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Gpnmb Is Induced in Macrophages by IFN-γ and Lipopolysaccharide and Acts as a Feedback Regulator of Proinflammatory Responses[1]

Vera M. Ripoll,* Katharine M. Irvine,* Timothy Ravasi,* Matthew J. Sweet,*† and David A. Hume[2]*

The process of inflammation requires the selective expression of a suite of genes in cells of the macrophage lineage. To identify candidate regulators of inflammation, we used cDNA microarrays to compare the transcriptome of inflammatory macrophages (thioglycolate-elicited peritoneal macrophages), bone marrow-derived macrophages, nonadherent spleen cells, and fibroblasts. We identified genes that were macrophage restricted and further elevated in inflammatory macrophages, and characterized the function of one such gene, *gpnmb*. *Gpnmb* mRNA expression was enriched in myelomonocytic cell lines and macrophage-related tissues and strongly up-regulated during macrophage differentiation. Epitope-tagged GPNMB expressed in RAW264.7 cells exhibited a perinuclear distribution and colocalized with the Golgi marker coat protein β. Upon activation of macrophages with IFN-γ and LPS, GPNMB translocated from the Golgi apparatus to vesicular compartments scattered toward the periphery. *Gpnmb* overexpression in RAW264.7 cells caused a 2-fold reduction in the production of the cytokines IL-6 and IL-12p40 and the inflammatory mediator NO in response to LPS. DBA mice, which have an inactivating point mutation in the *gpnmb* gene, exhibited reduced numbers of myeloid cells, elevated numbers of thioglycolate-elicited peritoneal macrophages, and higher levels of proinflammatory cytokines in response to LPS. Thus, GPNMB acts as a negative regulator of macrophage inflammatory responses. *The Journal of Immunology,* 2007, 178: 6557–6566.

Since the first description of the mononuclear phagocyte system, there has been an interest in characterizing molecules that better define this heterogeneous group of cells. The purpose of defining a cell lineage (e.g., macrophages) is to make predictions about their biological function (1). The identification and characterization of macrophage markers (e.g., membrane proteins) have provided useful information for describing both macrophage developmental stages and heterogeneity in different tissue microenvironments. Such information has helped to establish anatomic relations between macrophages and other cells in healthy and disease states, and has yielded insights into macrophage functional capacities and states of activation (2–5). In the past, mAb technology (1, 6, 7) as well as differential display PCR (8, 9) have enabled the identification of macrophage-restricted markers. Subsequently, strategies such as serial analysis of gene expression were used to identify transcripts that are differentially expressed between monocyte populations and other members of the mononuclear phagocyte system (http://bloodsage.gi.k.u-tokyo.ac.jp) (10–12). More recently, global transcription analysis using microarray technology has permitted the examination of the macrophage transcriptome (13). The interplay between macrophages and a range of pathogens and bacterial components has been investigated by microarray (14). Indeed, the remodelling of macrophage gene expression in response to such stimuli can be profound (1, 15). Such studies have also revealed the existence of groups of genes whose expression is coordinately regulated in response to pathogen challenge. Moreover, these reports have shown that different classes of pathogens stimulate the expression of overlapping gene sets to enable an appropriate immune response (16–18).

Gene expression studies in our laboratory have revealed the existence of a novel macrophage transcriptional phenotype. Clustering analysis of gene expression patterns of isolated mouse macrophages and 49 mouse tissues (13, 19) demonstrated that macrophage samples clustered independently from these tissues. Macrophages clustered closest to hemopoietic tissues such as the spleen, but were clearly distinct from the rest of the tissues. This conclusion was supported by recent publication of genome-wide promoter analysis using cap analysis gene expression (CAGE)[3] technology (20), which demonstrated that macrophage-specific promoters form a substantial cluster from other tissues and cell types. In the current study, we have conducted a gene expression profile comparison of two macrophage populations with nonmacrophage hemopoietic cells, and proliferating fibroblasts to identify additional candidate macrophage-restricted genes in the mouse. Within this gene set we also identified transcripts that were elevated in an

*Cooperative Research Centre for Chronic Inflammatory Diseases and Special Research Centre for Functional and Applied Genomics, Institute for Molecular Bioscience, University of Queensland, Queensland, Australia; and †School of Molecular and Microbial Sciences, University of Queensland, Queensland, Australia

