G-Protein Signaling Modulator 3 Regulation of Monocyte Survival

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Abstract:

G-Protein Signaling Modulator 3 (GPSM3), a GoLoco family protein that regulates G protein coupled receptors (GPCRs), is highly expressed in immune cells, particularly monocytes. Single nucleotide polymorphisms (SNPs) at the GPSM3 gene locus that were associated with a decreased incidence of autoimmunity in humans have been shown to correlate with reduced transcript and protein expression. Furthermore, Gpsm3-/- mice fail to develop inflammatory disease in two preclinical mouse models of rheumatoid arthritis, but the underlying cellular and molecular mechanisms are currently unknown. Our published and preliminary data suggest that Gpsm3 expression levels impact monocyte survival, which could explain why Gpsm3-/- mice fail to develop inflammatory arthritis. To directly test the impact of Gpsm3 on monocyte survival *ex vivo*, monocytes were differentiated from the bone marrow of wild type and Gpsm3-/- mice and treated with one of four chemokines known to regulate monocyte function (Ccl2, Cx3cl1, chemerin, and Cxcl12) under apoptotic stress. Monocyte survival versus apoptosis was then measured. Preliminary results suggested that Cx3cl1, chemerin and Cxcl12 all protect against apoptosis in wild type monocytes, but fail to protect the Gpsm3-/- monocytes. Future directions involve identifying potential leukocyte subpopulations, including macrophages, which are also dependent on Gpsm3 for survival. Overall, Gpsm3 is critical to the development of monocyte inflammatory responses and could impact future development of novel therapeutic targets for the treatment of arthritis in humans.

Introduction:

Monocytes are myeloid-derived leukocytes that differentiate into macrophages. Together, monocytes and macrophages make up one of the innate immune system's primary lines of defense against pathogens; but they are also involved in chronic inflammatory and autoimmune diseases[1]. These cells secrete the chemokines interleukin-1β, interleukin-6, tumor necrosis factor-α, interleukin-23, and interferon-γ which are involved in the pathogenesis of autoimmunity[2]. Furthermore, although monocytes and macrophages are not likely the originators of rheumatoid arthritis (RA), they have pro-inflammatory, destructive and remodeling capacities which play a substantial role in disease[3].

Monocyte and macrophage activation, survival, and chemotaxis can be regulated by G-protein coupled receptors (GPCRs)[4]. These seven transmembrane receptors are linked to a heterotrimeric G-protein composed of a Gα, Gβ, and Gγ subunit. The Gα subunit is activated through guanine nucleotide exchange; it is inactive when bound to GDP and in an activated state when bound to GTP. The Gβ and Gγ subunits heterodimerize and bind with high affinity to Gα when it's bound to GDP and, therefore, in its inactive form. Upon ligand binding, the receptor facilitates the release of GDP, after which Gα binds GTP and undergoes a conformational change that causes it to release Gβγ[5]. GTP bound Gα and Gβγ independently initiate signals. G-Protein Signaling Modulator 3 (GPSM3), also known as G18 or AGS4, is a GoLoco family protein that is highly expressed in monocytes. The functional GoLoco motifs of GPSM3 bind the GDP bound form of Gα and prevents the exchange of GDP for GTP, therefore affecting downstream signaling (**Figure 1**)[6]. However, the overarching effect of this interaction on cell function is unknown. GPSM3 also contains a motif that binds the Gβ subunit of the heterotrimeric G protein[5} and could, therefore, effect downstream signaling of Gβ like the phosphorylation of AKT for example. Additionally, GPSM3 has been shown to bind 14-3-3, a protein known to regulate cell functions such as signaling, proliferation, apoptosis and metabolic

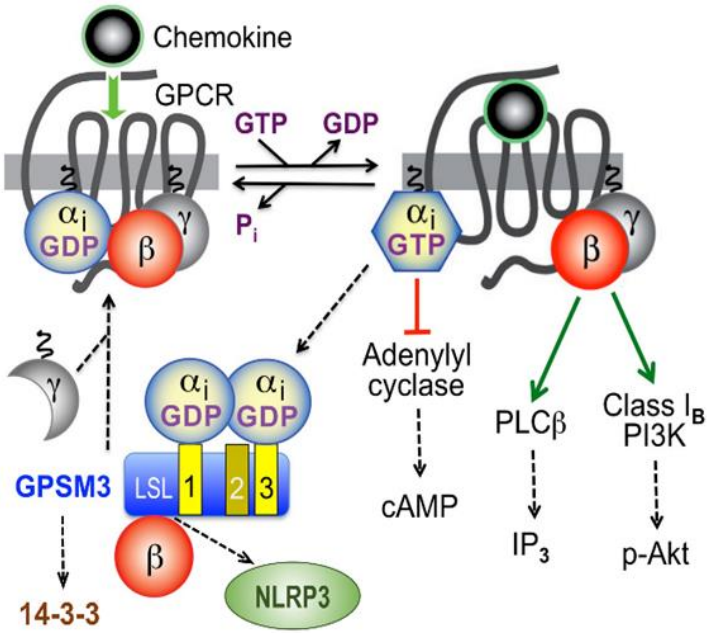

Figure 1. Model for GPSM3 function in regulating GPCRs. Two of the three GoLoco motifs within GPSM3 (yellow) bind to Gαi·GDP subunits. Additionally, the LSL motif within GPSM3 interacts with Gβ subunits. These interactions regulate ligand-activated signaling pathways, including Gαi-mediated inhibition of cAMP production by adenylyl cyclase, inositol phosphate production from Gβγ-mediated activation of phospholipase-Cβ, and/or cell survival signaling by Gβγ-mediated activation of class IB phosphatidylinositol-3'-kinase (PI3K) leading to PKB/Akt phosphorylation. GPSM3 also interacts directly with 14-3-3 proteins in the cytop*lasm and the NLRP3 inflammasome.*

pathways[7]. This interaction, which stabilizes GPSM3 itself, also keeps both proteins exclusively in the cytoplasm which impacts the functions of both proteins by regulating their subcellular localization. Lastly, GPSM3 has also been shown to interact with the C-terminal domain of NOD-like receptor family, pyrin domain containing 3 (NLRP3), negatively regulating the production of IL-1β through inflammasome activation[8].

Recent data has shown that two single nucleotide polymorphisms (SNPs) at the GPSM3 locus are associated with a reduced risk of developing autoimmunity[9], suggesting a potential role for GPSM3 in human autoimmunity. These SNPs have been associated with decreased transcript and protein levels of GPSM3 in humans containing the minor allele (unpublished data). Furthermore, our lab recently demonstrated that Gpsm3-/- mice are protected from both collagen antibody-induced arthritis (CAIA)[10] and collagen-induced arthritis (CIA) models (unpublished data). When compared to wild type mice, the Gpsm3-/- mice show significantly diminished paw swelling, as well as significant decreases in disease indicators such as inflammation, cartilage damage, and periosteum damage as determined by histopathology score[10].

