Brief Definitive Report

Murine CD9 Is the Receptor for Pregnancy-specific Glycoprotein 17

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Abstract

Pregnancy-specific glycoproteins (PSGs) are a family of highly similar secreted proteins produced by the placenta. PSG homologs have been identified in primates and rodents. Members of the human and murine PSG family induce secretion of antiinflammatory cytokines in mononuclear phagocytes. For the purpose of cloning the receptor, we screened a RAW 264.7 cell cDNA expression library. The PSG17 receptor was identified as the tetraspanin, CD9. We confirmed binding of PSG17 to CD9 by ELISA, flow cytometry, alkaline phosphatase binding assays, and in situ rosetting. Anti-CD9 monoclonal antibody inhibited binding of PSG17 to CD9-transfected cells and RAW 264.7 cells. Moreover, PSG17 binding to macrophages from CD9-deficient mice was significantly reduced. We then tested whether PSG17 binds to other members of the murine tetraspanin family. PSG17 did not bind to cells transfected with CD53, CD63, CD81, CD82, or CD151, suggesting that PSG17–CD9 binding is a specific interaction. We have identified the first receptor for a murine PSG as well as the first natural ligand for a member of the tetraspanin superfamily.

Key words: tetraspanins • macrophages • expression cloning • placenta • PSG

Introduction

Pregnancy-specific glycoproteins (PSGs) are a family of highly similar, placentally secreted proteins, originally isolated from the circulation of pregnant women (1). PSGs are members of the carcinoembryonic antigen (CEA) family and in humans, PSG concentration in the bloodstream increases exponentially until term (2). Low levels of PSGs are associated with pathological conditions including spontaneous abortion, intrauterine growth retardation, and preeclampsia (3, 4). Administration of anti-PSG Abs induced spontaneous abortion in primates, indicating that PSGs are essential for successful pregnancy (5). Recently, we demonstrated that human PSGs induced IL-10, IL-6, and TGFβ_1 expression in human monocytes (6). This suggests that PSGs, through induction of these Th2 cytokines, may have a role in protecting the fetus from attack by the maternal immune system.

Despite their isolation from maternal serum 30 years ago, the receptor(s) for these proteins has not yet been identified. PSGs have also been identified in nonprimates with hemochorial placentation including rats and mice. There are 14 murine PSG genes (*PSG14–29*; reference 7) and the cDNAs that have been characterized showed exclusive expression by the placenta (8).

We recently reported that murine PSG17 and PSG18 mimic the biological effects of human PSGs, by inducing cytokines in murine macrophages and the RAW 264.7 macrophage cell line (9) and unpublished data. In addition, we showed that PSG17 binds to RAW cells with high affinity (unpublished data). For the purpose of cloning the PSG17 receptor, we screened a RAW cell cDNA expression library by panning. Positive clones were sequenced, and database queries revealed that the receptor for PSG17 is the tetraspanin CD9.

Sequence analysis of CD9 suggests that like other tetraspanins, it has four hydrophobic transmembrane domains and two extracellular loops, with two short intracytoplasmic tails at the amino and carboxyl termini (10, 11). In the membrane, CD9 associates with other tetraspanin family members (12, 13) and with β_1 integrins (14–17). Other cell surface molecules present in tetraspanin complexes include HLA-DR and MHC class II glycoproteins, CD4, CD8, CD19, and a 135-kD protein designated CD9 partner 1 (12, 13, 18–21). Treatments with Abs to different tetraspanins have implicated these proteins in cell migration, proliferation, activation, and adhesion (18). With such potentially diverse roles, they have been described as "molec-

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ular facilitators." This is the first report to identify the binding partner for a PSG family member, as well as a biological ligand for a member of the tetraspanin superfamily.

Materials and Methods

Reagents. Recombinant PSG17N-Myc-His, which consists of the leader peptide, N1 domain of PSG17 (formerly known as Cea 2), followed by the Myc and 6X His tags was generated using a baculovirus expression system and purified as described previously (6, 9). Transfections were performed with Lipofectamine 2000 (Invitrogen) and transfection efficiency was monitored using a plasmid encoding the green fluorescent protein.

