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Reduction of *GPSM3* expression akin to the arthritis-protective SNP rs204989 differentially affects migration in a neutrophil model

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Abstract

G Protein Signaling Modulator-3 (*GPSM3*) is a leukocyte-specific regulator of G protein-coupled receptors (GPCRs), which binds inactivated $G\alpha_i$ -GDP subunits and precludes their reassociation with $G\beta\gamma$ subunits. *GPSM3* deficiency protects mice from inflammatory arthritis and, in humans, *GPSM3* single nucleotide polymorphisms (SNPs) are inversely associated with the risk of rheumatoid arthritis development; recently, these polymorphisms were linked to one particular SNP (rs204989) that decreases *GPSM3* transcript abundance. However, the precise role of *GPSM3* in leukocyte biology is unknown. Here we show that *GPSM3* is induced in the human promyelocytic leukemia NB4 cell line following retinoic acid treatment, which differentiates this cell line into a model of neutrophil physiology (NB4*). Reducing *GPSM3* expression in NB4* cells, akin to the effect ascribed to the rs204989 C>T transition, disrupts cellular migration toward leukotriene B4 (LTB4) and (to a lesser extent) interleukin-8 (*a.k.a.* IL-8 or CXCL8), but not migration toward formylated peptides (fMLP). As the chemoattractants LTB4 and CXCL8 are involved in recruitment of neutrophils to the arthritic joint, our results suggest that the arthritis-protective *GPSM3* SNP rs204989 may act to decrease neutrophil chemoattractant responsiveness.

Keywords

arthritis; chemotaxis; CXCL8; fMLP; G Protein Signaling Modulator-3; leukotriene B4; neutrophils; SNP rs204989

INTRODUCTION

G Protein Signaling Modulator-3 (*GPSM3*) is a GoLoco motif protein (1, 2) restricted in expression to mature monocytes, lymphocytes, and neutrophils; recent expression

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quantitative trait locus (eQTL) analyses of stimulated primary monocytes (3) and peripheral blood CD16+ neutrophils (4) have identified human genomic sequence variations that impinge upon *GPSM3* expression, including the single-nucleotide polymorphism (SNP) rs204990 (3) in a region directly upstream of the presumptive *GPSM3* transcription start site. Independent, genome-wide association studies of sequence variations associated with autoimmune disease risk have highlighted additional *GPSM3* SNPs as being inversely correlated (rs204989, rs204991) to the risk of developing rheumatoid arthritis (RA), ankylosing spondylitis, multiple sclerosis (refs. (5–7)), and systemic lupus erythematosus (SLE) (8). *GPSM3* polymorphisms are also associated with the risk of developing type 1 diabetes (rs204991; ref. (5)), atopic dermatitis (rs176095; ref. (9)), and childhood asthma (rs176095; ref. (10)). We recently reported (11) that SNPs rs204989, rs204990, and rs204991 constitute a haplotype block within which the rs204989 C>T transition is causal to reduced *GPSM3* promoter activity. The association between a promoter-weakening *GPSM3* SNP and reduced risk of RA is supported by the protection afforded to *Gpsm3*-deficient mice in an acute model of inflammatory arthritis (12). Histologic analysis of synovial tissue from *Gpsm3*-deficient mice revealed a marked acellularity (12), suggesting that their protection from developing pathologic inflammation relies on a potential cellular trafficking defect.

Neutrophils are leukocytes critical to the activation of inflammation in RA and many other autoimmune-related diseases (reviewed in (13)). Neutrophils elicit this function following their initial recruitment by early-phase proinflammatory chemoattractants. In RA, this early-phase recruitment is mediated primarily by leukotriene B4 (LTB4), followed by a LTB4 positive-feedback loop in which recruited neutrophils secrete LTB4, CXCL8, and additional chemoattractants (reviewed in (14)). To date, work to identify the role of *GPSM3* in disease pathophysiology has exclusively focused on circulating monocytes, which compose less than 10% of circulating leukocytes (15). However, mice made deficient in one of several monocyte chemokine receptors (*i.e.*, CCR1, CCR2, and CCR5) do not exhibit significant protection from models of RA inflammation (16); furthermore, mice depleted of Ly6C^{high} monocytes have no change in histological inflammation (17). Moreover, the initial class of leukocytes recruited to synovial tissues in human arthritis is thought to be neutrophils, which migrate in response to a sequential cascade of LTB4, IL-1 β , and CXCR2 agonists (14). Further support for the primary role of neutrophils in initiating RA inflammation comes from protection afforded to mouse strains that are deficient in various proteins required for the recognition or synthesis of LTB4, including the LTB4 receptor BLT1 (18), 5-lipoxygenase activating protein (19), 5-lipoxygenase (20), and leukotriene A4 hydrolase (20).