Collectively, these data suggest a potential function of GPSM3 in monocyte or macrophage survival and migration to affected areas, but the mechanism is unknown. We first hypothesized that GPSM3 regulated monocyte survival signals. In support of this theory, a recent study from our group showed that THP-1 monocytes treated with *GPSM3*-silencing shRNA were more sensitive than control cells to apoptosis following etopside treatment[10]. In addition, THP-1 cells showed a correlation between decreased levels of GPSM3 and diminished phosphorylation of AKT, an intracellular target of the Gβ pathway[6]. AKT phosphorylation is a downstream effect of GPCR signaling and has been shown to be part of a pro-survival pathway[11]; therefore, if decreased GPSM3 diminishes phosphorylation of AKT in primary monocytes as it does in THP-1 cells, this could be a possible mechanism by which GPSM3 regulates cell survival. Another intracellular pathway of GPCRs that has yet to be studied in this context is the pro-survival ERK pathway[12], another potential mechanism for GPSM3 regulation of cell survival signaling. Additionally, survival of *primary* monocytes from wild type and Gpsm3-/- mice, in response to more physiological stimulations of apoptosis, such as serum starvation or TNFα stimulation, has yet to be investigated, nor have the pro-survival effects of particular chemokines known to regulate monocyte function been evaluated. These questions are the focus of this thesis.

Here we investigated four chemokines known to regulate monocyte function through G-protein coupled receptors (Ccl2, Cx3cl1 (fractalkine), chemerin, and Cxcl12), for the ability to rescue both wild type and Gpsm3-/- primary monocytes from serum starvation which induces Fas-FasL mediated apoptosis[13]. Additionally, we further characterized the different populations within our heterogeneous bone marrow derived cell cultures. Upon this characterization, we were driven to also investigate the ability of a single, implicated chemokine to rescue the adherent population, which are macrophages[8], from TNFα induced apoptosis, a mechanism that does not require cell-cell contact [14] and so is likely a more efficient model for non-confluent, adherent cells. In both monocytes and macrophages, phosphorylation of Akt and

Erk was also compared to investigate potential molecular signaling pathways required for Gpsm3 regulation of apoptosis.

Materials and methods:

Mice. Experiments were performed using 8-12 week old wild type and Gpsm3 knockout (-/-) mice of both C57BL/6 and dba1 strains. Gpsm3-/- mice were derived as previously described. Parental strains came from the Texas Institute for Genomic Medicine and were generated by knocking out a single copy of Gpsm3 using genetrap insertion in embryonic stem cells. The resulting heterozygote mice were crossed to generate homozygous knockouts.[10] All work was compliant with IACUC-approved protocols and performed in the AAALAC-accredited vivarium of UNC.

Reagents. Ccl2 (cat# 479-JE), Cx3cl1 (cat# 472-FF), Cxcl12 (cat# 460-SD) and chemerin (cat# 2325-CM) chemokines were purchased from R&D Systems (Minneapolis, Minnesota). The flow cytometry antibodies for Ly6C-Brilliant Violet 421 (clone HK1.4), CD11b-PE (clone M1/70), and CD115-APC (clone AFS98) were purchased from Biolegend (San Diego, California), those for Ccr2-PE (clone 475301) and Cx3cr1-PE (goat polyclonal) were purchased from R&D Systems, and those for Cxcr4-PE (clone 2b11) and Cmklr1-PE (clone BZ332) were purchased from Thermo Fisher Scientific (Waltham, Massachusetts). Antibodies for western blotting, including phospho-Akt (ser473) (clone D9E), total Akt (clone C67E7), phospho-Erk (thr202/tyr204) (clone D13.14.4E), total Erk (clone 137F5), β-Actin (clone 8H10D10) and all secondary antibodies were purchased from Cell Signaling (Danvers, Massachusetts). Gapdh antibody (cat # 2275-PC) for western blotting was purchased from Trevigen (Helgerman, Connecticut).

Bone Marrow Derived Monocyte Culture. Primary monocytes were harvested from bone marrow as previously described[15]. Briefly, cells were collected from the bone marrow of a wild type and a Gpsm3-/- mouse by flushing, and filtered through a 70-micron filter to remove any

debris. The cells were treated with red blood cell lysis buffer, washed two times with HBSS and counted. They were then resuspended to a concentration of 10^6 cells/mL in RPMI-1640 media with 10% bovine growth serum, 50 μM 2-mercaptoethanol, 1% Pen-Strep, 20 mM HEPES, and 20 ng/mL M-Csf (R&D Systems, Minneapolis, MN), and cultured for five days in low-adhesion conditions, either in 50 mL conical tubes or on plates pre-coated with 20 μg/mL poly-HEMA (poly-2-hydroxyethylmethacrylate, Sigma-Aldrich cat# P3932) to promote monocyte differentiation.

To obtain the adherent macrophages, cells were initially cultured as described above. On day five, the cells were plated on non-tissue culture-treated plates and allowed to adhere for 30 minutes before being used in their intended assay. Bone marrow cells differentiated in this manner that are adherent have been determined to be macrophages[8]. To remove macrophages from the plates, cells were washed with PBS, followed by a wash with Versene (0.6 mM EDTA in PBS) and fully released with Accutase solution (Sigma Aldrich).

Apoptosis Assay. To test monocyte apoptosis, suspension cells were aspirated off the plates, washed twice with HBSS, and aliquoted into tubes at $0.5x10^6$ cells per tube. Apoptosis was induced by serum starving the monocytes overnight, which is known to induce spontaneous apoptosis through Fas-FasL mediated interactions[13]. To test the ability of four chemokines to rescue the cells from Fas-FasL induced apoptosis, the wild type and Gpsm3-/- samples were treated with 0.5 mL serum free media plus 100 ng/mL of either Ccl2, chemerin, Cxcl12, or Cx3cl1. The “live” control cells were left in complete media with 20 ng/mL M-Csf and the “dead” control cells were in serum free media with no chemokine added. After 20 hours, the cells were washed with HBSS and stained with FITC-Annexin V and propidium iodide per the instructions in the TACS Annexin V-FITC Apoptosis Detection Kit (Trevigen). Samples were analyzed using flow cytometry on a Cyan Analyzer from the UNC Flow Cytometry Core Facility. Data is

expressed as percentage of total cells positive for Annexin V, which includes those in both early and late stage apoptosis.

