Defective Chemokine Signal Integration in Leukocytes Lacking Activator of G Protein Signaling 3 (AGS3)*

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Background: Roles for AGS3/Gpsm1 in the immune system are not defined.

Results: Loss of AGS3 expression results in defective leukocyte chemotaxis, calcium mobilization, and ERK1/2 and Akt activation.

Conclusion: AGS3 is required for efficient chemokine receptor signal integration.

Significance: These studies extend the functional repertoire for AGS3 in the immune system, providing unexpected regulatory mechanisms for immune function.

Activator of G-protein signaling 3 (AGS3, gene name G-protein signaling modulator-1, Gpsm1), an accessory protein for G-protein signaling, has functional roles in the kidney and CNS. Here we show that AGS3 is expressed in spleen, thymus, and bone marrow-derived dendritic cells, and is up-regulated upon leukocyte activation. We explored the role of AGS3 in immune cell function by characterizing chemokine receptor signaling in leukocytes from mice lacking AGS3. No obvious differences in lymphocyte subsets were observed. Interestingly, however, AGS3-null B and T lymphocytes and bone marrow-derived dendritic cells exhibited significant chemotactic defects as well as reductions in chemokine-stimulated calcium mobilization and altered ERK and Akt activation. These studies indicate a role for AGS3 in the regulation of G-protein signaling in the immune system, providing unexpected venues for the potential development of therapeutic agents that modulate immune function by targeting these regulatory mechanisms.

Integration of signals emanating from chemokine receptors represents one of the most commonly used mechanisms for leukocyte distribution and recruitment to lymphoid organs and the periphery. Such signal integration involves not only the regulated expression of individual chemokine receptors and the stoichiometries of the core signaling triad of receptor, heterotrimeric G-protein, and effectors but also cell type-specific accessory proteins that modulate signals across this core signaling system. Such accessory proteins bestow upon leukocytes and other cells the ability to tightly control signaling pathways to maximize signal efficiency, strength, and duration while at the same time providing flexibility to quickly adapt to changes in environmental stimuli (1, 2).

Perturbations of heterotrimeric G-protein signal input or duration result in defective leukocyte development, trafficking, motility, and overall chemokine responsiveness (3–9). In addition, accessory proteins at the GPCR/G-protein interface also play key roles in regulating leukocyte function by modulating G-protein activity and responsiveness to chemokines. Many regulators of G-protein signaling (RGS) proteins are expressed in leukocytes (1, 10) and play important functions in regulating chemokine responsiveness. For example, RGS1 plays an important role in modulating lymphocyte motility and trafficking (4). RGS1−/− lymphocytes move more rapidly in response to chemokines, suggesting that modulating the duration of Goi activation in response to chemokines plays an important role in leukocyte activation and trafficking (4). Additional mechanisms for modulation of Goi activity are also likely important for spatio-temporal regulation of leukocyte responsiveness and for integration of signals from multiple chemokines at any given time.

Another group of accessory proteins, the activators of G-protein signaling (AGS) proteins, were identified in a yeast-based functional screen for receptor-independent activators of heterotrimeric G-proteins (11, 12) and can be broadly categorized into three groups based on their input into the G-protein activation/deactivation cycle (13). Group II AGS proteins are characterized by the presence of up to four G-protein regulatory

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‡ The abbreviations used are: GPCR, G-protein-coupled receptor; GPR, G-protein regulatory; RGS, regulators of G-protein signaling; AGS, activators of G-protein signaling; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; iDC, immature dendritic cell; mDC, mature dendritic cell; DMSO, dimethyl sulfoxide; LGN, Leu-Gly-Asn-enriched protein.
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(GPR) motifs (also referred to as LGN or GoLoco motifs (14, 15)), which bind free Gαi/o and subunits in the GDP-bound conformation and act as guanine nucleotide dissociation inhibitors (16, 17). GPR motif-containing proteins thus provide a novel mode of signal input to heterotrimeric G-proteins that may operate distinct from the superfamily of GPCRs and may also function as binding partners for Gαi/o subunits independent of heterotrimer formation. These theories have broad implications for signal processing and provide a mechanism for unexpected functions of G-proteins as signal transducers within the cell.