The murine CD53 and CD82 cDNAs were amplified by reverse transcription (RT)-PCR from RAW cell RNA and the murine CD81 cDNA was amplified from pBluescript containing the CD81 cDNA. The primer sets used for the amplifications were: for CD53: 5′ATGGGCATGAGCAGCCTGAAA and 5′TCACAGCCCTAAAGCCTGGC; for CD82: 5′CAGAATGGGGGCAGGCTGTG and 5′CAGCAACCTCAGTACTTGGGG; for CD81: 5′ATGGGGGTGGAGGGCTGC and 5′TCAGTACACGGAGCTGTTCCGG. After amplification with Vent DNA polymerase, the cDNAs were cloned into pEF6/V5-His Topo (Invitrogen), colonies were probed with oligonucleotides specific for each tetraspanin and the correct orientation was confirmed. The murine CD151 cDNA in pcDNA3.1 Zeo was obtained from Dr. L. Ashman, The Hanson Centre for Cancer Research, Adelaide, Australia, and the murine CD63 cDNA in pcDNA3.1/GS was purchased from Research Genetics.

PSG17N-Myc-His–coated plates were prepared as follows. Bacteriological culture dishes were layered with 10 μg/ml goat anti–mouse IgG, extra serum absorbed (XSA; KPL) in phosphate coating solution (KPL). The plates were rinsed with PBS and blocked with BSA buffer (KPL). Anti-myc mAb (1 μg/ml) (Invitrogen) was added to each dish followed by the addition of PSG17N-Myc-His with washes after each step.

Cell Culture. RAW 264.7 (American Type Culture Collection) cells were cultured in DMEM with high glucose, 5 mM sodium pyruvate (Irvine Scientific), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B (PSA; Quality Biological), and 10% fetal bovine serum (FBS). Human embryonic kidney (HEK) 293T cells (Edge BioSystems) were cultured in DMEM, 10% FBS, 50 μg/ml gentamicin, 250 μg/ml G418 (Calbiochem), and PSA. HEK 293 EBNA cells (Invitrogen) were grown in DMEM, 10% FBS, PSA, and 250 μg/ml G418. Baby hamster kidney (BHK)–21 cells (American Type Culture Collection) were sustained in DMEM with 10% FBS and PSA. All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Generation of the RAW 264.7 cDNA Library and Recovery of cDNA Clones by Panning. RNA was extracted from RAW 264.7 cells using TRIzol (Invitrogen) and was used to generate the cDNA expression library in the PEAK10CV vector (Edge BioSystems). The unamplified library yielded ~4.3×10^6 primary transformants. For the first round of screening, 1.1×10^6 clones were plated on Luria-Bertani (LB) broth/agar plates with 100 μg/ml ampicillin (Sigma-Aldrich). After 16 h the plates were flooded with LB broth, the pooled bacteria were pelleted, and plasmid DNA was isolated.

Pooled purified plasmids from the library were transfected into HEK 293 EBNA cells. Positive transfectants were selected using 0.5 μg/ml puromycin (Edge BioSystems). At 72 h after selection, the cells were dislodged in PBS and 0.5 mM EDTA, and resuspended in binding buffer (PBS, 2% BSA). The detached cells were panned in PSG17N-Myc-His coated Petri dishes at 1.0–1.5×10^7 cells per dish. Non-adherent cells were removed by extensive washing with PBS/BSA and adherent cells were transferred to poly-L-lysine coated 96-well plates. An additional two rounds of panning were performed before the episomal plasmids were isolated and transformed into ElectroMAX™ DH10B cells (Invitrogen). Individual plasmids were purified and transfected into HEK 293T cells, after which the transfected cells were screened for their ability to bind PSG17N-Myc-His by ELISA (see below). Inserts from the plasmids that conferred PSG17N binding were sequenced with the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems).

Detection of PSG17 Binding to Transfected HEK 293T Cells by ELISA. HEK 293T cells were seeded in poly-L-lysine coated 96-well plates at 5×10^4 cells per well and transiently transfected with plasmid DNA recovered after library screening as described above or encoding the CD53, CD63, CD81, CD82, or CD151 cDNAs. At 48 h after transfection, the cells were washed with binding buffer containing 0.01% sodium azide and PSG17N-Myc-His (10 μg/ml) or no ligand was added to each well. After 1 h of incubation at room temperature, the ligand was aspirated and the cells were washed five times with binding buffer without sodium azide. To detect binding of PSG17N-Myc-His, anti-myc-horseradish peroxidase (HRP)-conjugated mAb was added to the cells for 1 h at room temperature at a concentration of 1 μg/ml in binding buffer. Binding of the antibody to the ligand was detected after the addition of tetramethylbenzidine (TMB)-peroxidase substrate (KPL) followed by 2 N H_2SO_4. The color change was quantitated at 450 nm on a microplate spectrophotometer.

