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Uterine Macrophages Express the gp49B Inhibitory Receptor in Midgestation

Yukie Matsumoto, Lawrence L. Wang, Wayne M. Yokoyama, and Takeshi Aso

Mouse gp49B is an immunoreceptor tyrosine-based inhibitory motif-bearing receptor identified on mast cells and NK cells. In this report, however, we show that this receptor is expressed on macrophages accumulating in the uterine metrial gland in midgestation, along with gp49A that has a very homologous extracellular domain with gp49B but has a short cytoplasmic domain without ITIM. Culture of bone marrow cells in the conditioned medium of the metrial gland resulted in the selective proliferation of macrophages expressing both Fcy-activating receptors and gp49B inhibitory receptor. Stimulation of macrophages with immobilized IgG, but not with anti-FcγRII/III, induced a considerable amount of TNF-α and IL-10 production, suggesting that the high-affinity receptor for IgG (FcyRI) can transmit activating signals in cytokine production of macrophages. Furthermore, coligation of gp49B with FcyRI resulted in the inhibition of TNF-α production. Thus, our data provide evidence that gp49B is an endogenous negative regulator of macrophage activation and may regulate the function of macrophages during pregnancy. The Journal of Immunology, 2001, 166: 781–786.

In recent years, it has been found that the immune system is regulated by both activating and inhibitory receptors. Each group of receptors has characteristic cytoplasmic motifs. The activating receptors have immunoreceptor tyrosine-based activation motifs. These motifs become tyrosine phosphorylated by src family kinases triggered by the clustering of activating receptors, resulting in the recruitment and activation of additional tyrosine kinases and other molecules. On the other hand, the inhibitory receptors have immunoreceptor tyrosine-based inhibitory motifs (ITIMs) consisting of a 6-aa stretch I/VxYxxL/V. Ligand binding of the inhibitory receptors induces phosphorylation of ITIM and subsequent recruitment of cytoplasmic tyrosine phosphatases, SHP-1, SHP-2, and/or SHIP depending on the receptor, presumably resulting in the dephosphorylation of molecules involved in the activation pathways (1, 2).

On NK cells, these receptors fall into two general structural types, including type I membrane-oriented Ig-like molecules such as the human killer inhibitory receptors (KIRs) type II-oriented C-type lectin-like molecules. Many of these receptors are specific for MHC class I molecules on target cells and the ITIM-bearing receptors prevent NK cell activation. However, recent data indicate that inhibitory receptors are more widely expressed, particularly those receptors related to the KIR family.

In the mouse, Ig-like receptors include the gp49 family of molecules, gp49A and gp49B, gp49A is 89% identical to gp49B in the extracellular domain but has a short cytoplasmic tail lacking ITIMs. Whereas the function of gp49A is not known, gp49B is an inhibitory receptor. Originally isolated as a murine mast cell receptor, gp49B is now known to be present also on NK cells and macrophage-differentiated M1 myeloid leukemia cells (3–6). This receptor can suppress the release of secretory granule mediators from mast cells when it is coligated with the high-affinity Fc receptor for IgE (FceRI) (4). Similarly, gp49B can inhibit NK cell functions (7). Furthermore, the phosphorylated ITIMs of gp49B can associate and activate the cytoplasmic tyrosine phosphatase SHP-1 in IL-2-activated NK cells (8).

We previously reported that gp49 mRNA is expressed in the uterine endometrium just before implantation (6). In a subsequent study, we have found that the expression of this transcript is higher in the uterus at midgestation than around implantation. Coincident with this, it has been well established that pregnancy-related leukocytes accumulate in maternal uterine tissues up to midgestation. Although these maternal leukocytes normally act in defense against certain microbial and parasitic infections, they may play a homeostatic role in the control of cytokine balance and placentalation while being suppressed in their effector functions on fetal tissues (9, 10). The most abundant leukocyte in the midgestation uterus is the NK cell. Indeed, HLA-G-specific inhibitory receptors on human NK cells are postulated to protect fetal trophoblasts from NK cell-mediated lysis since trophoblasts do not express classical MHC class I molecules but do express nonclassical HLA-G molecules (11). We therefore postulated that murine uterine NK cells express gp49B. To our surprise, gp49 is expressed by uterine macrophages and not NK cells. We further determined that gp49B can inhibit macrophage function. Thus, we suggest that this receptor down-regulates macrophage function during pregnancy.