A member of the Group II AGS proteins, AGS3 (gene name, G-protein signaling modulator-1 (Gpsm1)) contains seven tetra- ricopeptide repeats, which are involved in protein-protein interactions and four G-protein regulatory (GPR)/GoLoco motifs (also referred to as LGN or GoLoco motifs (14, 16, 17). GPR motif-containing proteins thus provide a novel mode of signal input to heterotrimeric G-proteins that may function as binding partners for Gαi/o subunits free of Gβγ. Previous data suggest functional roles for AGS3 in such diverse processes as neuronal plasticity and addiction, autophagy, membrane protein trafficking, polycystic kidney disease, cardiovascular regulation, and metabolism (18–28). As part of an expanded approach to more fully understanding the in vivo role of AGS3 in G-protein signal processing, we previously reported the generation of a conditional AGS3-null mouse strain, which is a valuable model to dissect physiological functions of AGS3 (18, 23, 26).

Our goal in this study was to define the functional role of AGS3 in leukocytes, beginning with its role in chemokine receptor signal integration. Our data suggest that AGS3 assists in the integration of signals from the receptor to the chemotactic machinery, including calcium mobilization, ERK1/2 and Akt phosphorylation, and leukocyte motility. These data indicate key roles for AGS3 in the integration of chemokine receptor signaling and expand the functional repertoire of accessory proteins in the immune system.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant mouse G-M-CSF, CXCL12, and CCL19 were purchased from BioAbChem Inc. (Ladson, SC). AGS3-antisera generated by immunization of rabbits with a GST-AGS3 fusion protein encoding the GPR domain (Ala461–Ser650) of AGS3 was kindly provided by Dr. Dzwokai Ma (University of California, Santa Barbara, CA). Gαi1/2 and Gαi3 antisera were kindly provided by Dr. Thomas Gettys (Pennington Biomedical Research Center, Baton Rouge, LA). Protease inhibitor cocktail tablets (Complete Mini) were obtained from Roche Diagnostics (Mannheim, Germany). Other materials were obtained as described elsewhere (29, 30).

**Mice**—Generation of Gpsm1−/− mice3 used in this study was previously described (18). Wild-type and Gpsm1−/− female littermates at 6–12 weeks of age from Gpsm1+/+ intercrosses were used. Gpsm1+/− breeding pairs were generated from backcrosses onto C57BL/6 mice for more than 12 generations. Tissues and lysates were prepared and processed for immunoblotting as described (31).

**Flow Cytometry and Cell Sorting**—Single-cell suspensions from spleen and thymus were prepared from female mice (Gpsm1+/+ and Gpsm1−/−) littermates at 6 weeks of age by crushing freshly dissected tissues between frosted glass slides in PBS. After lysing red blood cells with ammonium-chloride-potassium (ACK) lysis buffer (170 mM NH4Cl, 170 mM Tris), cells were counted and washed with PBS with 1% BSA and 0.05% NaN3 (PBS-BSA). A total of 106 cells were first incubated with anti-FcγIII (CD16/CD32) for 30 min at 4 °C to block Fc receptors, and then cells were incubated with primary FITC or phycoerythrin-conjugated antibodies in PBS-BSA for 30 min at 4 °C (BD Pharmingen). Cells were washed twice in PBS-BSA, resuspended in 500 μl of PBS-BSA, and analyzed on a flow cytometer (BD Pharmingen). CD4 and CD8 mAbs were used to characterize thymus cell subsets. The following mAbs were used to characterize spleen cell subsets: total B cells (B220+, CD3−); follicular B cells (B220+, CD21intermediate+, CD23+); marginal zone B cells (B220+, CD21high+, CD23−); double positive T cells (CD4+, CD8+); and double negative T cells (CD4+, CD8−).