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[2] Address correspondence and reprint requests to Dr. David A. Hume, Institute for Molecular Bioscience, University of Queensland, St. Lucia, Queensland 4072, Australia. E-mail address: d.hume@imb.uq.edu.au

[3] Abbreviations used in this paper: CAGE, cap analysis gene expression; BFA, brefeldin A; BMM, bone marrow-derived macrophage; DC, dendritic cell; ER, endoplasmic reticulum; HMDM, human monocyte-derived macrophage; hprt, hypoxanthine-guanine phosphoribosyltransferase; f, forward; r, reverse; TEPM, thioglycolate-elicited peritoneal macrophage; β-COP, coat protein β.

inflammatory macrophage population (thioglycolate-elicited peritoneal macrophages (TEPM)) when compared with nonstimulated bone marrow-derived macrophages (BMM). One of the most highly macrophage-restricted transcripts, *gpnmb*, was subjected to detailed functional analysis, and we show in this study that this gene has an important regulatory function in macrophages, acting as a negative regulator of excessive inflammatory responses.

Materials and Methods

General reagents

LPS (from *Salmonella minnesota*; Sigma-Aldrich) was used at a final concentration of 10 ng/ml. Mouse rIFN-γ (R&D Systems) was used at a final concentration of 500 pg/ml. Human rCSF-1 (a gift from Chiron, Emeryville, CA) was used at a final concentration of 10^4 U/ml. Brefeldin A (BFA) (Sigma-Aldrich) was used at a final concentration of 5 μg/ml. G418 (geneticin; Invitrogen Life Technologies) was used at a final concentration of 250 μg/ml. The CpG-containing phosphorothioate-modified oligonucleotide 1668S (5′-TCCATGACGTTCCTGATG-3′) (21) was used at a final concentration of 0.1 μM.

Mouse strains

C57BL/6 and DBA *gpnmb* wild-type mice (DBA/2J Arc) were sourced from the Animal Resources Centre, Western Australia. DBA *gpnmb* mutant mice (DBA/2J) were obtained from The Jackson Laboratory. Animals were housed under specific pathogen-free conditions, and all experiments were conducted in accordance with local animal ethics guidelines.

Cell lines and cell culture

Unless otherwise stated, all cells were from the American Type Culture Collection and were routinely grown in complete medium consisting of RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 5–10% heat-inactivated FBS, 20 U/ml penicillin, 20 μg/ml streptomycin (Invitrogen Life Technologies), and 2 mM L-glutamine (glutamax-1; Invitrogen Life Technologies). Cells were cultured at 37°C in a humidified tissue culture incubator with 5% CO_2. BMM were obtained by culturing bone marrow cells, derived from the femurs of adult C57BL/6 mice, in the presence of 10^4 U/ml human rCSF-1 for 7 days. Bone marrow-derived dendritic cells (DC) were derived from culture of bone marrow cells with IL-4 (2.5 ng/ml; Bioscientific) plus GM-CSF (2.5 ng/ml; Bioscientific) for 10 days. TEPM were obtained by i.p. injection of 1 ml of 10% thioglycolate broth into C57BL/6 mice, followed by peritoneal lavage with 10 ml of PBS 5 days later. To obtain nonadherent spleen cells, splenocytes were strained through a 100 μM mesh cell strainer (BD Biosciences; Falcon) and cultured overnight on tissue culture plates (Iwaki). On the following day, nonadherent cells (lymphocyte-enriched population) were harvested. Embryonic skin fibroblasts were obtained from 18.5 days postcoitum C57BL/6 embryos. The skin of freshly dissected embryos was digested with 10% trypsin in EDTA for 1 h at 37°C. The tissue suspension was washed with complete medium and cultured on tissue culture plates (Iwaki) for 24 h. For PBMC isolation from C57BL/6 mice, red cells were lysed using erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA) and white cells were recovered by centrifugation. Granulocytes, monocytes, and B cells were isolated using R-PE-conjugated anti-Ly-6G (clone 1A8; BD Pharmingen), anti-CSF-1R (Serotec), and anti-CD19 (Serotec) Abs, respectively. Labeled cells were incubated with anti-PE MicroBeads (Miltenyi Biotec) and applied to LS columns, according to manufacturer's instructions.