The LTB4 receptor BLT1 and the CXCL8 receptors CXCR1 and CXCR2 are GPCRs linked to pertussis toxin-sensitive G_i-subfamily heterotrimers (21–23), and therefore sensitive to potential regulation by GoLoco motif-containing proteins (like *GPSM3*) that act as G α_i -selective guanine nucleotide dissociation inhibitors (1). The role of *GPSM3* in neutrophil function has not previously been assessed. Here, we identify the expression of *GPSM3* in an immortalized cell line that can be differentiated towards neutrophil-like function, and further identify a selective role for *GPSM3* in its migration toward proinflammatory

chemoattractants LTB₄ and CXCL8 – a discovery that helps strengthen the association between the promoter-weakening *GPSM3* SNP rs204989 and a reduced risk of RA.

RESULTS

GPSM3 expression is induced in ATRA-differentiated NB4* cells

GPSM3 protein expression was undetectable within whole cell lysates of the human acute promyelocytic leukemia NB4 cell line. However, upon treatment of NB4 cell cultures with all-trans retinoic acid (ATRA), expression of *GPSM3* transcript (Fig. 1A) and encoded protein (Fig. 1B) increased in a differentiation-dependent manner concurrently with the established differentiation marker CD11b. This ATRA-dependent induction of GPSM3 expression also paralleled the acquisition of other markers of differentiated neutrophil function: specifically, the activity of intracellular oxidoreductase enzymes that can reduce nitroblue tetrazolium to colored formazan dye (Fig. 1C) and morphologic changes (Fig. 1D; *e.g.*, invagination of the nucleus), both of which have been reported previously in ATRA-differentiated NB4* cells (24).

GPSM3 deficiency does not impair ATRA-induced differentiation of NB4 cells

We established stable, monoclonal daughter lines from the parental NB4 cell line using previously validated short hairpin RNA (shRNA)-expressing lentiviral vectors directed against *GPSM3* (25). After ATRA treatment, all daughter lines exhibited equivalent upregulation of CD11b and equivalent expression of G β subunits (Fig. 2A, right panel); the latter result suggests that the established interaction between GPSM3 and neo-synthesized, free G β subunits (25) does not affect steady-state G β levels in this cell line. In addition, all daughter lines exhibited equivalent induction of intracellular oxidoreductase activity upon ATRA treatment (Fig. 2B, right panel; Supplemental Table S1).

GPSM3 deficiency impairs migration towards LTB₄ and CXCL8, but not fMLP

NB4* monoclonal lines with successful knockdown of GPSM3 expression were observed to have negligible spontaneous migration behaviors (Supplemental Fig. S1A) and similar overall migratory responsiveness to 10% FBS placed in the lower chamber of the Transwell apparatus (Fig. S1B). However, the NB4* lines with reduced GPSM3 expression were found to have disrupted migration towards LTB₄ and (to a lesser extent) CXCL8 in Transwell migration assays (Fig. 3) when compared both to scrambled shRNA control lines and to a line expressing an ineffective shRNA (*i.e.*, daughter line NB4*-shRNA22.2; Fig. 2A&B). In contrast to the diminished migratory responses towards LTB₄ and CXCL8 elicited by GPSM3 knockdown, no significant differences were observed in migration towards 100 ng/mL of formylated peptide (fMLP; Fig. 3C,D). Additional dose-response studies failed to demonstrate any difference in fMLP-induced migration upon GPSM3 knockdown (*e.g.*, Fig. S3 employing from 1 to 250 ng/mL fMLP concentrations). The observed reductions in migratory responses to chemoattractants LTB₄ (Fig. 3A&B) and CXCL8 (Fig. 3E&F) were not correlated with any concomitant decrease in mRNA expression of the high-affinity LTB₄ receptor 1 (*LTB4R1*; Fig. 3A *inset*) or the CXCL8 receptors *CXCR1* and *CXCR2* (Supplementary Figure S1). For example, compared to the scrambled shRNA control lines, only the NB4*-shRNA19.5 line exhibited a significant difference in *LTB4R1* mRNA

expression (Fig. 3A inset, = +56.5%, $p = 0.0134$) and this difference was an increase in expression.