For adherent macrophages, cells were aliquoted directly from the 50 mL conical tube on day five of differentiation onto non-tissue culture-treated 6-well plates (Corning Incorporated, cat# 351146) and allowed to adhere for 30 minutes. Any cells remaining in suspension after the 30 minutes were considered monocytes and removed. The adherent cells were washed with HBSS. Since the Fas-FasL induced apoptosis using serum free media likely requires cell to cell contact, it is not as efficient in non-confluent, adherent cells which do not come into contact, compared with suspension cells that freely interact. To induce apoptosis in the adherent macrophages, 10 mM TNFα (R&D Systems) was added to the serum free media[14]. Cxcl12 was added to the sample well at 100 ng/mL to test its ability to rescue the macrophages from TNFα induced apoptosis. After twenty hours, the samples were released with Accutase solution and transferred to tubes for Annexin/PI staining and analysis by flow cytometry.

Phenotyping. Cells, either suspension or adherent, were washed twice with HBSS, aliquoted into flow cytometry tubes, blocked for 15 minutes with rat serum and Fc block (BD Biosciences) and stained with 2 μL of antibody for Ly6C, CD11b, CD115, Ccr2, Cx3cr1, CxcR4 and Cmklr1 in 100 μL HBSS for 30 minutes. Samples were washed twice, resuspended in 400 μL of HBSS and analyzed using flow cytometry on a Cyan Analyzer from the UNC Flow Cytometry Core Facility. The UNC Flow Cytometry Core Facility is supported in part by P30 CA016086 Cancer Center Core Support Grant to the UNC Lineberger Comprehensive Cancer Center.

Western Blot Analysis. After the five days of differentiation, primary suspension monocytes were washed, counted, and resuspended to a concentration of $1x10^6$ in 50 μL of HBSS so that one million cells could be aliquoted into multiple 1.5 mL tubes. The cells were

then treated with 100 μL of a solution containing 100 ng/ml Ccl2 or chemerin chemokine in HBSS over the indicated time course. Ice cold lysis buffer (1% (final) Triton-X100 in HBSS) was added to halt the reaction at the appropriate time and lyse the cells. Cells were incubated in lysis buffer for 25 minutes at 4 degrees Celsius and then centrifuged for 5 minutes at 3000 rpm to pellet nuclei. The protein-containing supernatant was transferred to fresh tubes and a BCA Protein Assay Kit (Thermo scientific, cat # 23227) was utilized to normalize the protein concentration according to manufacturer's instructions. Lysates were resolved on precast Any kD Mini-PROTEAN TGX Gels (Bio-Rad Laboratories) in running buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS at pH 8.3 (Bio-Rad). The proteins were transferred to 0.45 μm pore size PVDF membrane (Millipore) using transfer buffer containing 25 mM Tris, 192 mM glycine and 20% methanol. Immunoblotting was done for phosphorylated Akt (ser473), total Akt, phosphorylated Erk (thr202/tyr204) and total Erk. Blots were blocked with 5% milk in TBS/T (25 mM Tris, 135 mM NaCl, 2.7 mM KCl, at pH 7.6, plus 0.1% Tween) for two hours at room temperature, incubated in primary antibody at a concentration of 1:2000 in TBS/T with 5% milk overnight at 4 degrees Celsius, washed three times for 10 minutes in TBS/T, incubated in HRP-linked secondary antibody at a concentration of 1:5000 in TBS/T with 5% milk for one hour at room temperature, and washed two times in TBS/T and once in TBS before imaging. Cells were imaged using Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare) on a G-box Gel Documentation System (Syngene, USA). Phospho-blots were stripped for 15 minutes using Restore PLUS Western Blot Stripping Buffer (Thermoscientific) and re-probed for total protein. Blots were stripped again and re-probed (1:10,000) for GAPDH or β-Actin as a loading control.

For adherent macrophages, the procedure above was followed with the exception that the cells were lysed on the plate by adding lysis buffer directly and lysing for 30 minutes at 4°C

with shaking. Lysates were collected into tubes and then spun down and analyzed as detailed above.

Results:

Preliminary Phenotyping of bone marrow derived monocytes indicate receptor expression

Initially, our cultured bone marrow derived monocytes (BMDMs) were analyzed for monocyte marker and chemokine receptor expression using flow cytometry (**Table 1**). Cells were stained individually with each antibody so percentages are of total population. This was done to confirm that our cultured cells were monocytes and that they expressed the chemokine receptors of interest.

Table 1. Wild type and Gpsm3-/- Bone Marrow Derived Monocytes (BMDM) phenotype.

	Ly6C	CD11b	Ccr2	Cmklr1	Cx3cr1
Wild type	80.5	71	51	41	9.5
Gpsm3-/-	79	70	56	30.5	15.5

Percentage of total wild type and Gpsm3-/- monocytes expressing the indicated markers and receptors. Data is shown as the mean of percent positive, n=2.

Gpsm3-/- monocytes lack chemokine protection from Fas-FasL induced apoptosis.

Since previous studies have shown that THP-1 monocytes treated with *GPSM3-/-* silencing shRNA were more sensitive to apoptosis than control cells[10], we hypothesized that primary monocytes from a Gpsm3-/- mouse would follow the same trend when compared to cells from a wild type mouse. Furthermore, since cell survival can be regulated by GPCRs, we hypothesized that monocytes from Gpsm3-/- mice would have altered survival responses through certain pro-survival GPCRs. We first used serum starvation, which induces apoptosis through Fas-FasL interaction, to induce death in wild type and Gpsm3-/- cells. We then added treatments with either Ccl2, Cx3cl1, chemerin, or Cxcl12 to test the ability of these chemokines to increase cell survival. Overnight incubation in serum-free media induced apoptosis in both

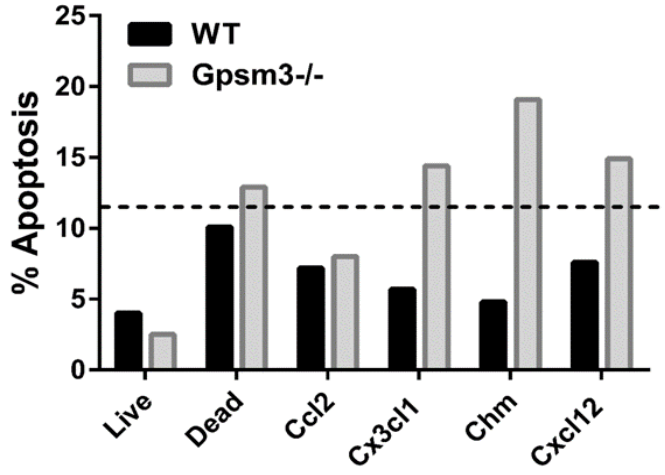

Figure 2. Monocytes can be rescued from Fas-FasL induced apoptosis by chemokines. Percentage of apoptotic monocytes after overnight incubation in the specified conditions determined by flow cytometry as described in the Methods. Live refers cells in complete media with M-Csf and dead refers to cells in serum free media alone, without any chemokine, Dotted line represents mean apoptosis for dead controls. Chm= chemerin. n=1.

wild type and Gpsm3-/- monocytes at comparable levels, although slightly less in the wild type cells (**Figure 2**). Ccl2 did rescue the cells from apoptosis, however, it increased the survival of both the knock out cells and wild type cells equally. In contrast, Cx3cl1, chemerin, and Cxcl12 all showed a substantial ability to protect wild type cells from apoptosis while failing to protect Gpsm3-/- cells (**Figure 2**). This suggests that Gpsm3 is necessary for survival signaling with Cx3cl1, chemerin and Cxcl12, but not necessarily signaling with Ccl2.