Primary human monocyte-derived macrophages (HMDM) were prepared from buffy coat cells from duplicate donors. Cells were isolated by a Ficoll gradient, and monocytes were positively selected using MACS $CD14^+$ magnetic beads (Miltenyi Biotec), according to manufacturer's instructions. Monocytes were cultured overnight in IMDM medium (Invitrogen Life Technologies) supplemented with 10% FCS, 20 U/ml penicillin (Invitrogen Life Technologies), 20 μg/ml streptomycin (Invitrogen Life Technologies), and 2 mM L-glutamine (Invitrogen Life Technologies). HMDM were generated by differentiation of monocytes for 7 days in the presence of 10^4 U/ml human rCSF-1. THP-1 monocyte-like cells were cultured in complete medium supplemented with 10 mM HEPES, 1 mM sodium pyruvate, and 50 mM 2-ME, and were differentiated in 30 ng/ml PMA (Sigma-Aldrich) for 4 days.

RNA isolation and quantitative PCR

Total RNA was extracted using the RNeasy Midi Kit (Qiagen), according to the manufacturer's instructions. Genomic DNA was removed using DNA-Free (Ambion), and cDNA was synthesized using Superscript III (Invitrogen Life Technologies). A negative cDNA synthesis control was performed following the same conditions as above, but with no enzyme addition. cDNA was quantitated using SYBR Green (Applied Biosystems) in 20-μl reactions in a 96-well plate using an ABI Prism 7000 sequence detection system (Applied Biosystems), as per the manufacturer's instructions. Data were collected and analyzed using the ABI Prism software. Gene expression was determined relative to hypoxanthine-guanine phosphoribosyltransferase (*hprt*) mRNA using the comparative threshold method. Primers (f, forward; r, reverse) used were as follows: *gpnmb* f: agcacaaccaattacgtggc, r: cttcccaggagtccttcca; *csf1r* f: ccagagcccccacagataa, r: agcttgctgtctccacgtttg; *il-6* f: cagaattgccatcgtacaactcttttctca, r: aagtgcatca tcgttgttcataca; *p40il-12* f: ggaagcacggcagcagaata, r: aacttgagggagaagtagg aatgg; *hprt* f: gcagtacagccccaaaatgg, r: aacaaagtctggcctgtatccaa; *hgpnmb* f: tccgacgaaaccttcctcaa, r: tccccgaagctccacttcta; *hhprt* f: tcaggcagtataatccaa agatggt, r: agtctggcttatatccaacagttc; *hcsf1r* f: cctcgcttccaagaattgc, r: gcccga agtccccaatct.

Northern blotting

RNA samples were run under denaturing conditions and transferred to a nylon ζ-probe membrane (Bio-Rad). The probe used for Northern detection of *gpnmb* mRNA was the ~1.8-kb full-length cDNA (forward primer, tcggagtcagcatggaaagt; reverse, gagtgtccttggcttgtcct). The 18S ribosomal RNA was detected using the oligonucleotide probe catggtaggcacggcgactaccat, as described previously (22). cDNA probes were labeled with ^{32}P-dCTP using the Megaprime DNA labeling system (Amersham Biosciences), according to the manufacturer's instructions. The 18S oligonucleotide probe was labeled using T4 polynucleotide kinase (New England Bioscience), according to the accompanying instructions. Hybridizations were performed overnight at 65°C in hybridization solution containing the peak-labeled probe fractions. Following hybridization, membranes were washed and exposed at −80°C using Fuji Super RX films (Fuji).

Generation of RAW264.7 cells stably transfected with gpnmb *expression construct*

The coding region of mouse *gpnmb* was PCR amplified from cDNA (forward primer, tcggagtcagcatggaaagt; reverse primer, gagtgtccttggcttgtcct) and cloned into the pGene/V5His vector (Invitrogen Life Technologies). Stable transfections of RAW264.7 cells were performed by electroporation of 10 μg of endotoxin-free plasmid into 5×10^6 cells using a Gene-Pulser electroporator (Bio-Rad) at 280 V and 960 μF capacitance. Cells were cotransfected with the selection plasmid pPNT-Neo at a 3:1 ratio, plated into tissue culture plates, and grown for 48 h before selection with G418 (geneticin; Invitrogen Life Technologies). Stable transfectants were maintained in the presence of the selective agent (geneticin) during routine culture. The cell lines generated were RAW264.7/*gpnmb* and RAW264.7/pGene (empty expression vector).