Materials and Methods

Mice and acquisition of tissues
C57BL/6 mice were obtained from Sankyo Laboratory Service (Tokyo, Japan). Female mice at 10–16 wk of age were mated overnight and checked for vaginal plugs in the morning. The morning a vaginal plug was found was designated as day 1 of pregnancy. Mice were killed by cervical
dislocation, and uteri were removed at the time points indicated. One uterine horn was cut open, and embryos, placenta, and uteri were collected for RNA preparation, the other one was processed as individual implantation sites, embedded in Tissue-Tek OCT compound (Sakura, Tokyo, Japan) and stored at −80°C until cryostat sectioning.

Northern blot analysis and RT-PCR

Total RNA was prepared using Isogen (Nippon gene. Tokyo, Japan). Twenty micrograms of total RNA was separated in a denaturing gel containing 2.2 M formaldehyde and transferred onto a Biodyne A membrane (Biodyne, Pall BioSupport, NY) by vacuum blotting, and then cross-linked by UV irradiation in a Stratalinker (Strategene, La Jolla, CA). The gp49 probe-represented nt 631-1168 of gp49B1 cDNA was labeled with a random primer labeling kit (Amersham, Arlington Heights, IL), and hybridization was performed in QuikHyb solution (Strategene) for 1 h at 68°C. To identify which isotype of gp49, gp49A, or gp49B is expressed, 3 μg of total RNA was reverse transcribed using Superscript first-strand cDNA synthesis kit (Life Technologies, Rockville, MD) followed by PCR amplification. Amplification was performed in a GeneAmp PCR System 9700 for 25–28 cycles (30 s at 95°C, 30 s at 58°C, and 30 s at 72°C) using a set of primers as follows: forward, GTGGGACCAAAAATGAAAGCC; and reverse, TGGGCGTA(C/G)ACAATTCCCTG.

Amplified DNA was separated in 25% acrylamide gel followed by ethidium bromide staining.

In situ hybridization

In situ hybridization was performed as described previously (12). To generate antisense and sense probes, a 1166-bp fragment of gp49B1 cDNA (nt 3–1168) cloned into pSPT18 and pSPT19 plasmids were linearized with EcoRI and HindIII, respectively, and transcribed using Sp6 RNA polymerase, followed by limited alkaline hydrolysis, to reduce their average size into 200 bp.

Cell preparation and flow cytometry

Uterine mononuclear cells were prepared as follows. Uterine horns were excised from C57BL/6 mice on day 13 of pregnancy. Each horn was opened along the antimesometrial side and placenta were stripped off from the uterine myometrium with a fine forceps. Metrial glands remaining on uterine strips were peeled off with a scalpel, minced in fine pieces, and shaken in 5 mM EDTA-PBS. Dispersed cells were obtained by filtration through a 38-μm pore size stainless steel mesh. After hemolysis and centrifugation in 40% Percoll, the uterine mononuclear cells collected at the bottom were subjected to flow cytometry. The cells were first incubated with mouse IgG (10 μg/ml) to block Fc-mediated binding except for staining of Fcy receptors and then stained with other Abs described below. Anti-gp49 (G2.3) was generated as described previously and labeled with FITC. This Ab reacts with both gp49A and gp49B (7). Anti-gp9 (B23.1), a gift from P. A. LeBlanc (University of Alabama, Tuscaloosa, AL), also recognizes both gp49A and gp49B (4, 7). PE-conjugated anti-NK1.1 (PK136), PE-streptavidin, and isotype controls were purchased from PharMingen (San Diego, CA). Biotinylated anti-F4/80 (A3-1) was purchased from Serotec (Oxford, U.K.). APC-conjugated mouse IgG2a was purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). A total of 2 × 10^5 cells was counted and analyzed by FACScalibur (Becton Dickin-son, Mountain View, CA) after gating out dead cells with propidium iodide staining.