Ccl2 activation of Akt and Erk is not altered in primary Gpsm3-/- monocytes.

The phosphorylation of Akt and Erk are downstream effects of GPCR signaling and, therefore, have the potential to be affected by Gpsm3 interaction with Gα or Gβ subunits. Phosphorylated Akt has been shown to be part of a pro-survival pathway[10]. Since Ccl2 rescued wild type and Gpsm3-/- cells from apoptosis equally, we hypothesized that there would be no difference in Akt activation through phosphorylation at serine 473 or in Erk activation through phosphorylation at threonine 202 and tyrosine 204. To measure this we stimulated wild type and Gpsm3-/- monocytes with 100 ng/ml Ccl2 and then used Western Blotting to measure the level of phosphorylated Akt or Erk over six time points. In congruence with the apoptosis assay, no difference in phosphorylation of Akt was found with Ccl2 stimulation between wild

type and Gpsm3-/- monocytes when normalized to the GAPDH load control (**Figure 3A**). Additionally, there was not a significant difference in Erk activation; if anything, the Gpsm3-/- monocytes had slightly higher levels of phosphorylated Erk (**Figure 3B**).

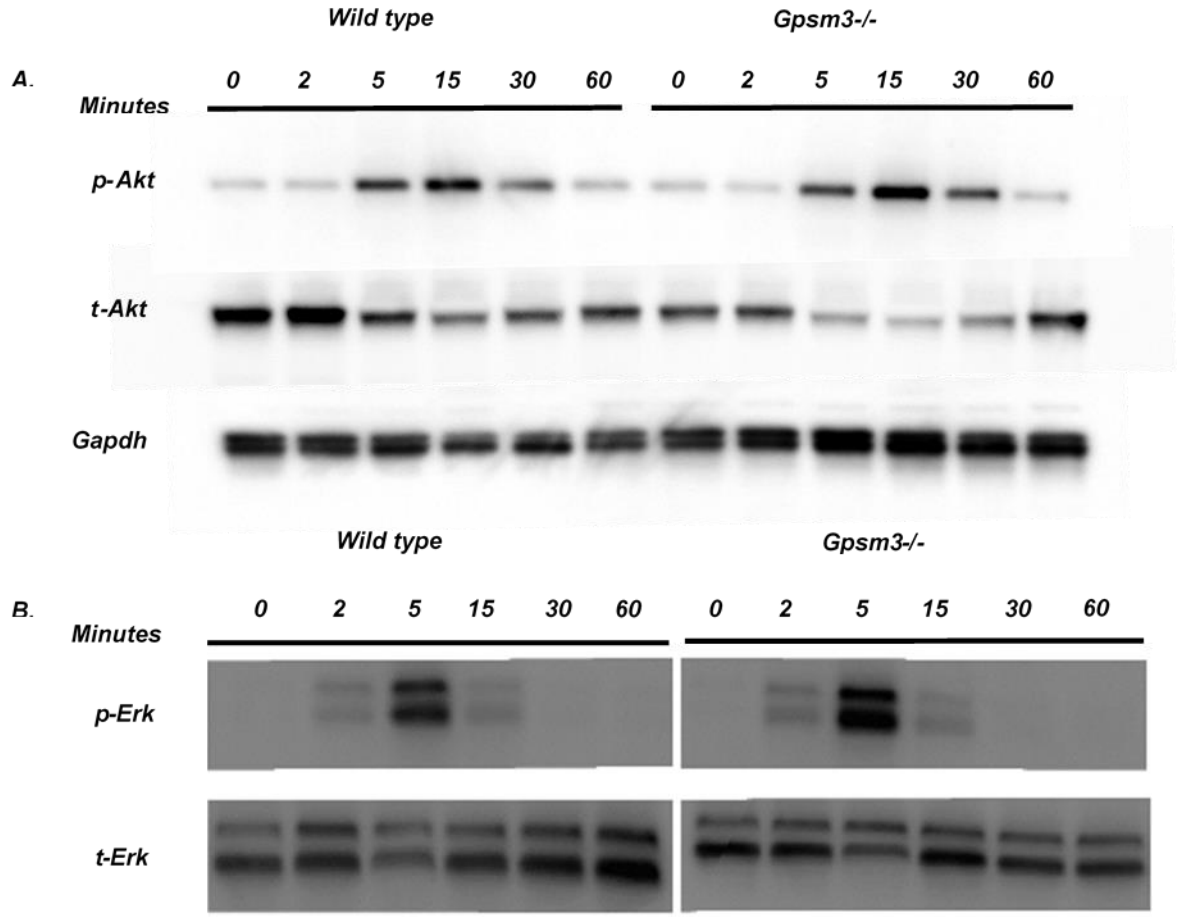

Figure 3. Akt and Erk activation through Ccl2 stimulation is not diminished in Gpsm3-/- monocytes. Western blot analysis for Ccl2 activation of Akt through serine 473 phosphorylation (A) and Erk through phosphorylation of threonine 202 and tyrosine 204 (B) in wild type and Gpsm3 -/- monocytes relative to total protein levels. Gapdh was used as a load control in A.

Monocyte stimulation with chemerin results in differential signaling through Erk.

Since chemerin rescued wild type but not Gpsm3-/- cells from apoptosis, we hypothesized that the Gpsm3-/- monocytes would show reduced activation by chemerin of Akt through phosphorylation of serine 473 and Erk through phosphorylation of threonine 202 and tyrosine 204. To measure this, we performed a time course stimulation with chemerin of wild

type and Gpsm3-/- monocytes and then used western blotting to measure the level of phosphorylated Akt or Erk over six time points of stimulation. In contrast to our hypothesis based on the apoptosis assay where chemerin rescued the wild type cells but not the Gpsm3-/- cells, there was also very little difference in phosphorylated Akt levels between wild type and Gpsm3-/- cells (**Figure 4A**). Similar to the Ccl2 activation, Gpsm3-/- cells actually had slightly higher levels of phosphorylated Akt over the time course tested (**Figure 4A**), which opposes our hypothesis based on the known correlation between phosphorylation of Akt and cell survival[6, 16] Chemerin-treated wild type and Gpsm3-/- cells did, however, show a substantial difference in not just the maximum level of phosphorylated Erk, but also the time course over which it was phosphorylated. Maximum activation occurred at 15 minutes in the Gpsm3-/- monocytes, but at