Protein isolation and Western blotting

Total protein extracts were prepared using a SDS-boiling method. Cells were lysed on the culture plate with 1 ml of boiling lysis buffer (66 mM Tris-HCl (pH 7.4), 2% SDS) per 1×10^7 cells. For supernatant precipitation, medium was collected, and after centrifugation at $350 \times g$ for 10 min, supernatants were filtered through a 0.2-μM membrane (Millipore). Four times the sample volume of cold acetone (−20°C) was added. Samples were mixed and incubated for 60 min at −20°C. Samples were centrifuged at $15{,}000 \times g$ for 30 min, the acetone was evaporated, and protein pellets were resuspended in 1× SDS-loading dye.

Protein concentration of total lysates was determined using the bicinchoninic acid assay kit (Pierce), according to the manufacturer's instructions. Proteins were routinely resolved and analyzed by SDS-PAGE using precast NuPAGE Bis-Tris 12% gels (Invitrogen Life Technologies). SDS-PAGE gels were transferred to methanol-activated Immobilon-P (Millipore) polyvinylidene difluoride membrane, blocked, and probed with anti-V5 Ab (Serotec) or anti-TNF-α polyclonal Ab (Genzyme). Appropriate HRP-conjugated secondary Abs (Cell Signaling Technology) and ECL Plus system (Amersham Biosciences) were used for HRP detection.

Immunofluorescence

A total of 5×10^5 RAW264.7 cells was plated onto glass coverslips and grown overnight. Cells were fixed with 4% w/v paraformaldehyde, permeabilized with Triton X-100, and washed with blocking solution. Coverslips/Cell monolayers were incubated with the appropriate primary Ab (anti-V5 Ab), anti-coat protein β (β-COP) Ab (a gift from R. Teasdale, Institute for Molecular Bioscience, Queensland, Australia) diluted in blocking solution. Cells were washed three times with blocking solution

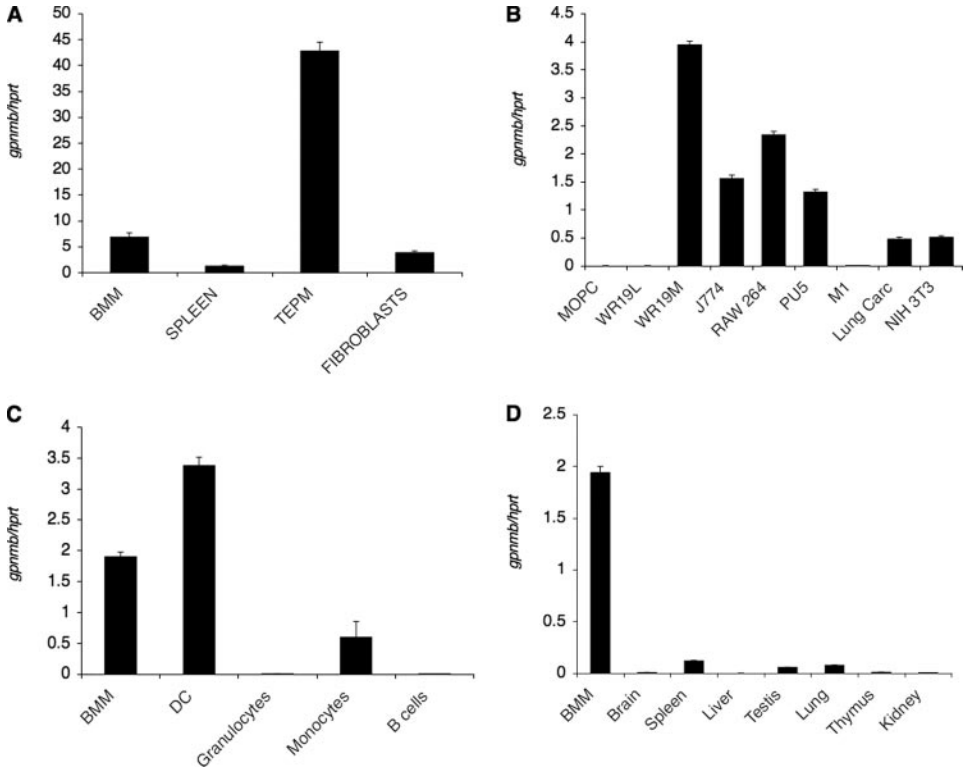

FIGURE 1. *Gpnmb* expression is highly restricted to TEPM, BMM, macrophage-related tissues, and myelomonocytic cell lines. Total RNA from C57BL/6 TEPM, BMM, spleen nonadherent cells, and fibroblasts (*A*); myelomonocytic and other cell lines (*B*); C57BL/6 BMM, DC, monocytes, and B cells (*C*); and C57BL/6 various tissues (*D*) was prepared. Levels of *gpnmb* mRNA relative to *hprt* were estimated by real-time PCR using SYBR Green amplification. Data points represent the mean of triplicates, and SDs are displayed. Results are representative of at least three experiments using independent RNA samples.