Cell culture and stimulation

Bone marrow cells were aspirated flushed from femurs with PBS. After hemolysis, cells were cultured for 5–7 days in the conditioned medium of the metrial gland prepared as follows. Briefly, metrial glands separated from a mouse on day 13 of pregnancy were minced and cultured in 20 ml of RPMI 1640 medium supplemented with 10% FCS, penicillin-streptomycin, and 5 × 10^{-5} M 2-ME for 24 h, and the supernatant was passed through a 38-μm filter to remove cell debris. Bone marrow-derived macrophages thus obtained were stimulated with immobilized mouse IgG or 2.4G2 (anti-FcγRIII/I). Mouse IgG or 2.4G2 was immobilized on 96-well flat-bottom culture plates (Sumilion, Tokyo, Japan) by incubating for 2 h at 37°C. After stimulation for 24 h, TNF-α and IL-10 in the supernatant were measured by ELISA using a Quantikine M kit (Genzyme, Minneapolis, MN). To examine the inhibitory effect of gp49B, the cells were stimulated with immobilized IgG plus B23.1 or IgG plus isotype control rat IgG. In some experiments, mAb G2.3 and its isotype control hamster IgG were used. All data are presented as means ± SEM from triplicate cultures.

Results

Gp49 mRNA is expressed in the uterine metrial gland in midgestation

Starting on day 7 of pregnancy, we examined the expression of gp49 mRNA in the uterus of midgestation by Northern blot analysis (Fig. 1A). The levels of its expression increased gradually, with the highest level around days 12–14 of pregnancy. However, the two isoforms of gp49, gp49A and gp49B, are >90% homologous at the nucleic acid level and they cannot be distinguished by our Northern blot probe. To identify which isoform is expressed in the uterus, we performed competitive RT-PCR using a set of primers that can amplify both gp49A and gp49B fragments from the 3’ end of exon 7 to the 5’ end of exon 8. These two fragments were different in size because of the deletion of two cytosines in gp49B. Both gp49A and gp49B transcripts were detected in the uterus in almost equal amounts (Fig. 1B). In addition, coexpression of these two isoforms was detected in bone marrow-derived mast cells. Thus, gp49A and gp49B transcripts are expressed in the pregnant uterus.

gp49 is present on uterine macrophages

To determine the cellular location of gp49 mRNA, we examined sections of uteroplacental units on day 13 of pregnancy by in situ hybridization. With the antisense probe, a strong signal was detected in the metrial gland also known as the mesometrial lymphoid aggregate of pregnancy (Fig. 2). The sense probe detected no signal (data not shown). These results are consistent with the data shown in Fig. 1 in which higher expression of gp49 was detected in the extracted mRNA of uterus than placenta, indicating that the major portion of the metrial glands remains on the uterus when individual implants are peeled off.

To investigate what population of the metrial gland expresses gp49, we performed flow cytometry. At first, we stained uterine mononuclear cells with G2.3 and anti-NK1.1 (the most specific

FIGURE 1. A. Northern blot analysis of gp49 mRNA in the uterus and placenta of midgestation. Total RNA isolated from uteri and placentas on days 7–16 of pregnancy was probed with gp49B cDNA. Uteroplacental units that consist of maternal decidua were substituted for placental samples on gestation days 7 and 8. The 28S RNA marker is the control for the amount of RNA loaded. B. Identification of gp49 isoforms by RT-PCR analysis. Total RNA prepared from the uterus on day 13 (lane 4) or bone marrow-derived mast cells after a 4-wk culture in the presence of 50 U/ml IL-3 (lane 5) was reverse transcribed and amplified as described in Materials and Methods. A 25-bp DNA ladder (lane 1) and controls amplified with gp49A cDNA (lane 2) and gp49B cDNA (lane 3) are shown.
marker of mouse NK cells) because NK cells are the most abundant population of the metrial gland and because human uterine NK cells express KIRs, structurally related to gp49. However, most of the cells expressing gp49 were negative for NK1.1 and the major population of NK1.1+ cells around channel 10 was negative for gp49 (Fig. 3A). Next, we stained metrial gland cells with G2.3 and anti-F4/80 (a marker of mouse macrophages). When cells with high autofluorescence were gated out, 84.9% of the uterine mononuclear cells were double positive for gp49 and F4/80. By contrast, gp49 single positive or F4/80 single positive cells were 2.4 and 7.2%, respectively. These data suggest that gp49 is present on uterine macrophages (Fig. 3B). Furthermore, the cells could be stained also with B23.1, another anti-gp49 mAb, although the intensity of its staining was lower than that with G2.3 (data not shown). Since G2.3 and B23.1 react with both gp49A and gp49B transfectants by flow cytometry, the levels of surface expression of each isoform could not be determined. However, it is reasonable to conclude that the gp49B inhibitory receptor is present on the surface of uterine macrophages because gp49B transcripts were present.