DISCUSSION

Before its mRNA was cloned (26), GPSM3 protein was first detected in neutrophil detergent-resistant fractions by mass spectrometry (27). More recently, RNA-seq datasets indicate that human neutrophils have greater *GPSM3* transcript expression than all other types of circulating leukocytes (*e.g.*, NCBI GEO accession number GSE62408 (28)). These findings sparked our interest in determining whether GPSM3 regulates aspect(s) of neutrophil biology, especially in light of the GPSM3 SNP rs204989 being associated with decreased GPSM3 expression (11) and protection against the development of rheumatoid arthritis (5–7) – an autoimmune disease in which neutrophil function is central to its initiation. Using RNA-interference to negate the *GPSM3* upregulation found to occur normally in the promyelocytic NB4 cell line upon ATRA differentiation, we have now established that GPSM3 is required for the full migratory response of this neutrophil-like cell line toward a pathophysiologically relevant concentration of LTB4 (29); in addition, GPSM3 also appears to play a role in migratory responsiveness toward CXCL8, although only observed with the highest degree of GPSM3 deficiency (*i.e.* the NB4*-shRNA19 monoclonal lines in which GPSM3 protein was barely detectable by immunoblotting). Migratory responses to LTB4 and CXCL8 are believed to be dependent on the relative abundance of GPSM3 protein in these cells, given that the NB4*-shRNA20 monoclonal lines 20.2 and 20.5 were seen to have an intermediate level of GPSM3 knockdown efficiency and, in parallel, an intermediate migratory phenotype toward LTB4 and no significant defect in migration toward CXCL8.

Given the primary coupling of LTB4 and CXCL8 receptors to $G\alpha_i$ -containing heterotrimers (21–23) and the inhibitory influence exerted by the GoLoco motifs of GPSM3 on $G\alpha_i$ signaling and heterotrimer recycling (26), reduced GPSM3 levels in NB4* cells likely increases the rate of inactive $G\alpha_i$ subunit reassociation with free $G\beta\gamma$ heterodimers. As signaling by the free $G\beta\gamma$ heterodimer is central to the migratory response (22, 23), any accelerated deactivation by increased $G\alpha_i$ reassociation would lead to a diminished migration phenotype. Given the diminished NB4* cell line migration toward the RA-relevant chemoattractants LTB4 and CXCL8, we surmise that the synovial acellularity seen in anti-collagen antibody challenged GPSM3-deficient mice (12) and the inverse association between RA development and the *GPSM3* expression-lowering SNP rs204989 (11) could be the result of reduced neutrophil recruitment by these particular chemoattractants.

During infections, neutrophil responsiveness to bacterial-derived formylated peptides is mediated via formyl peptide receptors (30): *i.e.*, GPCRs coupled to $G\alpha_i$ -containing heterotrimers (31) in a similar fashion to LTB4 and CXCL8 receptors. However, GPSM3 knockdown in NB4* cells does not appear to affect migration initiated through the formyl peptide receptor. We therefore conclude that GPSM3 knockdown does not globally affect the chemotactic machinery of NB4* cells, but instead leads to a selective abrogation of responsiveness to certain chemoattractants, like LTB4 and CXCL8 shown here. Future investigations will be directed at identifying the specific signaling mechanism(s) regulated

by GPSM3 that differentially affect LTB₄- and CXCL8- vs fMLP-induced migration; one such mechanism might involve direct coupling by GPSM3 of Gα_i subunits to select chemokine-responsive GPCRs (*i.e.*, in the absence of Gβγ subunits), as has been speculated on recently by Blumer and colleagues (32). The effect of GPSM3 deficiency on NB4* LTB₄-induced migration, coupled with the established role that LTB₄ plays in the early pathogenesis of RA, supports the therapeutic potential of GPSM3-targeted inhibitors in the treatment of RA, as we have proposed previously (11); the potential to inhibit GPSM3 pharmacologically and thereby reduce neutrophil migration to the inflamed joint while sparing fMLP responsiveness is attractive as it suggests a means of reducing RA pathology without compromising responsiveness to bacterial infections.