and exposed to the appropriate fluorophore-conjugated secondary Ab (Alexa fluor 647 donkey anti-rabbit IgG, Alexa fluor 568 goat anti-mouse IgG (Invitrogen Life Technologies). Fluorescence microscopy was conducted on an Olympus AX-70 unit, and images were captured with NIH Image 1.62 software using a Dage-MTI CCD300RC charge-coupled device camera.

Flow cytometry

A total of 2×10^6 cells was harvested, fixed, and stained with the primary Ab (mouse anti-V5 tag mAb) or directly conjugated Ab (RPE-conjugated rat anti-mouse TNF-α, IL-6, or IL-12p40; BD Pharmingen). Cells were then washed and incubated with the fluorophore-conjugated secondary Ab (diluted in blocking solution containing 0.1% saponin). Immunostained samples were detected using a FACSCalibur (BD Biosciences). For intracellular staining of proinflammatory cytokines, cells were treated with 5 μg/ml BFA (Sigma-Aldrich) 15 min after LPS activation.

ELISA

A total of 5×10^4 cells/well in 200 μl of complete medium in 96-well tissue culture plates was primed overnight with 500 pg/ml IFN-γ, then stimulated on the following day for 8 or 24 h with 10 ng/ml LPS. Levels of IL-6, TNF-α, and IL-12p40 in culture supernatants were estimated by ELISA (BD Pharmingen). To ensure equal cell numbers were plated for different cell lines, cell viability was determined in parallel using the MTT assay (Sigma-Aldrich).

Results

Gpnmb *mRNA expression is enriched in macrophages*

To identify new candidate regulators of the inflammatory process, we sought genes that were expressed in a macrophage-restricted fashion and were further enriched in inflammatory macrophages. Microarray expression analysis was performed on RNA from two mouse macrophage populations (BMM and TEPM), as well as proliferating fibroblasts and nonadherent spleen cells, which contain a mixture of most other hemopoietic cell types. BMM are actively proliferating in response to the growth factor, CSF-1, so the comparison with fibroblasts also eliminated many cell-cycle/proliferation-associated genes from consideration. Primary data derived from a common reference comparison of these cell populations on Compugen oligonucleotide arrays may be downloaded

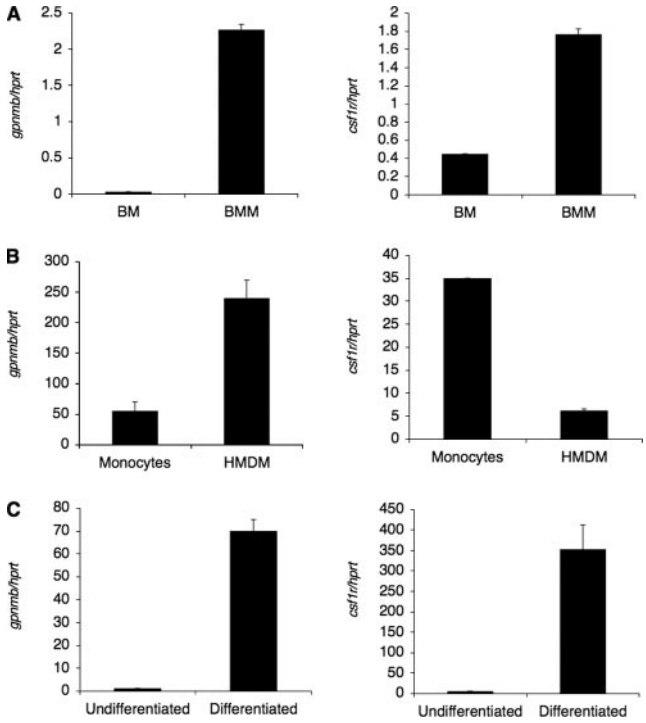

FIGURE 2. The expression of *gpnmb* is induced during macrophage differentiation. Mouse bone marrow progenitors (*A*) and CD14-positive human monocytes (*B*) were induced to differentiate into BMM or HMDM in the presence of CSF-1 (10^4 U/ml) over 7 days. RNA was extracted and cDNA was prepared. THP-1 monocyte-like cells were differentiated in the presence of 30 ng/ml PMA for 4 days (*C*). Levels of *gpnmb* and *cfs1r* mRNA relative to *hprt* were estimated by real-time PCR using SYBR Green amplification. Data points represent the mean of triplicates, and SDs are displayed. Results are representative of at least three experiments.