Competition experiments were performed in CD9-pEF6/V5-His (Invitrogen) transfected HEK 293T cells by adding increasing concentrations of anti-murine CD9 mAb KMC8.8 (BD PharMingen) or an isotype-matched control (rat IgG_{2a}) for 1 h at room temperature before treatment with 5 μg/ml PSG17N. Absorbance was normalized to background binding of PSG17N in the presence of anti-CD9 mAb to empty plasmid transfected HEK 293T cells.

In Situ Rosetting Assay. For the in situ rosetting assay, HEK 293T cells transiently transfected with empty vector or CD9-pEF6/V5-His were seeded at low density into poly-L-lysine coated 60-mm dishes (Becton Dickinson). Attached cells were washed in binding buffer (PBS-2% BSA). PSG17N-Myc-His (90 pM) or binding buffer alone was added to the dishes for 1 h at room temperature. The cells were washed four times with PBS to remove any unbound ligand before the addition of 1 μg/ml anti-myc mAb in binding buffer for 1 h at room temperature. As a control, the anti-myc mAb was omitted from some plates. Dishes were washed again with PBS before the addition of 15 μg of rabbit anti–mouse Ig coated beads (Bio-Rad Laboratories). Unbound beads were removed by washing extensively with PBS and receptor positive cells were viewed by microscopy.

Flow Cytometry. HEK 293T or BHK-21 cells were transfected with murine CD9-pEF/V5-His or empty vector. 48 h after transfection, the cells were washed twice with wash buffer (PBS; 3% FBS; 0.01% sodium azide), before the addition of 10 μg PSG17N-Myc-His for 30 min at room temperature. After two washes, cells were sequentially incubated for 30 min on ice with 0.5 μg anti-myc mAb (Invitrogen), 0.5 μg biotin-labeled goat anti–mouse $IgG_{2a\kappa}$ (BD PharMingen) and 0.5 μg streptavidin-FITC (BD PharMingen) with two washes between each incuba-

tion. For inhibition experiments, 10^6 RAW 264.7 cells were preincubated with 1 μg of Fc block (BD PharMingen) and various concentrations of anti-CD9 mAb or isotype control mAb for 30 min on ice. After several washes, 2 μg of PSG17N-Myc-His were added to each tube followed by 0.7 μg of anti-myc mAb, and PE-labeled rat anti–mouse IgG1 (BD PharMingen). Binding of PSG17N-Myc-His to thioglycollate-induced peritoneal macrophages isolated from CD9-deficient mice or wild-type mice was determined after 24 h treatment of the macrophages with 10 ng/ml PMA followed by incubation with Fc block, PSG17N or a control myc-tagged protein, anti-myc mAb, and PE-labeled rat anti–mouse IgG1 as described above for the RAW cells. PSG17N binding to HEK 293T, BHK 21, RAW 264.7 cells and macrophages was analyzed by flow cytometry using an EPICS XL-MCL flow cytometer (Beckman Coulter) and the percent binding was determined with the System II Software program (Beckman Coulter). Overlays were produced with the WinList program (Verity Software House).

Alkaline Phosphatase Binding Assays. The PSG17-alkaline phosphatase (PSG17-AP) fusion protein was generated by cloning the full length PSG17 cDNA into the AP-Tag 4 vector. CD9 binding assays with heat stable AP-PSG17 were performed as described by Flanagan and Cheng (22). Briefly, HEK 293T cells transiently transfected with murine CD9 or empty vector were cultured in poly-L-lysine–coated six-well plates. Increasing concentrations (43, 86, and 129 nM) of AP-PSG17 or the AP control protein were added to each well in triplicate for 90 min at room temperature. The cells were thoroughly washed, and the concentration of bound protein was measured from cleared, heat-inactivated cell lysates with dephosphorylation of p-nitrophenyl phosphate, which was quantitated by absorption at 405 nm using an ELISA plate reader.