METHODS

Cell lines and culture conditions

The human acute promyelocytic leukemia cell line (NB4) was obtained from the Leibniz Institute German Collection of Microorganisms and Cell Cultures. Preliminary studies were performed on NB4 cells that were a generous gift from Dr. Sherman Weissman. Cells were maintained at 37°C in a humidified, 5% CO₂ atmosphere in an ATCC-modification of RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU penicillin, 100 µg/ml streptomycin, and 2 µg/ml puromycin. GPSM3 expression was stably knocked-down via lentiviral transduction (WVU IBC protocol 15-04-08) as described previously (11). The shRNA hairpin sequences for shRNA19 (TRC clone ID: TRCN0000036819), shRNA20 (TRCN0000036820), and shRNA22 (TRCN0000036822) are available from The RNAi Consortium (<http://www.broadinstitute.org/rnai/public>) and listed in Table S2. Additional NB4 cell lines were established expressing a scrambled shRNA control (Addgene plasmid 1864), with the scrambled-hairpin sequence as reported by Addgene (<https://www.addgene.org/1864/>; listed in Table S2). Lentiviral transduction was performed at a multiplicity of infection of 10 using spinoculation (ref. (33)); transduced cells were selected with 2 µg/ml puromycin and monoclonal populations established by limiting dilution. The resultant NB4 cell lines stably expressing *GPSM3*-targeted shRNA with the most effective knockdown of GPSM3 were selected for this study. One monoclonal cell line expressing the scrambled shRNA control (“scr1”) was lost to poor growth under selection pressure. All NB4 lines were confirmed free of *Mycoplasma spp.* and *Acholeplasma spp.* contamination using the Lookout® Mycoplasma PCR Detection kit (Sigma-Aldrich, St. Louis, MO; see Fig. S4). NB4 cell line differentiation was performed as previously reported (34) by treatment with complete media containing 1 µM all-trans retinoic acid (Acros Organics, Geel, Belgium). Cell density was maintained between 2 × 10⁵ and 1 × 10⁶ cells/mL.

Nitroblue Tetrazolium (NBT) reduction

Cells (5 × 10⁵) were resuspended in PBS with 1 mg/ml NBT and 300 nM PMA, then incubated at 37°C and 5% CO₂ for 30 minutes. Cells were then washed in PBS twice and mounted on slides for visualization of 500 cells per cell population per experiment. Formazan-positive (black), -intermediate (gray), and -negative (colorless) cells were scored under brightfield microscopy. Investigator was blinded to the transgene status of each experimental condition.

Wright-Giemsa staining

Cells were fixed with 100% methanol for 1 minute, then allowed to air-dry briefly. Cells were then stained using Wright-Giemsa stain for 2 minutes, after which 1.5 volumes of PBS, pH 6.8 was added, and the cells were allowed to incubate for another 2 minutes. Cells were washed and visualized on an EVOS FL Auto microscope (Thermo Scientific, Waltham, MA).

cDNA synthesis and quantitative reverse transcriptase PCR (qRT-PCR)

RNA was extracted from ATRA-differentiated NB4* cells with the RNeasy kit (Qiagen). Briefly, cells were centrifuged at $200 \times g$ for five minutes, and the supernatant was removed. Cells were then washed with $1 \times$ PBS and centrifuged again to remove supernatant. The cell pellet was resuspended in $600 \mu\text{l}$ of the manufacturer's lysis buffer (RLT) with 1% β -mercaptoethanol. RNA isolation was performed using an automated QIAcube workflow according to the manufacturer's suggested protocol to minimize variability between preparations. Eluted nucleic acids were quantified by absorbance at 260 nm using a Nanodrop 2000 (Thermo Scientific) and assessed for purity using a 260/280 nm cut-off ratio 1.8. Immediately following isolation, a sample of eluted nucleic acids (250 ng) from each group of cells was used to synthesize complementary DNA (cDNA) with the Thermo Verso Reverse Transcriptase Kit (Thermo Scientific). cDNA was then diluted 1:5 and used immediately for qRT-PCR analyses. Samples of cDNA, derived from equal mass of input RNA from each sample, were amplified in duplicate (with an additional, independent, "no reverse transcriptase-treated" control) using the USB VeriQuest Fast SYBR Green qPCR Master Mix (Affymetrix, Cleveland, OH) and commercially-validated PCR primers (Qiagen, Hilden, Germany). Cycling conditions were established following the manufacturer's suggested protocol. Relative quantification of gene expression was performed using the C_T method (35) with β -microglobulin chosen as the stable housekeeping gene. Primer sequences for *GPSM3* and β -*microglobulin* were as previously described (11); all other primer pairs were procured from Qiagen.

Transwell migration assay

shRNA-expressing NB4 cell lines were serum-starved in HBSS + 1% BSA at 37°C for one hour and labeled with calcein-AM cell-permeant fluorescent dye (ex. 494 nm/em. 517 nm). Cells were washed and suspended in HBSS + 1% BSA + 10 mM HEPES, and 2.5×10^6 cells/ml were loaded into the upper well of Falcon™ HTS FluoroBlok 96-Multiwell Insert System chemotaxis chambers (Corning, NY). Cells were allowed to migrate toward vehicle or 100 nM LTB4, 100 ng/ml fMLP, 100 ng/ml CXCL8 (R&D Systems, Minneapolis, MN), or 10% FBS (ThermoFisher, Waltham, MA) in the lower chamber. Fluorescence was measured at 2-minute intervals over the course of 60 minutes at 37°C using a Flexstation 3 microplate reader (Molecular Devices, Sunnyvale, CA). All data are representative of biological triplicates with technical replicates (*i.e.*, $n = 6$).