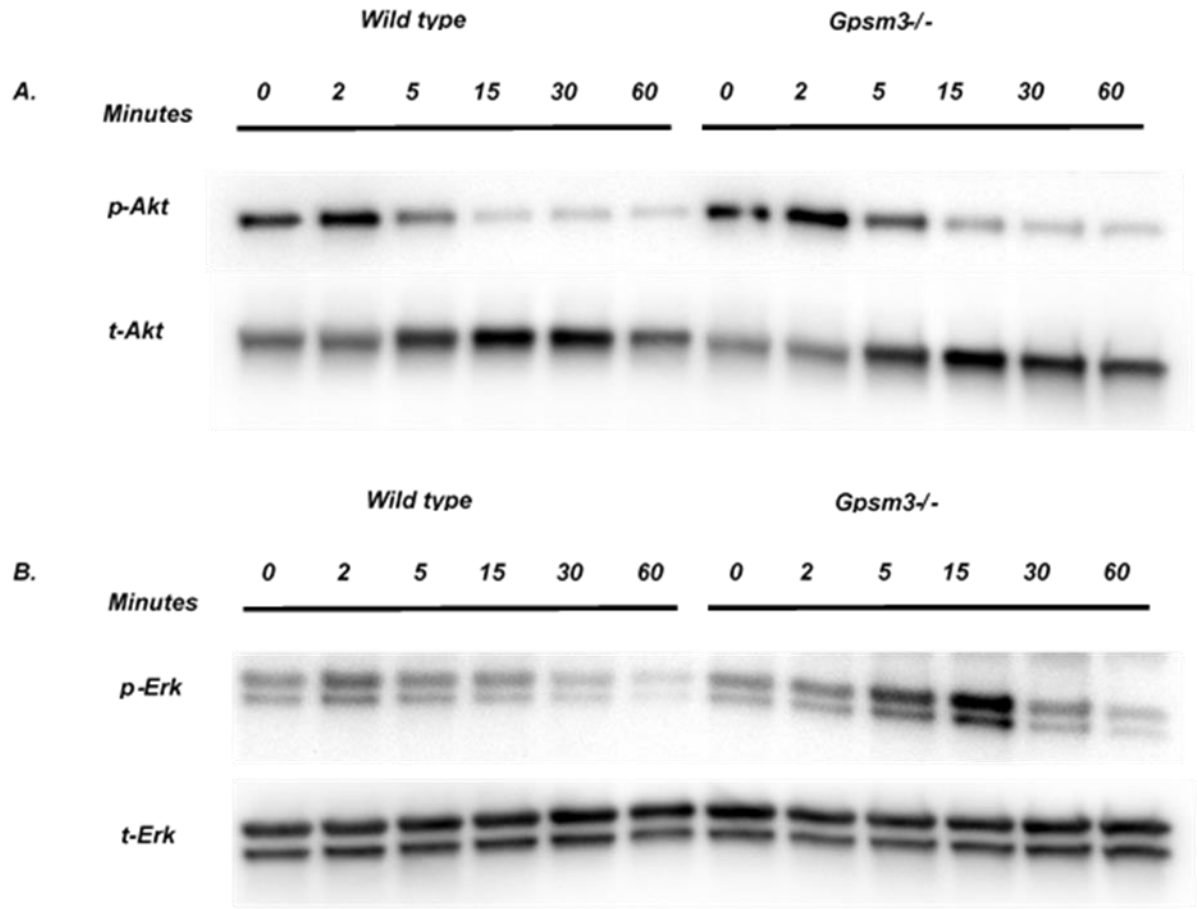

Figure 4. Akt and Erk activation through chemerin stimulation is not diminished in Gpsm3-/- monocytes. Western blot analysis for chemerin activation of Akt through Serine 473 phosphorylation (A) and Erk through phosphorylation of Threonine 202 and Tyrosine 204 (B) in wild type and Gpsm3 -/- monocytes relative to total protein levels.

2 minutes in the wild type (**Figure 4B**). Contrary to our hypothesis based on the pro-survival nature of the Erk pathway[12], Erk activation was substantially increased in Gpsm3-/-monocytes (**Figure 4B**). This suggests that the differential regulation of apoptosis by Gpsm3 must be downstream from the phosphorylation of either Erk or Akt or through an alternate pathway.

Cultured BMDMs are heterogeneous.

When trying to repeat the apoptosis data from Figure 2, we had varying results where sometimes none of the chemokines rescued either wild type or Gpsm3-/- cells. We also observed that some cells were adhering to the plates even though they were coated with poly-2-hydroxyethylmethacrylate (poly-HEMA) to prevent adherence. These observations led us to hypothesize that the population of cells we were culturing was heterogeneous. To test this, we extended our cell phenotyping of M-Csf differentiated BMDMs to determine the expression of three common surface markers associated with monocytes, CD11b, Ly6C and CD115, as well as to check for expression of chemokine GPCR receptors by flow cytometry. Summarized in **Table 2**, only 4-6% of the cells were positive for all three monocyte markers, 62-75% were double positive for CD11b and Ly6C, and 15-23% were double positive for CD11b and CD115. This experiment was repeated in another mouse model and the average for the three populations were 8%, 51% and 15.3% respectively for n=3 (data not shown).

Table 2. Non-adherent Bone Marrow Derived Monocytes (BMDM) are heterogeneous.

	Cell %	Ccr2	Cmklr1	Cxcr4	Cx3cr1
$CD11b^{+}$ $Ly6C^{+}$ $CD115^{+}$	6/4	54 / 64	28 / 40	38 / 48	3 / 6
$CD11b^{+}$ $Ly6C^{+}$ $CD115^{-}$	62/75	0 / 0	0 / 0	0 / 0	0 / 0
$CD11b^{+}$ $Ly6C^{-}$ $CD115^{+}$	23/15	81 / 81	60 / 60	74 / 72	6 / 8.5

Percentage of non-adherent wild type/Gpsm3-/- cells positive for all three monocyte markers, CD11b, Ly6C, and CD115, those positive for only CD11b and Ly6C, and those only positive for CD11b and CD115. Within these populations, the percentage of cells expressing the receptors Ccr2, Cmklr1, Cxcr4, and Cx3cr1 are also listed. These are the receptors for Ccl2, chemerin, Cxcl12, and Cx3cl1 respectively.

Adherent bone marrow macrophages (BMM) consist of Ly6C^{hi} and Ly6C^{low} populations.