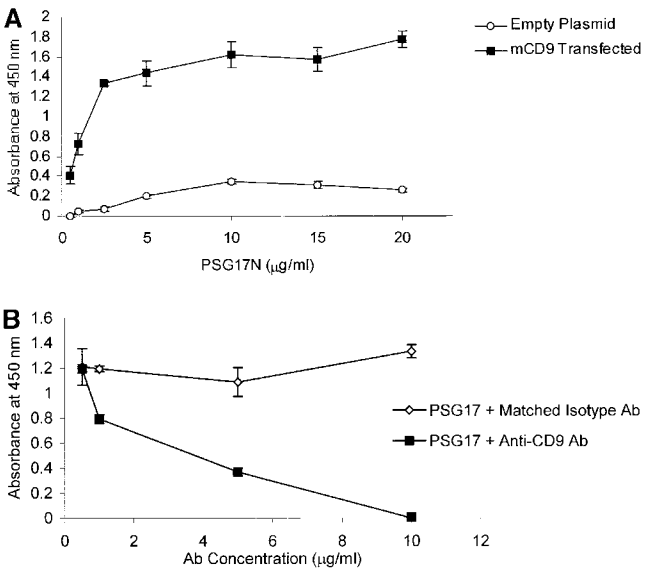

Figure 1. Binding of PSG17N to murine CD9-transfected 293T cells and inhibition of PSG17 binding to CD9 expressing cells by anti-CD9 mAb. (A) HEK 293T cells were transfected with CD9-pEF6/V5-His or empty plasmid after which they were incubated with varying concentrations of PSG17N-Myc-His. (B) HEK 293T-CD9-pEF/V5-His transfected cells were treated with increasing concentrations of anti-CD9 mAb or an isotype-matched control mAb before the addition of PSG17N-Myc-His (5 μg/ml). Bound PSG17N-Myc-His was detected after treatment with HRP-conjugated anti-myc mAb and TMB/peroxidase substrate. The data is expressed as mean absorbance ± SE. Each data point represents five identical wells and the experiment was repeated three independent times with similar results.

Results and Discussion

Identification of CD9 as the Cellular Receptor for PSG17. To isolate the PSG17 receptor, a RAW cell cDNA library was generated (Edge BioSystems) and plasmid DNA was transfected into HEK 293 EBNA cells, 72 h before panning on PSG17N-coated dishes. After three rounds of panning, plasmid DNA was isolated and electroporated into electrocompetent *Escherichia coli*. Plasmids isolated from individual bacterial colonies were transfected into HEK 293T cells and their ability to confer PSG17 binding was determined by ELISA. All positive clones were sequenced and database queries identified a perfect match between these cDNAs and murine CD9.

To confirm binding of PSG17 to CD9, CD9-pEF6/V5-His–transfected HEK 293T cell were treated with PSG17N-Myc-His and analyzed by ELISA. PSG17N-Myc-His was shown to bind CD9-transfected cells in a dose response manner with a binding plateau at 10 μg/ml (Fig. 1 A). Rosetting experiments further confirmed binding of PSG17N-Myc-His to CD9. CD9-pEF6/V5-His–transfected cells treated with PSG17N-Myc-His and anti-myc mAb followed by rabbit anti–mouse Ig immunobeads showed a rosetting pattern. This pattern was not observed in the control dishes, lacking PSG17N treatment, anti-myc mAb, or expression of CD9 (data not shown).

To demonstrate specific binding between PSG17N and murine CD9, competition ELISAs were performed in HEK 293T cells transfected with empty vector or CD9-pEF6/V5-His (Fig. 1 B). 48 h after transfection, anti-CD9 mAb or a matched isotype control mAb were added followed by PSG17N-Myc-His. The addition of 10 μg/ml anti-CD9 mAb reduced PSG17N binding to background levels. PSG17 binding to CD9 was also analyzed by flow cytometry (Fig. 2). HEK 293T cells were transfected with CD9-pEF6/V5-His or empty vector. To determine extent of expression of CD9 in the transfected cells, the cells were treated with biotin-labeled anti-CD9 mAb or a matched isotype control biotin-labeled mAb. Approximately 70% of the cells stained positive for CD9 (data not shown). The percentage of cells that stained positive for PSG17 binding to CD9-transfected cells ranged from 66–87% while there was <1% binding to the empty vector transfected cells (Fig. 2 A).

Screening of the library by panning and the binding experiments described above were all performed using HEK 293 cells. To examine whether PSG17 binds to CD9 on cells of a different species, we transfected BHK-21 cells with CD9-pEF6/V5-His. The results show significant binding of the PSG17N to CD9-transfected BHK-21 cells (~97%) compared with controls (<5%) suggesting that CD9 expression may be sufficient for PSG17N binding in any cell type (Fig. 2 B).