**Primary Cells**—Bone marrow cells from WT or Gpsm1−/− mouse femurs and tibiae were filtered through a 40-μm nylon cell strainer, red blood cells were lysed with ammonium-chloride-potassium lysis buffer, and cells were resuspended in DC media (RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin, and 20 ng/ml recombinant mouse GM-CSF) and plated 4–5 × 105 cells/ml in a 100-mm tissue culture dish. On day 4, 10 ml of fresh DC media was added to each dish. On day 8, nonadherent and loosely adherent cells were harvested, washed, and replated in fresh DC medium containing 10 ng/ml recombinant mouse GM-CSF to generate immature dendritic cells (iDC). On day 9, cells were treated with or without 200 ng/ml LPS for the indicated times or for 24 h to generate mature dendritic cells (mDC). Spleens of WT or Gpsm1−/− mice were gently crushed between frosted glass slides in serum-free RPMI. Red blood cells were lysed with ammonium-chloride-potassium lysis buffer, and splenocytes were washed in PBS (Ca2+, Mg2+-free) supplemented with 0.1% BSA and 2 mM EDTA and then resuspended at 5 × 107 cells/ml or 1 × 108 cells/ml for subsequent B cell or T cell isolation, respectively. Cell isolation was performed according to Invitrogen Dynabeads protocol for untouched B cell isolation or negative T cell isolation.

**Chemotaxis**—Corning Transwell 24-well inserts (6.5-mm diameter, 5.0-μm pore size) or 96-well inserts (5.0-μm pore size) were used for all chemotaxis assays. For dendritic cell chemotaxis, serum-free RPMI with or without 250 ng/ml CXCL12 or CCL19 was added to the lower chamber, and ~3 × 106 cells/ml were loaded into the upper chamber. Where indicated, dendritic cells were preincubated with 10 μM gallein (final concentration) or vehicle (0.02% DMSO final concentration) in serum-free RPMI for 30 min at 37 °C prior to measuring chemotaxis. In these cases, 10 μM gallein (final concentration) and vehicle (0.02% DMSO final concentration) were included in both upper and lower chambers as described previously (32).
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For lymphocytes, serum-free RPMI supplemented with 0.05 μM β-mercaptoethanol with or without 300 ng/ml CXCL12 or CCL19 was added to the lower chambers, and ~1 × 10⁷ lymphocytes/ml were added to the upper chamber. Chemotaxis chambers were incubated at 37 °C, 5% CO₂ for 20 h for dendritic cells and 5 h for lymphocytes. The upper chamber was then removed, and cells migrating to the lower chamber were counted. The percentage of cells migrating to chemokine was determined by subtracting the average cells migrating to vehicle alone and then comparing with the actual number of input cells.

**Determination of Intracellular Calcium Levels**—Freshly harvested splenocytes of WT and *Gpsm1*⁻/⁻ mice were seeded into clear bottom, black-walled 96-well plates at 1 × 10⁶ cells/well in 100 μl of serum-free, phenol red-free RPMI, and cells were serum-starved for at least 1 h. Cells were incubated in the Calcium 5 assay kit dye for 1 h at 37 °C, and then the plate was allowed to come to room temperature for 15 min prior to analysis. CXCL12 or CCL19 was added at a final concentration of 200 ng/ml by the FLIPRtetra instrument, and measurements were taken every second for at least 300 s. Bone marrow cells were harvested from WT and *Gpsm1*⁻/⁻ female mice between 6 and 10 weeks of age and cultured to dendritic cells as described above. DCs were seeded at 2.5 × 10⁸ cells/well and serum-starved for ~1 h followed by incubation in the calcium dye for 1 h. CXCL12 was added at a final concentration of 200 ng/ml by the FLIPRtetra, and measurements, reported as relative light units, were taken every second for at least 300 s. Negative control and response over baseline corrections were applied using the ScreenWorks 3.1 software.

**Immunoblotting**—Single-cell suspensions from spleen and thymus were prepared by crushing freshly dissected tissues between frosted glass slides in PBS. After lysing red blood cells with ice-cold 0.17 M NH₄Cl, 0.17 M Tris for 5 min at room temperature followed by centrifugation at 500 × g for 5 min at room temperature, cells were pelleted and resuspended in 1% Nonidet P-40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40) on ice for 20 min followed by centrifugation at 10,000 × g for 30 min at 4 °C. Protein concentration was determined by a Pierce BCA protein assay. Protein samples were loaded on denaturing 10% polyacrylamide gels and then were transferred to polyvinylidene difluoride membranes for immunoblotting as described (31). Cell pellets processed for phosphorylated proteins were lysed in 1% Nonidet P-40 buffer with protease and phosphatase inhibitors (50 mM NaF, 5 mM sodium pyrophosphate, 40 mM β-glycerophosphate, and 200 μM Na₃VO₄) on ice for 20 min followed by centrifugation at 10,000 × g for 30 min at 4 °C. Samples were subjected to SDS-PAGE, and proteins were transferred to PVDF membranes and immunoblotted for anti-phospho-Akt (Ser⁴⁸³) (Life Technologies), anti-phospho-ERK (Tyr⁴⁰²) (Santa Cruz Biotechnology, Dallas, TX), or total ERK (Abcam, Cambridge, MA) and total Akt (Cell Signaling Technology, Boston, MA) antibodies. Densitometric quantification of the immunoblotted bands was performed using Image densitometry software (Version 1.46r, National Institutes of Health, Bethesda, MD). Selected bands were quantified based on their relative intensities and normalized to total ERK or total Akt.