Antibodies and Western blot

Antibodies targeting GPSM3 (UNC Immunology Core; clone 35.5.1; 1:200 dilution), CD11b (Developmental Studies Hybridoma Bank; clone H5A4; 1:200 dilution), pan-Gbeta

(T-20 [cat# sc-378]; Santa Cruz Biotechnology; 1:500 dilution) and β -actin (Sigma [cat# A3854]; 1:2000 dilution) were purchased from the indicated sources. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG was purchased from Sigma. Cells were lysed with ice-cold RIPA buffer with protease inhibitor (ThermoScientific, Waltham, MA), sonicated on ice, then clarified by centrifugation at $20,000 \times g$ for 5 minutes. Protein content was quantified by the Bradford protein assay (ThermoScientific, Waltham, MA) using BSA as a standard. Lysates were diluted in Laemmli buffer (BioRad, Hercules, CA) and resolved on 8–16% pre-cast SDS-polyacrylamide gels (BioRad), transferred to nitrocellulose membranes, immunoblotted with primary (overnight, 4°C) and HRP-conjugated secondary (1:2000 dilution, 2 h at room temperature) antibodies, and visualized by chemiluminescence (ECL, ThermoScientific, Waltham, MA).

Statistical analyses

Regression analyses of Transwell migration assay data were performed using 4 parameter logistic nonlinear regression analyses over the first 30-minute period of migration with post-hoc comparison by F-test to confirm if one model adequately represents all datasets. Analyses were confirmed to accept the null hypothesis of the Shapiro-Wilk normality test ($p < 0.05$). Statistical analyses including initial rate determinations were performed in GraphPad Prism 6.0 using one-way ANOVA with Bonferroni or Dunnett's post-hoc tests where indicated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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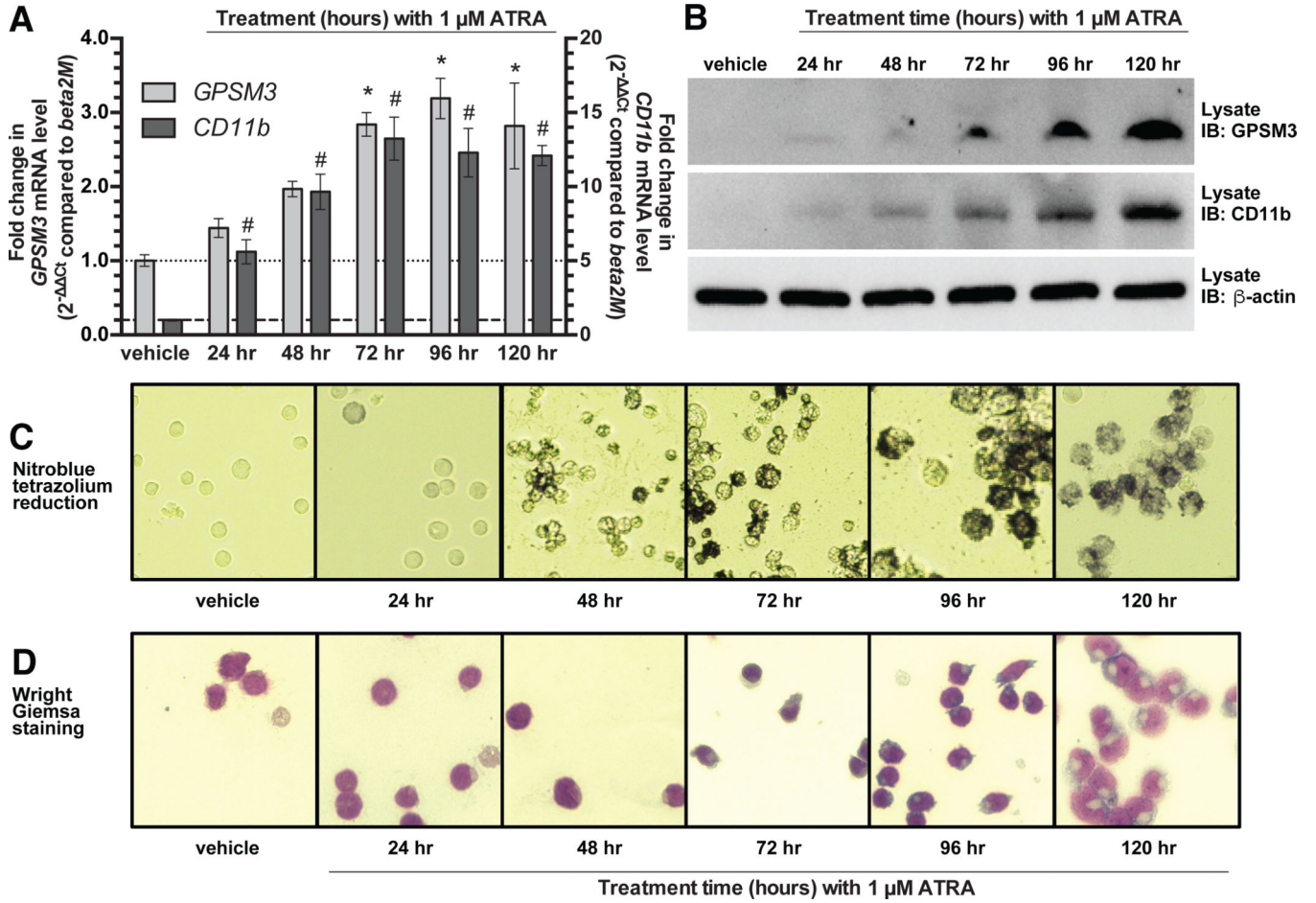