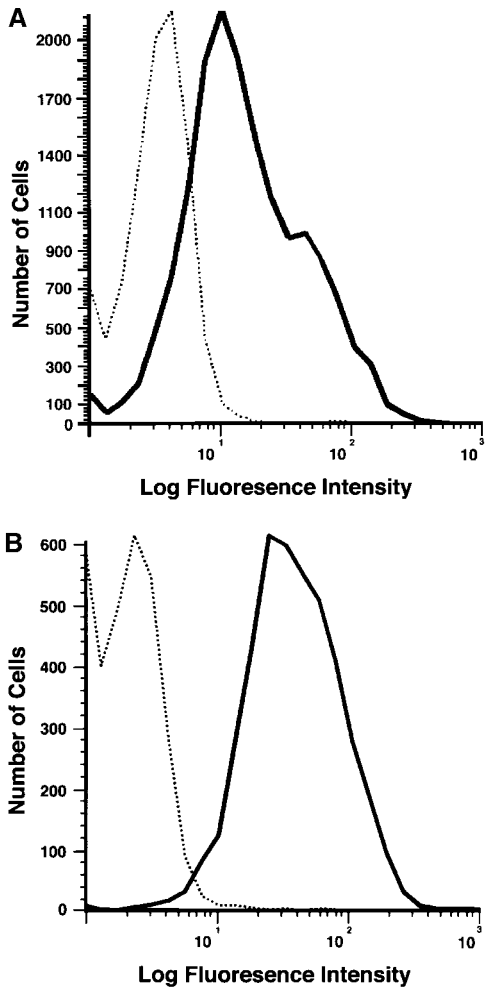

Figure 2. FACS® analysis of PSG17N-Myc-His binding to CD9-transfected HEK 293T cells and BHK-21 cells. HEK 293T cells (A) and BHK-21 (B) cells transfected with empty plasmid (dotted line) or murine CD9-pEF6/V5-His; solid line) were treated with PSG17N-Myc-His, anti-myc mAb, biotin-conjugated goat anti–mouse IgG_{2ak}, and FITC-conjugated streptavidin.

Binding of Alkaline Phosphatase-PSG17 (AP-PSG17) to CD9-expressing Cells. For the library screening and the binding experiments described above we used a truncated form of PSG17 consisting only of the N1-domain.

Table I. *Full-length PSG17 Binds to CD9-transfected Cells*

Protein treatment	AP-control bound	AP-PSG17 bound
nM	*pM*	*pM*
43.5	49.0 ± 20.8	701.7 ± 43.3
87.1	54.8 ± 17.3	3,564.3 ± 39.8
130.6	61.2 ± 8.7	5,956.4 ± 74.5

HEK 293T cells transfected with CD9-pEF6/V5-His were treated with increasing concentrations of AP-PSG17 or the AP-control protein. Each experiment was done in triplicate and the data points represent the mean concentration bound.

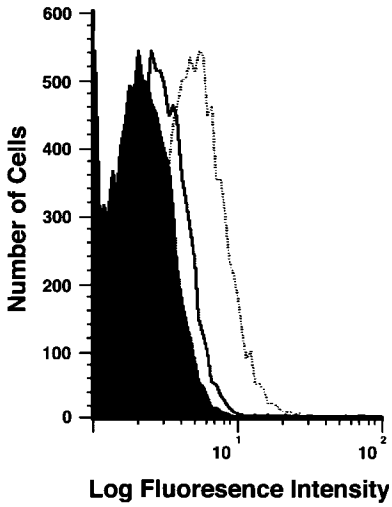

Figure 3. FACS® analysis of PSG17 binding to RAW 264.7 cells pretreated with anti-CD9 mAb KMC8.8. RAW 264.7 cells were incubated with Fc block after which they were treated with anti-CD9 mAb KMC8.8 (dotted line), an isotype match control mAb (filled line), or no Ab (shaded area) before the addition of PSG17N-Myc-His (dotted and filled lines), or a control myc-tagged protein (shaded area). Binding was detected by the sequential addition of anti-myc mAb and PE-labeled rat anti–mouse IgG1.