**RESULTS**

Increasing evidence indicates a growing number of cellular and physiological roles for accessory proteins such as AGS3 and other proteins containing the GPR motif in dynamic signaling systems such as the central nervous system (CNS) where signal modulation and adaptation of G-protein signaling systems are key to the responsiveness of the system (19–21, 28, 33). The dynamic processing of signals in the immune system also involves highly specialized, spatially integrated, G-protein signaling mechanisms (1, 34). As an initial approach to define the role of GPR proteins in such modes of signal integration, we studied the role of the GPR protein AGS3 in chemotactic signaling in immune cells.

**Analysis of Protein Expression and Leukocyte Populations from AGS3/Gpsm1⁻/⁻ Mice**—To explore potential functional roles for AGS3 in leukocytes, we took advantage of a recently developed AGS3/Gpsm1-null mouse model (18).³ AGS3 (*M*⁻/⁻ ~74,000) was abundant in cells isolated from the thymus and spleen of wild-type mice, but as expected, was completely absent from the same tissues in *Gpsm1*⁻/⁻ mice (Fig. 1). Immunoblot analysis revealed no obvious compensatory alterations in expression of the AGS3/Gpsm1-related protein LGN/
Expression analysis of Gα2 and Gα3 subunits, which are the predominantly expressed Gα subunits in hematopoietic cells (35–37), indicated that Gα2 and Gα3 expression was largely unaffected with a modest increase in expression of Gα2 in Gpsm1+/− splenocytes and thymocytes (Fig. 1).

AGS3 and other proteins with the GPR motif together with Gαi and Gβγ play key roles in asymmetric cell division of many types of stem and progenitor cells, thus influencing cell fate decisions (38, 39). In addition, defects in Gαi signaling result in aberrant lymphocyte differentiation (7, 35, 40). Therefore, we hypothesized that loss of AGS3 may result in defective lymphocyte differentiation. However, analysis of lymphocyte subsets from spleens and thymi of naive Gpsm1−/− mice indicated no statistically significant differences in the relative distribution of B and T cell and other leukocyte subsets as compared with wild-type mice (Table 1).

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Leukocyte Stimulation Enhances AGS3 Protein Levels—As we observed no obvious defects in lymphocyte subset differentiation, we next explored whether AGS3 levels were affected by stimulation of leukocytes. Indeed, dramatic up-regulation of AGS3 expression was observed in primary B cells stimulated with either LPS or IgM and primary T cells stimulated with anti-CD3 and interleukin (IL)-2 (Fig. 2A). In addition, AGS3 levels were also markedly increased over time in primary bone marrow-derived dendritic cells (BMDCs) after treatment with LPS (Fig. 2B). Up-regulation of AGS3 upon immune cell activation suggests a role for AGS3 in regulating immune responses; furthermore, these results indicate that AGS3 levels are responsive to changes in the cellular environment, which is consistent with previous reports of regulation of AGS3 expression by cell stress and maladaptation (19–21, 23, 24, 26).