Figure 1. GPSM3 transcript and protein expression are increased during all-trans retinoic acid (ATRA) differentiation of NB4 cells toward a neutrophil-like physiology
(A) *GPSM3* and *CD11b* mRNA levels in NB4 cells, as measured by qRT-PCR, both increase over treatment time with 1 μ M ATRA; relative expression was normalized to cells treated with vehicle (DMSO) for 120 hours. *, significantly different to *GPSM3* transcript level in vehicle control ($p < 0.05$); #, significantly different to *CD11b* transcript level in vehicle control ($p < 0.05$). **(B)** Levels of *GPSM3* protein and the differentiation marker *CD11b* both increase with ATRA-induced differentiation. **(C)** ATRA-induced differentiation over time increases the neutrophil-specific ability to reduce nitroblue tetrazolium to formazan, as observed by a steady increase in formazan-positive (dark) NB4 cells over time. **(D)** Wright-Giemsa stained NB4* cells indicating the acquisition over time of ATRA-induced morphological changes mimicking the nuclear invagination seen in neutrophil-like precursor cells.

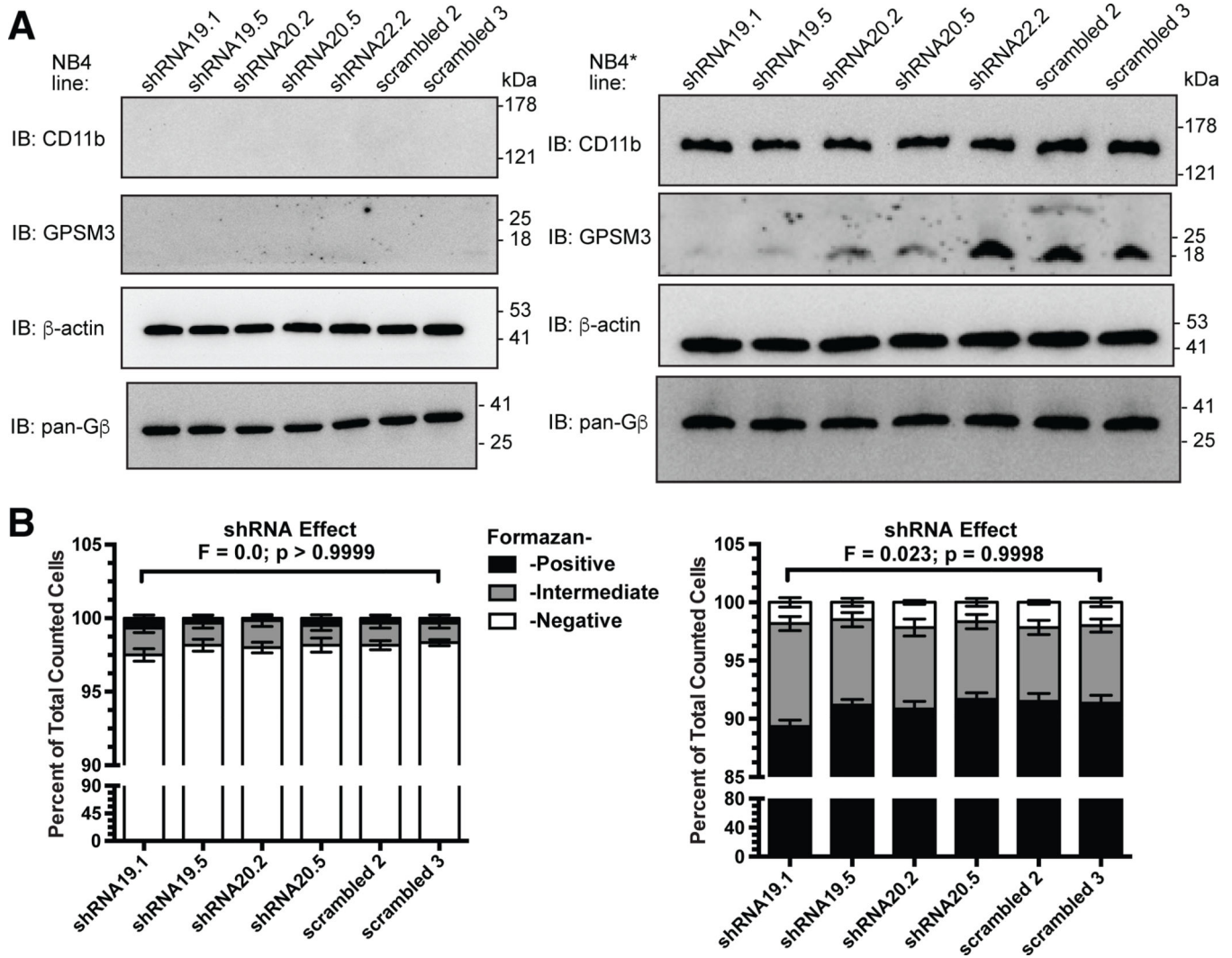


Figure 2. GPSM3 deficiency does not disrupt markers of NB4* cell differentiation
 (A) Immunoblot revealing undetectable GPSM3 and CD11b immunoreactivity within whole cell lysates from all shRNA-expressing monoclonal cell lines when these lines were mock-differentiated with vehicle only (0.01% DMSO; *left* panels). Immunoblot revealing decreased GPSM3 immunoreactivity after 120 hours of all-trans retinoic acid (ATRA) treatment of NB4* cells stably expressing GPSM3-targeted shRNAs shRNA19 or shRNA20 (*right* panels); GPSM3 knockdown was not seen to affect ATRA induction of CD11b levels nor steady-state levels of G β subunits. (B) Nitroblue tetrazolium reduction assay indicating that >90% of cells within each NB4-shRNA monoclonal cell line are incapable of reducing nitroblue tetrazolium after mock-differentiation with vehicle only (0.01% DMSO; *left* bar graph). Following differentiation with all-trans retinoic acid, >87% of differentiated NB4* cells were capable of reducing nitroblue tetrazolium, irrespective of expressed shRNA species (*right* bar graph).