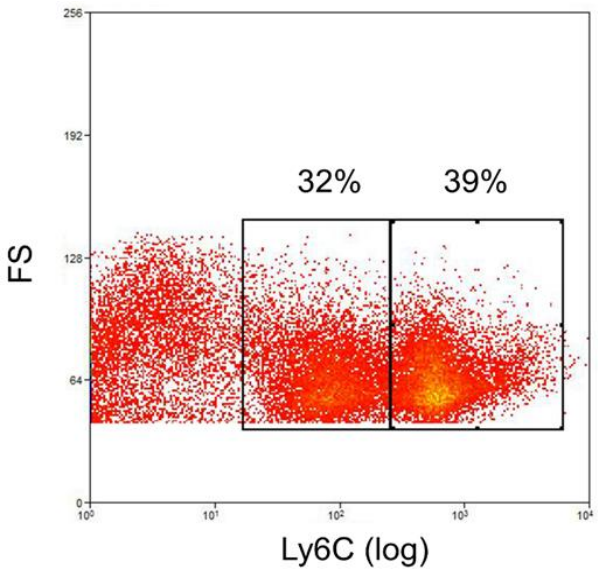

Figure 5. Adherent Bone Marrow Derived Monocytes (BMDM) consist of Ly6C^{hi} and Ly6C^{low} populations. Flow cytometry data for adherent population of bone marrow derived monocytes, or macrophages. Two distinct populations were evident, a Ly6C^{hi} and a Ly6C^{low} population. This image is representative of four separate runs. Mean for the Ly6C^{low} population was 37.75%, mean for the Ly6C^{hi} population was 35.75%, n=4. Mean total cells positive for Ly6C is 73.5%

The CD11b marker is also found on neutrophils, but in combination with Ly6G rather than Ly6C, and so we believe the last population in **Table 2** could represent neutrophil contamination. Of the cells determined to be monocytes, only the small percentage that were positive for CD115 were positive for the chemokine GPCRs**.** Additionally, the fact that up to 65% of the non-adherent cells do not express the chemokine receptors could explain why there was variability upon repeating the apoptosis assays. For this reason, we decided to further characterize the adherent population in our cell culture that was evident even on the low adherence plates. We considered this adherent population to be bone marrow macrophages based on the previously described methods for obtaining macrophages[8]. To characterize these cells, we stained for Ly6C and the chemokine GPCRs. We found two distinct Ly6C^{hi} and Ly6C^{low} populations. Importantly, contrary to the suspension cells, 73.5% of the cells were Ly6C positive and so there was less neutrophil contamination in this population (**Figure 5**). In addition, both the Ly6C^{hi} and Ly6C^{low} populations were also positive for Ccr2, Cxcr4, and Cx3cr1 (**Table 3**). Since Gpsm3 regulation is presumably through its interaction with the G-proteins of GPCRs, the presence of these GPCRs is necessary to evaluate the effects on survival of knocking out this protein. Due to the more homogeneous nature of the adherent population and the observation that they all express the

GPCRs for our chemokines of interest, we concluded that it would be best to use the adherent macrophages to examine Gpsm3 function in apoptosis and signaling.

Table 3. GPCR expressed predominantly on Ly6C- non-adherent BMDM and both Ly6C^{h}i and Ly6C^{low} adherent BMM populations.

	Ccr2	Cxcr4	Cx3cr1
Suspension Ly6C^{+}	6	26	2
Suspension Ly6C^{-}	52	21	19
Adherent Ly6C^{hi}	31	24	15
Adherent Ly6C^{low}	67	32	27

Percentage of cells expressing receptors for adherent, Ly6C high and Ly6C low populations compared to receptor expression on the Ly6C positive and Ly6C negative population, n=1.

TNFα induced apoptosis is rescued by CXCL12 in wild type but not Gpsm3-/- macrophages.

Ccr2 was the most predominant receptor on the adherent macrophage population (**Table 3**), however, since our initial data indicated that Ccl2 rescued both wild type and Gpsm3-/- cells equally, we hypothesized another chemokine might have differential effects on cell survival. Cxcr4 was the next highest expressed receptor on the macrophages and Cxcl12, the ligand for Cxcr4, did differentially impact wild type and Gpsm3-/- monocyte (BMDM) survival, as shown by our initial data. We, therefore, hypothesized that Cxcl12 survival signals would depend on Gpsm3 regulation. Therefore, we tested the ability of Cxcl12 to impact macrophage survival. TNFα was added to serum free media to induce apoptosis in the adherent cells. We chose to use this method to cause apoptosis because FasL-induced apoptosis using serum starvation requires cell to cell contact which would likely be less effective when the cells are adhered to the plate. Overnight incubation in 10 mM TNFα in serum free media induced apoptosis in both wild type and Gpsm3-/- monocytes at comparable levels; however, while Cxcl12 rescued the wild type macrophages to a level between the live and dead control, it did not rescue the Gpsm3-/- macrophages at all (**Figure 6**).

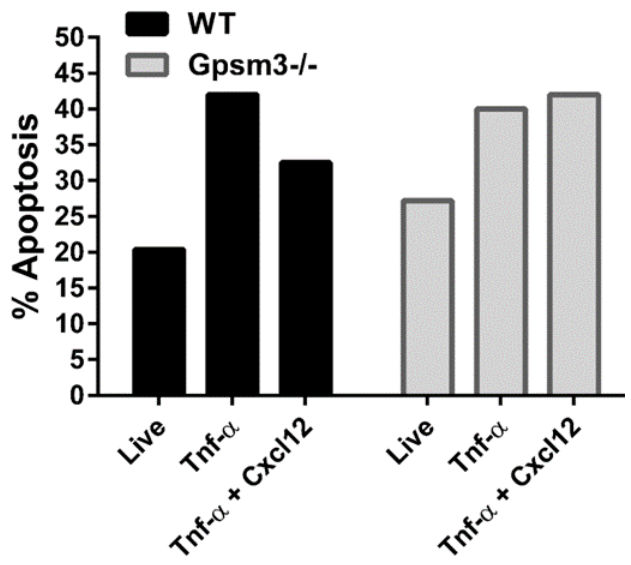

Figure 6. Cxcl12 rescues wild type bone marrow macrophages (BMM) from TNFα induced apoptosis. Percentage of apoptotic macrophages after overnight incubation in the specified conditions determined by flow cytometry as described in the Methods. Live refers cells in complete media with M-Csf and dead refers to cells in serum free media with 10mM TNFα, without any chemokine, n=1.

Gpsm3 may impact Cxcl12 signaling to Akt in macrophages.