**Generation of gp49+ macrophages from bone marrow cells**

Functional studies of uterine macrophages are technically difficult because of the small amount of cells. Fortunately, in the course of our studies, we found that gp49 was constitutively expressed on myeloid cells in bone marrow. We therefore speculated that immigrants of these myeloid precursors might differentiate into macrophages in the presence of factors secreted from the metrial gland. Previous studies demonstrated that several factors, including M-CSF, were detected in the conditioned medium of metrial gland explants (13), supporting that the supernatant can induce differentiation of macrophages and such cells would be useful for further functional studies. When bone marrow cells were cultured in metrial gland conditioned medium for 5 days, the cells increased 3-fold in number. When gated on the small cells with low side scatter (Fig. 4A), 80% of them were gp49+F4/80+ and about 70% of the cells showed high levels of F4/80 staining (Fig. 4B). F4/80low gp49+ cells were detected also in bone marrow before culture. In addition, most of the large cells with high side scatter were positive for gp49 and all of them showed high levels of F4/80 staining (Fig. 4C). Concerning the intensity of staining, the large cells showed slightly lower levels of gp49 and F4/80 staining than the small cells. On the other hand, when we stained the cells with APC-labeled mouse IgG2a to examine the expression of Fcγ receptors (Fig. 4, D and E), the large cells showed higher levels of staining than the small cells, although both of them showed heterogeneous intensity of staining. Thus, the bone marrow-derived macrophages express gp49 and Fcγ receptors, although expression levels are different depending on their activation.
Inhibition was evaluated by comparing TNF-α production of the cells stimulated with IgG plus B23.1 with that of the cells stimulated with IgG plus isotype control rat IgM. As shown in Fig. 5, the production of TNF-α was reduced in a dose-dependent manner when the cells were stimulated on the plate immobilized with the fixed concentration of IgG and incremental concentrations of B23.1. About 20% inhibition of TNF-α production was detected when the cells were stimulated at the ratio of 1:3 with IgG and B23.1. On the other hand, the production of TNF-α was nearly constant even when immobilized control rat IgM was increased. Although B23.1 recognizes both gp49A and gp49B (7), the inhibitory effect is most likely mediated through gp49B since it has an ITIM whereas gp49A does not, and attempts to cross-link isolated gp49A had no obvious functional effect (data not shown). Thus, gp49B functions as an inhibitory receptor on macrophages.

Discussion

In this study, we found that an inhibitory receptor gp49B was present on macrophages in the uterine metrial gland of mice in midgestation. Although gp49B was initially identified on mast cells and successively on NK cells, LeBlanc and Biron (14) reported that many macrophage populations expressed a molecule detected by mAb B23.1, which was then isolated and defined to be gp49B. Therefore, it is not surprising that uterine macrophages are also positive for gp49B. At first, however, we hypothesized that it was present on uterine NK cells because of our recent finding that this receptor is present on peripheral NK cells (5) and because NK cells are the most abundant population in the metrial gland. Furthermore, in humans, two KIRs, KIR2DL4 and p49, were shown to interact with HLA-G molecules preferentially expressed on fetal extravillous trophoblasts. Functional transfer of KIR2DL4 into an NK cell line results in inhibition of lysis of target cells expressing HLA-G, suggesting that KIR2DL4 on maternal decidual NK cells may have a role in preventing the elimination of fetal tissues as non-self (15, 16). On the other hand, in mice, little has been reported on inhibitory receptors of uterine NK cells, except that an inhibitory receptor Ly49G2 is expressed in early gestation but disappears by midgestation (17). Evidence that uterine NK cells in midgestation show low cytolytic activity despite high levels of perforin and surface Ags of activated NK cells (18, 19) suggested the possibility that gp49B could inhibit their cytolytic activity.
Surprisingly however, we found that gp49B is not present on the major population of metrial gland NK cells.