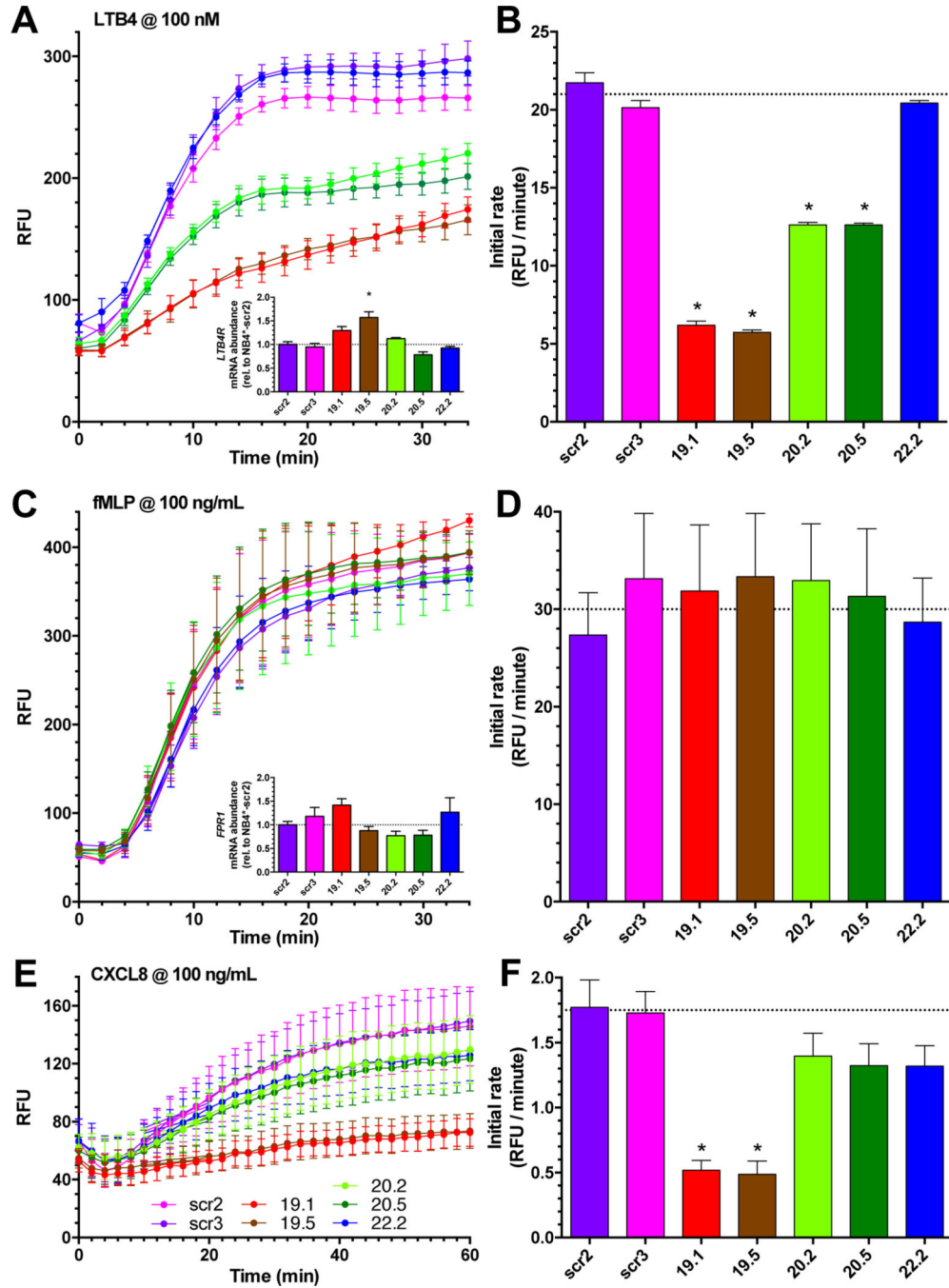


Figure 3. GPSM3 deficiency selectively disrupts NB4* cell migration toward LTB4 and CXCL8
 (A) Real-time Transwell migration towards 100 nM LTB4 over 30 min by indicated NB4* cell lines (after 120 hours ATRA differentiation). Significant differences in migration for all four GPSM3-deficient NB4* lines (19.1, 19.5, 20.2, 20.5; each $p < 0.0001$) compared to scrambled controls were determined by one-way ANOVA analyses of area under the curve with Bonferroni post-hoc. (A inset) Post-differentiated NB4* transcript abundance of the LTB4 receptor BLT1 (*LTB4R*); significance ($p < 0.05$) determined by Kruskal-Wallis test (unequal group variances, Brown-Forsythe $p < 0.05$) with Dunn's post-hoc. All data plotted

as mean \pm S.E.M. **(B)** Plot of initial migration rates (RFU/min) derived from data in panel A. **(C)** Real-time Transwell migration towards 100 ng/mL fMLP over 30 minutes by indicated NB4* cell lines (after 120 hours of ATRA differentiation). No significant differences were observed between cell lines ($F = 0.4255$; $p = 0.9826$). **(C inset)** Post-differentiated NB4* transcript abundance of the fMLP receptor (*FPR1*); significance ($p < 0.05$) determined by Kruskal-Wallis test (unequal group variances, Brown-Forsythe $p < 0.05$) with Dunn's post-hoc. All data plotted as mean \pm S.E.M. **(D)** Plot of initial migration rates (RFU/min) derived from data in panel C. **(E)** Real-time Transwell migration towards 100 ng/mL CXCL8 (*a.k.a.* IL-8) over 60 minutes by indicated NB4* cell lines (after 120 hours of ATRA differentiation). Significant differences in migration for two of the GPSM3-deficient NB4* lines, namely 19.1 ($F = 47.87$; $p < 0.0001$) and 19.5 ($F = 40.97$; $p < 0.0001$), compared to scrambled controls were determined by one-way ANOVA analyses of area under the curve with Bonferroni post-hoc. Post-differentiated NB4* transcript abundance of the CXCL8 receptors *CXCR1* and *CXCR2* are presented in Supplemental Figure S2. **(F)** Plot of initial migration rates (RFU/min) derived from data in panel E.