Defective Chemotaxis in AGS3/Gpsm1−/− Leukocytes—Gαi signal integration is a critical component of chemokine signaling (e.g. (41)). We therefore sought to determine the effect of the loss of AGS3 on chemokine-directed signaling events. As an initial approach to address this question, we analyzed the chemotactic responses of leukocytes isolated from WT and Gpsm1−/− mice. B and T lymphocytes isolated from Gpsm1−/− mice showed nearly a 50% reduction in chemotaxis toward either CXCL12/SDF-1α or CCL19 as compared with WT lymphocytes (Fig. 3, A and B). Similarly, BMDCs from AGS3-null mice showed ~30% reduction in migration toward the chemokine CXCL12 (Fig. 3C, left panel). There was no significant difference in random migration between WT and Gpsm1−/− lymphocytes or BMDCs, suggesting that the chemotactic defect in Gpsm1−/− cells was not due to an overall decrease in cell motility. Chemokine receptor expression levels were not altered in Gpsm1−/− BMDCs or lymphocytes as compared with WT cells as determined by flow cytometry, indicating that the chemotactic defect in Gpsm1−/− leukocytes was not due to loss of chemokine receptor expression.

Treatment of BMDCs with inflammatory signals such as LPS induces differentiation from iDCs into mDCs. Chemokine receptor expression levels and subsequent chemokine responsiveness in DCs are very sensitive to this maturation process,

![Table 1](magnify)

| Genotype | Gpsm1+/+ | Gpsm1−/− |
|----------|----------|----------|
| Total splenic cell populations | | |
| B220+ | 49 ± 1 | 50 ± 2 |
| Non B, Non T | 10 ± 1 | 12 ± 1 |
| CD3+ | 38 ± 1 | 35 ± 2 |
| Percentage of cells from splenic B220+ population | | |
| Follicular | 50 ± 3 | 50 ± 3 |
| Marginal Zone | 14 ± 3 | 13 ± 5 |
| Percentage of cells from splenic CD3+ population | | |
| CD4+ | 55 ± 2 | 53 ± 1 |
| CD4+ CD8+ | 1.2 ± 0.1 | 1.2 ± 0.2 |
| CD4− CD8+ | 6.3 ± 1.1 | 7.8 ± 0.4 |
| CD8+ | 38 ± 2 | 37 ± 1 |
| Percentage of cells from thymic CD3+ population | | |
| CD4+ | 8 ± 2 | 7 ± 2 |
| CD4+ CD8+ | 85 ± 4 | 86 ± 3 |
| CD4− CD8+ | 3.4 ± 0.5 | 3.3 ± 0.3 |
| CD8+ | 3.3 ± 0.7 | 3.2 ± 0.7 |

![Figure 2](magnify)

**FIGURE 2.** AGS3 is up-regulated in lymphocytes and BMDCs upon stimulation. A, purified B and T lymphocytes from C57BL/6J mice were isolated as described under “Experimental Procedures.” Purified B cells were stimulated with 20 μg/ml anti-IgM F(ab′)2 fragment or 1 mg/ml LPS for 12 h. Purified T cells were stimulated with 0.1 μg/ml anti-CD3 and 20% IL-2 for 24–48 h. After treatment, cells were washed and lysed in SDS sample buffer and subjected to SDS-PAGE and immunoblotting with AGS3 and actin-specific antisera. B, T lymphocytes isolated from Gpsm1−/− mice were incubated with 0.5–18 μg/ml LPS for the times indicated in the figure. After treatment, cells were washed and lysed with 1% Nonidet P-40 buffer and subjected to SDS-PAGE and immunoblotting with AGS3 and actin-specific antisera as described under “Experimental Procedures.”

4 M. Branhom-O’Connor, W. G. Robichaux, III, and J. B. Blumer, unpublished observations.
and DCs respond by rapidly changing their chemokine receptor expression profiles and chemokine sensitivity (42, 43). Although CXCR4 expression increases during maturation, chemotactic responses to its agonist CXCL12 are apparently reduced (44–46). In addition, CCR7 levels increase dramatically upon maturation, and DC responsiveness to the CCR7 agonist CCL19 is significantly enhanced (42–47). We measured WT and Gpsm1−/− BMDC chemotaxis to CXCL12 and CCL19 before and after treatment with LPS for 24 h. As shown in Fig. 3C, our results for WT BMDC chemotaxis before and after maturation (iDC and mDC, respectively) are consistent with previously published observations (44–46) in that iDCs preferentially responded to CXCL12 (Fig. 3C, left panel) and mDCs preferentially responded to CCL19 (Fig. 3C, right panel). One would predict that if AGS3 is up-regulated upon LPS treatment (Fig. 2) and if AGS3 plays a positive role in integrating chemokine signals, then the chemotactic defect in DCs from Gpsm1−/− mice would be greater for mDCs as compared with iDCs. As expected, Gpsm1−/− iDCs exhibited an ~25% decrease in chemotaxis to CXCL12 (Fig. 3C, left panel); however, Gpsm1−/− mDCs showed an ~40% decrease in chemotaxis to CCL19 as compared with WT mDCs (Fig. 3C, right panel). These data are consistent with the hypothesis that chemokine signal integration is sensitive to AGS3 levels and that AGS3 plays a positive role in chemokine signal processing.