Since Cxcl12 rescued the wild type macrophages but not the Gpsm3-/- macrophages, we hypothesized that similar to the results in the THP-1 cells,[6] there would be diminished phosphorylation of Akt, a pro-survival mediator, in the Gpsm3-/- macrophages. We also wanted to compare Ccl2 stimulation in the macrophages to the monocytes, hypothesizing that there would again be no difference in Akt phosphorylation. First, we stimulated wild type and Gpsm3-/- macrophages for 4 minutes, previously determined to be the time point of maximum stimulation, with either Ccl2 or Cxcl12 and then performed immunoblotting to test the response. Ccl2 stimulated a very low level of Akt phosphorylation in both wild type and Gpsm3-/- macrophages, but perhaps slightly lower in the wild type (**Figure 7A**). Cxcl12 did show a significant level of Akt phosphorylation in both cell types, with a slightly higher activation in the Gpsm3-/- macrophages when normalized to the Actin control, which appears to go against our hypothesis. To more closely examine Cxcl12 signaling in macrophages, we repeated the stimulation over a time course to see if there was a difference in phosphorylation of Akt over time in response to Cxcl12. Again, phosphorylation of Akt was actually slightly increased in the Gpsm3-/- cells at the 4-minute time point (**Figure 7B).** This gives further evidence that the

regulation of apoptosis by Gpsm3 is either downstream of Akt phosphorylation or through another mechanism altogether, perhaps due to its binding affinity for 14.3.3.

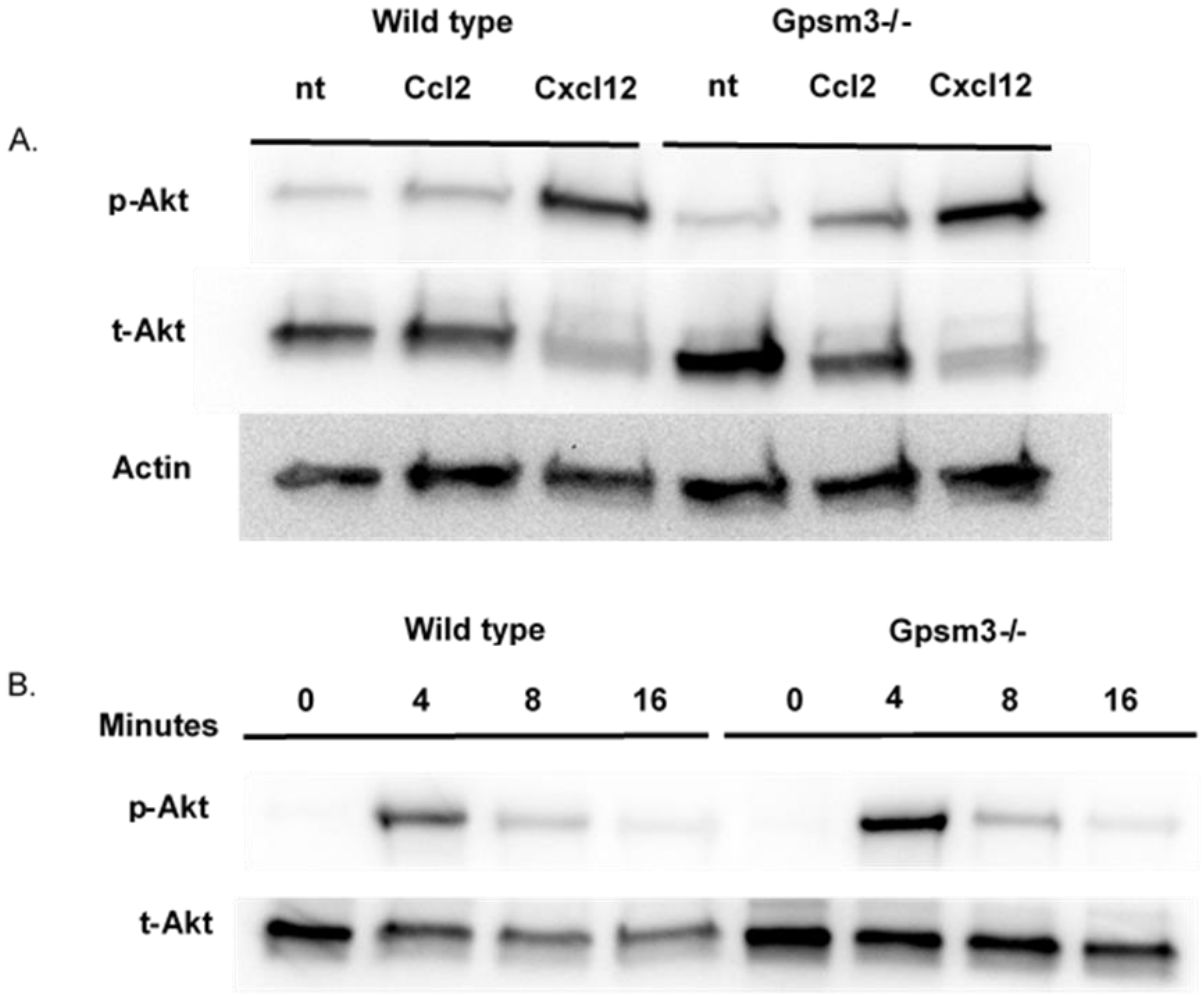

Figure 7. Western blot analysis for Cxcl12 and Ccl2 activation of Akt through Serine 473 phosphorylation in wild type and GPSM3 -/- macrophages relative to total Akt levels. A. Phosphorylated Akt levels in adherent macrophages after a 4-minute stimulation with Ccl2 and Cxcl12 as shown compared to unstimulated cells. nt stands for no treatment. B. Phosphorylated Akt levels in adherent macrophages stimulated for the indicated amount of time.

Discussion:

Previous studies have demonstrated that silencing GPSM3 in THP-1 cells makes them more susceptible to apoptotic death following etopside treatment[10]. The current study takes

this one step further by using primary monocytes, both wild type and Gpsm3-/-, and tests the ability of chemokines to rescue the cells from serum starvation which induces Fas-FasL mediated apoptosis[13], a more physiologically relevant model of apoptosis than etoposide treatment. While Ccl2 appeared to rescue wild type and Gpsm3-/- cells equally from Fas-FasL mediated apoptosis, Cx3cl1, Chemerin, and Cxcl12 all appeared to increase survival of wild type cells while failing to do so in the Gpsm3-/- cells (**Figure 2**). This suggests that Gpsm3 is required for pro-survival signaling by Cx3cl1, chemerin and Cxcl12, but not Ccl2. Since monocytes play a large role in autoimmune disease, their exhibiting increased apoptosis when lacking Gpsm3 could limit the number of cells available to contribute to pathogenesis. This could be a substantial contributor to the disease protection that Gpsm3-/- mice experience in rheumatoid arthritis models[10], which has also been predicted by SNP analysis in humans[9].

