were tested for their sensitivity to NK-mediated lysis. In addition, 721.221 cells coated with the mouse IgG MAb L243 were tested for sensitivity to antibody-dependent cellular cytotoxicity. Human cells are not expected to express a ligand for gp49 and are therefore suitable targets to evaluate the basal level of mouse NK cell activity. Figure 5A shows no detectable difference in the killing of target cells beyond those differences attributable to intermouse variability. Subtle changes in the lytic activity of NK cells from gp49B⁰ mice would have been difficult to detect due to this variability. The extent of degranulation by BMMC upon FcεRI cross-linking was identical in the two strains of mice (Fig. 5B).

Conclusion. gp49B⁰ mice developed normally, without any obvious phenotypic differences from wild-type mice. Therefore, gp49B expression is not essential for survival of mice in a clean environment. Analysis of activated NK and mast cells isolated from gp49B⁰ mice revealed that these cells were phenotypically and functionally similar to the same cells derived from wild-type mice. We conclude that gp49B is not required for the development or the in vitro activity of NK and mast cells. It is possible that gp49B⁰ mice have compensated for the lack of this receptor and that the in vivo function of gp49B is not readily observed in such mice. Alternatively, the main function of gp49B on NK and mast cells may be to control immune responses during certain infections or allergic reactions. Backcrossing of the gp49B⁰ mice to a congenic strain is required to test this possibility.

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