A possible explanation for AGS3 function in chemokine signal integration is that by binding Go subunits prior to reformation of Gαβγ heterotrimer, AGS3 could enhance or prolong Gβγ signaling. As an initial approach to address this question, we treated WT and Gpsm1−/− dendritic cells with gallein, a Gβγ inhibitor. Although the chemotactic response to chemokine was reduced in Gpsm1−/− dendritic cells, the magnitude of inhibition of chemokine-induced chemotaxis by the Gβγ antagonist gallein was similar in both WT and Gpsm1−/− dendritic cells (Fig. 3D). These data suggest that the reduced chemotaxis observed in Gpsm1−/− cells may result from the loss of a positive modulatory role of AGS3 on Gβγ signaling.

**Impaired Chemokine-mediated Signal Processing in AGS3/Gpsm1−/− Leukocytes—** To further define the effects of loss of AGS3 on chemokine signaling, we investigated chemokine-induced mobilization of intracellular calcium in WT and Gpsm1−/− leukocytes (Fig. 4). An increase in intracellular calcium plays a critical role in cell motility and chemotaxis (48, 49).
Indeed, Gpsm1<sup>-/-</sup> splenocytes and BMDCs showed diminished chemokine-induced calcium responses (Fig. 4), consistent with the chemotactic defect in Gpsm1<sup>-/-</sup> cells as compared with WT cells.

In addition to calcium mobilization, phosphorylation of ERK1/2 and Akt is also attributed to chemokine signaling. Stimulation of chemokine receptors such as CXCR4 activates the kinase ERK1/2 by stimulating its phosphorylation (50), which in turn is an important regulator of cell motility in many cell types including lymphocytes and dendritic cells (41, 51). Interestingly, Gpsm1<sup>-/-</sup> BMDCs showed significant impairment of CXCL12-stimulated phosphorylation of ERK1/2 as well as Akt (Fig. 5). In addition, pretreatment with pertussis toxin blocked CXCL12-induced ERK1/2 and Akt phosphorylation in BMDCs from both WT and Gpsm1<sup>-/-</sup> mice to the same extent (Fig. 5). GM-CSF-stimulated ERK1/2 phosphorylation, acting through a tyrosine kinase receptor pathway, was not affected by the loss of AGS3 expression in Gpsm1<sup>-/-</sup> BMDCs.<sup>4</sup> Splenocytes from Gpsm1<sup>-/-</sup> mice also showed significant defects in both CXCL12-stimulated and CCL19-stimulated ERK1/2 and Akt phosphorylation (Fig. 6).

**DISCUSSION**

Accessory proteins for G-protein signaling systems have revealed surprising diversity in modes of heterotrimeric G-protein signal integration, including but not limited to the modulation of signal strength, duration, location, termination, and the formation of signal transduction complexes (for review, see Refs. 2, 13, 16, and 17). Although functional roles for the accessory protein AGS3 have been described in asymmetric cell division, neuronal plasticity and addiction, autophagy, polycystic kidney disease and renal injury, cardiovascular regulation, and metabolism (18–28, 33), the data described in this study are the first to demonstrate a role for AGS3 in the regulation of chemokine responses in hematopoietic cells. Indeed, there are relatively few reports of GPCR signal modulation by GPR motif-containing proteins (21, 28, 52–56), underscoring the significance of the current study, which, in contrast to the previous studies, makes use of primary cells obtained from genetic null (Gpsm1<sup>-/-</sup>) mice.