To investigate possible pathways for the role of Gpsm3 in survival, phosphorylated Akt and Erk levels in response to Ccl2 and chemerin stimulation were measured over a time course in both wild type and Gpsm3-/- BMDMs. Both Akt and Erk are intracellular signal transduction proteins that have been associated with cell survival, particularly in cancer cells which are resistant to apoptosis[11, 12]. Since Gpsm3 possesses a motif that binds the Gβ subunit of GPCRs[16], and Gβγ is an upstream regulator of these pathways, a downstream effect on Akt or Erk phosphorylation is a possible mechanism for the role of Gpsm3 in cell survival. Ccl2 treatment resulted in no significant difference in Akt or Erk phosphorylation between wild type and Gpsm3-/- monocytes, which corresponded to its similar effect on apoptosis in wild type and Gpsm3-/- monocytes (**Figure 3**). In contrast, chemerin effected both Akt and Erk phosphorylation in Gpsm3-/- monocytes slightly more than in wild type cells; the increase was slight in phospho-Akt and was more distinct for phospho-Erk (**Figure 4**). These results indicate that Gpsm3 binding to Gβ may actually be inhibitory such that its removal slightly increases the downstream effects of the Gβ pathway, such as phosphorylation of Akt. Gpsm3 also appears to

have a role in Erk signaling; however, the regulatory mechanism and downstream functional impact is still not clear. The conflict between reduced survival in Gpsm3-/- monocytes but increased phosphorylation of Akt and Erk, documented to be pro-survival, suggests that Gpsm3 is regulating apoptosis through an alternative mechanism, which does not involve classical Akt or Erk pathways, or may function independently. Interestingly, total Akt was reduced in the samples where Akt was phosphorylated even though the loading controls were even (**Figures 3 and 7**). Although less pronounced, this is slightly evident in the Erk blots as well. One possible explanation for this is that the phospho-protein antibody is not being completely stripped and so is interfering with the binding of the total protein antibody. This has been noted by other investigators in our department. Furthermore, following phosphorylation of serine 473, Akt has been shown to be poly-ubiquitinated and marked for proteasome degradation[17], which could impact total Akt levels following chemokine stimulation. Another potential explanation is that these proteins are being phosphorylated and then shuttled to the nucleus[18-21]; since we lyse with 1% Triton-X100, the nuclear envelope is not being lysed and so nuclear proteins are not included on the blot. To test the theory of antibody interference, the total Akt blot in **Figure 7B** was done *prior to* phospho-Akt immunoblotting. The stimulated lanes are still slightly reduced in the wild type, which supports the theory that the protein may be shuttled to the nucleus after phosphorylation. Future directions include using a stronger lysis buffer (e.g., RIPA) to completely solubilize both the cell membrane and nuclear membrane.

Varying results upon trying to repeat the apoptosis data drove us to look more closely at our cultured suspension cell population. As a result, we have characterized, and thus made important distinctions between, the heterogeneous suspension cell population and the more homogeneous adherent cell populations. We concluded that the adherent macrophage population appears to be more compelling for the purposes of this study. The suspension population was found to contain a large amount (15-23%) of contaminating neutrophils, which

did express the relevant chemokine GPCRs, while only a very small proportion (4-6%) of monocytes expressed these receptors (**Table 2**). The majority (62-75%) of these cultured BMDM cells were monocytes that did not express these GPCRs at all. Therefore, any differential survival in this cell population had to be a result of the smaller population of cells, including neutrophils, which could explain the varying results when using a heterogeneous cell population. The adherent cells proved to be a much more homogeneous population with reliable receptor expression. Almost all of these cells (73.5%) are Ly6C positive (**Figure 5**) and a significant portion of both the $Ly6C^{hi}$ and $Ly6C^{low}$ populations express the relevant chemokine receptors (**Table 3**). Macrophages, which develop from monocytes, are also critical mediators of inflammation. For this reason, we decided to turn our focus to these macrophages.

Since serum starvation induces Fas-FasL mediated apoptosis, which requires cell to cell contact [13] and so is likely less efficient in cultured adherent cells that are non-confluent and may not come into direct contact, we used TNFα to induce apoptosis in the adherent macrophages. TNFα is the natural ligand for cell surface receptors, TNFαR1 and TNFαR2, which trigger cell death[14]. We tested the ability of Cxcl12 chemokine to differentially increase survival of wild type versus Gpsm3-/- macrophages because it had shown promise in the monocyte trial (**Figure 2**) and its receptor, Cxcr4, was the second most expressed receptor on these cells (**Table 3**). The most abundant GPCR, Ccr2, the receptor for Ccl2, had shown no dependence on Gpsm3 in the monocytes and thus was used initially as a control. As shown in **Figure 6**, Treatment with TNFα did induce apoptosis in both wild type and Gpsm3-/- macrophages; however, while Cxcl12 rescued wild type cells to a level between the live and dead controls, it did not show any rescue in the Gpsm3-/- cells. This suggests that Gpsm3 regulation continues after monocytes differentiate into macrophages. If both Gpsm3-/- monocytes and macrophages exhibit increased cell death *in vivo* as they have shown *ex vivo,*

this provides further evidence that the reduction in disease pathogenesis could be attributed to dysregulated survival of the Gpsm3-/- immune cells.

Akt activation was investigated by a four-minute stimulation with both Ccl2 and Cxcl12. While the response was quite low from Ccl2, it was substantial from Cxcl12 with noticeably increased levels of phosphorylated Akt in the Gpsm3-/- cells when normalized to Actin control (**Figure 7A**). Furthermore, the time course stimulation with Cxcl12, also showed increased levels of Akt phosphorylation (**Figure 7B**). This further supports the theory that Gpsm3 could have an inhibitory effect on Akt phosphorylation and so regulation of cell survival by Gpsm3 might act through an alternative mechanism. For example, 14-3-3 is a downstream substrate of Akt that also has been shown to bind directly to Gpsm3 (**Figure 1**)[7, 22]. 14-3-3 proteins also have a role in regulating cell cycle and apoptosis [23, 24] and could be functionally important in macrophage inflammation, but investigation into this potential pathway was beyond the scope of this study.

In summary, our apoptosis data does support the hypothesis that Gpsm3-/- monocytes and macrophages show dysregulated GPCR survival responses, but the mechanism requires further investigation. It does not appear that activation of Akt or Erk is diminished, but rather is *increased* in the Gpsm3-/- cells, which implies that this is likely not the pathway through which Gpsm3 directly regulates cell survival. Future directions include repeating the apoptosis data in the macrophages to obtain significance. Additionally, other pathways should be explored in attempt to work out the mechanism through which Gpsm3 regulates cell survival, perhaps further investigating the interactions between Gpsm3 and 14-3-3 or NLRP3, and by testing other known signal mediators, such as caspase-3.[20] Finally, the Gpsm3-/- mice do not appear to have reduced lifespans; however, they have not been immunologically challenged. Given the role of macrophages in innate immunity, it would be worthwhile to investigate whether these

mice have any deficits in the immune response to infectious disease. This would be an important piece of information before looking into this protein as a potential drug target.

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