Macrophages constitute another major population of uterine leukocytes. They increase in number during pregnancy and are localized in the metrial gland by midgestation (20). The production of M-CSF in the uterus in midgestation is \( >1000 \)-fold of that in the nonpregnant uterus, indicating that the uterine environment during pregnancy is suitable for differentiation of macrophages (21). Various roles have been proposed for uterine macrophages, including immunosuppression, Ag presentation, cytokine secretion, and phagocytosis, but little has been well elucidated so far. To determine the significance of uterine macrophages, the fertility of M-CSF-deficient mice (\( \text{op/op} \) mice) has been examined. Although the females show a low rate of pregnancy success, this is due to failure of ovarian follicular development and ovulation. The phenotype of gross deficiency of macrophages in the uteri of \( \text{op/op} \) mice is improved by gestation day 7, suggesting that other factors can substitute for M-CSF (22, 23). On the other hand, in the CBA \( \times \) DBA/2 model with high rates of spontaneous abortion, increased infiltration of activated macrophages expressing class II Ag can be detected in maternal decidua of 20–30% embryos before the first macroscopically detectable signs of resorption (24). In addition, the decidual cells at resorbing sites show increased infiltration of activated macrophages expressing class II MHC molecules, and this may substitute for M-CSF (22, 23). On the other hand, in the nonpregnant uterus, indicating that the uterine environment during pregnancy is suitable for differentiation of macrophages (21).

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Katz et al. (4) have demonstrated that the gp49B on the surface of bone marrow-derived mast cells inhibits the FceRI-induced degranulation response. Consistent with the inhibitory function on mast cells, coligation of gp49B with FcγRI by immobilized B23.1 and IgG resulted in a B23.1-dependent, dose-related inhibition of TNF-\( \alpha \) production of macrophages. This inhibition was not the result of a decrease in the amount of immobilized IgG as a result of IgM addition, because in preliminary studies we confirmed that the amount of immobilized IgG was nearly constant when fixed in the conditions used here. In fact, the evidence is that the TNF-\( \alpha \)-production of the cells stimulated with mouse IgG and various amount of isotype control rat IgM was constant. Instead of B23.1, another anti-gp49 G2.3 showed no inhibitory effect (data not shown). We suspected that these discrepant results might be due to the difference in the isotype of the mAbs. B23.1 is an IgM isotype and therefore it does not bind FcγRI, whereas G2.3 may have associated also with FcγRI through its Fc portion since its isotype is IgG. Thus, gp49B on macrophages can inhibit proinflammatory cytokine production and thus may be relevant to control of macrophage activity during pregnancy.

It seems that both gp49A and gp49B messages are expressed in uterine macrophages, although this may not necessarily reflect the surface expression of each molecule. Macrophages have another pair of receptors, paired Ig-like receptor (PIR) A and PIR-B, which are very homologous in their extracellular domains but distinct in cytoplasmic domains. PIR-A may function as an activating receptor in association with the Fcγ chain (29) whereas PIR-B may mediate an inhibitory signal through its own ITIMs. The coexpression of PIR-A and PIR-B suggests the regulation of macrophage activation through their interaction with the putative common ligand. Assuming that gp49A is an activating receptor, paired gp49 receptors may also regulate macrophage activation by themselves. Although the function of gp49A has not yet been understood, the functional analysis of gp49A will be crucial to elucidate whether the relative expression level of gp49A and gp49B on cell surface contributes to the regulation of cellular activation.

In conclusion, gp49B inhibitory receptor is present on macrophages accumulating in the uterine metrial gland in midgestation. Coligation of gp49B with FcγRI inhibits FcγRI-mediated TNF-\( \alpha \) production of macrophages in vitro. This finding suggests that gp49B may be an endogenous negative regulator of inflammatory cytokine production, which may contribute to the pathophysiology of inflammatory autoimmune disorders including recurrent spontaneous abortion caused by immune complexes.

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**References**