In this study we demonstrate that the loss of AGS3 expression results in aberrant chemokine-directed cell motility, Ca<sup>2+</sup> flux, and ERK and Akt activation. These data suggest expanding functional roles for accessory proteins that, like AGS3, contain the GPR motif in the integration of chemokine receptor signals. As compared with the dramatic defects in chemokine responsiveness in Go<sub>ax</sub>-null mice (3–9), the ~30–40% reduction in agonist-induced chemotaxis rather than a complete loss of chemotaxis in Gpsm1<sup>-/-</sup> leukocytes is consistent with its effect as a modulatory protein. With respect to the role of AGS3 in chemokine receptor signal integration, three distinct hypotheses present themselves: 1) the chemokine receptor couples to the AGS3-G<i><sub>ai2</sub></i> complex in a manner analogous to Go<i><sub>a</sub></i>βγ heterotrimer and releases Go<i><sub>a</sub></i>-GTP to modulate the chemotactic response; 2) the Go<i><sub>a</sub></i>-AGS3 module itself initiates the formation of an additional, noncanonical signaling complex distinct from Gβγ that modulates chemokine-directed signaling events; and/or 3) by virtue of its GPR motifs, AGS3 influences Go<i><sub>a</sub></i> and Gβγ subunit interactions to prolong or enhance Gβγ signaling to facilitate directed migration.

In the first hypothesis, the chemokine receptor couples to the Go<i><sub>a</sub></i>-AGS3 complex (29) to integrate downstream signaling events involving both Go<i><sub>a</sub></i> and Gβγ. In heterologous systems, the Go<i><sub>a</sub></i>-AGS3 module appears to directly engage with and is regulated by Go<i><sub>a</sub></i>-coupled GPCRs (29) including CXCR4. It is not immediately clear how such a signaling system may directly impact GPCR-initiated G-protein signaling events; however, studies of Go<i><sub>a</sub></i> subunit selectivity for chemokine-induced responses (5, 35, 57, 58) may suggest that Go<i><sub>a</sub></i> subunits (particularly Go<i><sub>a16</sub></i>) do have a direct impact on chemokine signaling in addition to regulating Gβγ, and thus the AGS3-Go<i><sub>a</sub></i> module may be operative within this context.

<sup>5</sup> W. G. Robichaux III and J. B. Blumer, unpublished observations. Using a recently developed bioluminescence resonance energy transfer (BRET) platform in HEK293 cells, we observed that when coexpressed with CXCR4, AGS3-RLuc–Go<i><sub>a</sub></i>ε-YFP BRET signals were reduced by ~40% by the CXCR4 agonist CXCL12. We also observed a Go<i><sub>a</sub></i>ε-dependent, agonist-regulated complex between AGS3-RLuc and CXCR4-Venus. Both effects were blocked by either the CXCR4 antagonist AMD3100 or by pertussis toxin pretreatment.
FIGURE 5. Gpsm1−/− dendritic cells exhibit defects in CXCL12-stimulated phosphorylation of ERK1/2 and Akt. Single cell suspensions of Gpsm1+/+ and Gpsm1−/− cultured dendritic cells were pretreated in the absence or presence of 100 ng/ml pertussis toxin (PTX) for 18 h prior to the addition of 200 ng/ml CXCL12 as described under "Experimental Procedures." At the indicated times, cells were lysed in 1% Nonidet P-40 lysis buffer containing protease and phosphatase inhibitors, and lysates (50 μg/lane) were subjected to SDS-PAGE, transferred to PVDF, and immunoblotted with anti-phospho-Akt (Ser483) (pAkt), anti-phospho-ERK (Tyr204) (pErk1/2), or total Akt- or total ERK-specific antibodies. Representative immunoblots are shown in the left panels, and densitometric analysis of at least three independent experiments (represented as means ± S.E.) are shown in the right panels. *, p < 0.05.