We examined binding of the full length PSG17 to murine CD9 using a rAP-PSG17 fusion protein. HEK 293T cells transiently transfected with CD9-pEF6/V5-His were treated with increasing concentrations of AP-PSG17 or an AP control protein (Table I). AP-PSG17 binding to CD9 transfected cells was concentration dependent while binding of the AP-control protein remained at baseline levels.

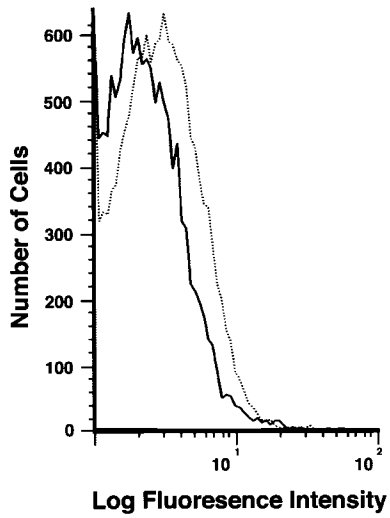

Figure 4. FACS® analysis of PSG17 binding to peritoneal macrophages isolated from CD9-deficient and wild-type mice. Peritoneal macrophages isolated from wild-type mice (dotted line) or CD9-deficient mice (solid line) were sequentially incubated with Fc block, PSG17N-Myc-His, anti-myc mAb, and PE-labeled rat anti–mouse IgG1.

Murine PSG17 Does Not Bind to the Murine Tetraspanins CD53, CD63, CD81, CD82, and CD151. To determine whether PSG17 used other members of the tetraspanin family as binding partners, murine CD53, CD63, CD81, CD82, and CD151 were transfected into HEK 293T and the cells were incubated with PSG17N-Myc-His. PSG17N did not show significant binding to the five tetraspanins tested in the ELISA assay (data not shown). At this point, we cannot conclude whether other members of the tetraspanin family, which we have not yet tested, could serve as receptors for murine PSG17 or whether the binding of PSG17 to the murine tetraspanins tested is of low affinity and cannot be detected in the assay we employed.

CD9 Is the Receptor for PSG17 in RAW 264.7 Cells and Macrophages. Scatchard analysis revealed that PSG17 has a single binding site in RAW 264.7 cells and murine macrophages (unpublished data). To determine whether this binding site is CD9, RAW 264.7 cells were pretreated with anti-CD9 mAb KMC8.8 or an isotype match control mAb before the addition of PSG17N-Myc-His and binding was determined by flow cytometry. Preincubation with 40 μg/ml of anti-CD9 mAb resulted in a significant reduction in median fluorescence intensity when compared with the median fluorescence intensity of the cells pretreated with the control isotype match mAb (Fig. 3). In addition, the extent of the inhibition of PSG17 binding to RAW 264.7 cells by the anti-CD9 mAb was dependent on the concentration of the anti-CD9 mAb employed (data not shown).

Binding of PSG17 to macrophages isolated from CD9-deficient mice was reduced to almost background levels, defined as the binding of a control myc-tagged protein (data not shown), and was significantly lower than the binding of PSG17 to macrophages of wild-type mice (Fig. 4). Based on these and our previous results (unpublished data), we conclude that CD9 is the only receptor in macrophages and that binding of PSG17 to CD9 results in the secretion of cytokines.

Signaling molecules associated with CD9 include phosphatidylinositol 4-kinase and protein kinase C (23, 24). PSG17 could signal induction of cytokines by means of a variety of interactions with any of these molecules or others (18). To date, the proteins associated with CD9 in murine macrophages have not been identified although a functional association of CD9 with Fcγ receptors in murine macrophages has been reported (25).

$CD9^{-/-}$ have reduced female fertility linked to a defect in egg/sperm fusion (26, 27). $CD9^{-/-}$ mice produce some viable fetuses, indicating that CD9 is important for fertilization but is not essential for pregnancy success. Whether all murine PSGs use CD9 as their cellular receptor remains to be investigated. Different murine PSGs may have evolved to use different receptors and no single PSG may be essential for pregnancy success. In addition, we anticipate that pregnancy complications associated with lack of PSG-mediated cytokine secretion would be demonstrated in matings of mice with different genetic backgrounds.

Because CD9 is expressed in several cells of the immune system, including: macrophages, dendritic cells (data not shown), and T cells (28), PSGs have a potential role in regulating both the innate as well as the adaptive immune responses during pregnancy.

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