FIGURE 6. Gpsm1−/− splenocytes exhibit defects in CXCL12- and CCL19-stimulated phosphorylation of ERK1/2 and Akt. A and B, single cell suspensions of Gpsm1+/+ and Gpsm1−/− freshly isolated splenocytes were treated with 200 ng/ml CXCL12 (A) or CCL19 (B) as described under "Experimental Procedures." At the indicated times, cells were lysed in 1% Nonidet P-40 lysis buffer containing protease and phosphatase inhibitors, and lysates (50 μg/lane) were subjected to SDS-PAGE, transferred to PVDF, and immunoblotted with anti-phospho-Akt (Ser483) (pAkt), anti-phospho-ERK (Tyr204) (pErk1/2), or total Akt- or total ERK-specific antibodies. Representative immunoblots are shown in the left panels, and densitometric analysis of at least three independent experiments (represented as mean ± S.E.) are shown in the right panels. *, p < 0.05; **, p < 0.01.
The second hypothesis is that the AGS3-G<sub>α</sub><sub>i</sub>-GDP complex generated subsequent to receptor activation by AGS3 binding to G<sub>α</sub><sub>i</sub>-GDP prior to reassociation with G<sub>β</sub><sub>γ</sub> acts in the context of a novel, noncanonical signaling complex that integrates with G<sub>β</sub><sub>γ</sub>-regulated events to influence chemotaxis. Interestingly, a recent study suggests that the G<sub>α</sub><sub>i</sub>-AGS3 module acting through the AGS3 binding protein mammalian inscuteable (mInsc) and the Par3-atypical PKC (aPKC) complex is involved in regulating directed migration of neutrophils (57). These observations would suggest that G<sub>α</sub><sub>i</sub>-GDP bound to GPR motif(s) would either function as an active signaling entity (38) or serve as a platform for additional signal input as proposed previously (16).

However, cellular responses to chemokine receptor activation are generally thought to be predominantly due to the actions of G<sub>β</sub><sub>γ</sub> subunits, stemming from original observations by Neptune and Bourne (59), Charo and co-workers (60), and in Dicyostelium (61). G<sub>β</sub><sub>γ</sub>-mediated stimulation of PI3Kγ (62, 63), phospholipase Cβ (PLCβ) (64), ERK1/2 (65, 66), and exchange factors for small GTPases Rac and Cdc42 (67) appear to underlie the requirement of G<sub>β</sub><sub>γ</sub> for regulating chemotactic-directed cell motility. More recently, small molecule inhibitors of G<sub>β</sub><sub>γ</sub> further implicate a role for G<sub>β</sub><sub>γ</sub> in mediating chemokine responses in multiple cell types (32, 71) (Fig. 3D).

Within this context, the third hypothesis is that AGS3 may influence interactions between G<sub>α</sub><sub>i</sub> and G<sub>β</sub><sub>γ</sub> subunits and thus impair a positive modulatory effect on cellular responses to chemokines. As AGS3 GPR motifs compete with G<sub>β</sub><sub>γ</sub> for G<sub>α</sub><sub>i</sub> binding (12, 29, 72, 73), one possibility is that AGS3 may “grab” free G<sub>α</sub><sub>i</sub>-GDP prior to reassociation with G<sub>β</sub><sub>γ</sub> and thus enhance or prolong G<sub>β</sub><sub>γ</sub>-regulated effector activation (13, 16), and this hypothesis does have some support in the broader context of GPR proteins influencing G<sub>α</sub><sub>i</sub>-GDP subunit interactions (12, 23, 24, 26, 33, 53–56). The loss of AGS3 may thus lead to reduced chemokine-mediated G<sub>β</sub><sub>γ</sub> signaling, which is the most likely explanation for the observed defects in Gpsm1<sup>−/−</sup>-leukocyte chemotaxis (Fig. 3), calcium mobilization (Fig. 4), and activation of ERK1/2 and Akt (Figs. 5 and 6), all of which are known G<sub>β</sub><sub>γ</sub> effector pathways as discussed above. Taken together, the data strongly suggest that AGS3 plays a modulatory role on chemokine-mediated G<sub>β</sub><sub>γ</sub> signaling. As other GPR proteins are also expressed in immune tissues and cells, including LGN/Gpsm3 (Fig. 1) (31, 74), AGS4/Gpsm3 (75),<sup>a</sup> and RGS14 (76), it is possible that these proteins may be partially masking the effects of the loss of AGS3 in this process. Defining the roles of these GPR proteins in chemokine signal integration may reveal additional functional capacity of the GPR motif in this context and is a focus of current efforts.

This study extends our previous work in defining functional roles of GPR proteins in the intact animal using the AGS3/Gpsm1<sup>−/−</sup> mouse model (18, 23, 24, 26). This model should continue to provide a valuable platform for further exploration into the functional impacts of GPR-G<sub>α</sub><sub>i</sub> module regulation on leukocyte function by examining other GPR proteins expressed in the immune system.

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