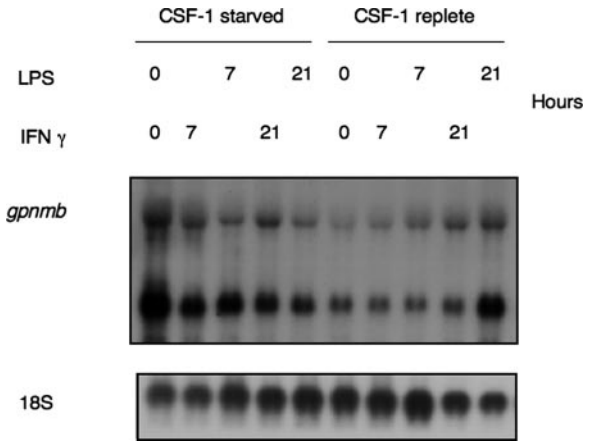

FIGURE 3. CSF-1, LPS, and IFN-γ regulate *gpnmb* expression in BMM. C57BL/6 BMM were prepared and maintained overnight in the presence or absence of CSF-1 (10^4 U/ml). The following day, cells were stimulated with LPS (10 ng/ml) or IFN-γ (500 pg/ml) for 0, 7, or 21 h. RNA was prepared and used to determine *gpnmb* levels of expression by Northern blotting using a ^{32}P-labeled probe encoding full-length *gpnmb*. The membrane was stripped and reprobed for 18S ribosomal RNA. *Gpnmb* expression is representative of two experiments using independent RNA samples.

from our web site at www.macrophages.com. A total of 119 genes was expressed at least 2-fold higher in both macrophage populations than in either fibroblasts or nonadherent spleen cells, and, of these, 40 genes were more highly expressed in TEPM than in BMM. The validity of this approach for the identification of macrophage-enriched/specific genes was internally controlled by the presence within the gene list of most well-known macrophage functional markers, such as *csf1r* (CSF-1 receptor), *Itgam* (CD11b), *lyzs* (lysozyme), *lgals3* (lectin, galactose-binding 3), *msr1* (macrophage scavenger receptor 1), and *cd14* (data not shown).

We selected the *gpnmb* gene, which was both macrophage restricted and more highly expressed in TEPM, for detailed analysis, because this expression profile is consistent with a potential role in inflammatory processes. The pattern of *gpnmb* expression and enrichment in TEPM was confirmed by real-time PCR (Fig. 1*A*). *Gpnmb* mRNA was also highly expressed in macrophage-like cell lines (RAW264.7, J774, WR19M, PU5.1), particularly in WR19M, but was not detectable in the immature myeloblastic cell line M1 or the lymphocyte-like cell lines MOPC 31C and WR19L. Expression of *gpnmb* was detected in fibroblasts and Lewis lung carcinoma, but at lower levels than in macrophages (Fig. 1*B*). We next compared *gpnmb* mRNA expression within the myeloid compartment in mice. Fig. 1*C* shows that *gpnmb* mRNA was highly expressed in BMM, DC, and monocytes, but not in granulocytes. Consistent with the cell line data (Fig. 1*B*), primary B cells, which share a similar gene expression profile with macrophages, did not express high levels of *gpnmb* mRNA (Fig. 1*C*). The detection of *gpnmb* mRNA in tissues was also consistent with a macrophage-enriched expression profile, being elevated in the spleen and lung compared with other tissues (Fig. 1*D*).

The expression of gpnmb *during macrophage differentiation was conserved across species*

To confirm the association of *gpnmb* with macrophage differentiation, the effect of CSF-1 on *gpnmb* expression in mouse bone marrow and human monocytes was examined. *Gpnmb* mRNA was induced during the process of macrophage differentiation in both mice (CSF-1-induced differentiation of bone marrow cells for 7 days; Fig. 2*A*) and humans (CSF-1-induced differentiation of CD14-positive monocytes for 7 days; Fig. 2*B*). By contrast, *csf1r* expression was induced during macrophage differentiation in mice,

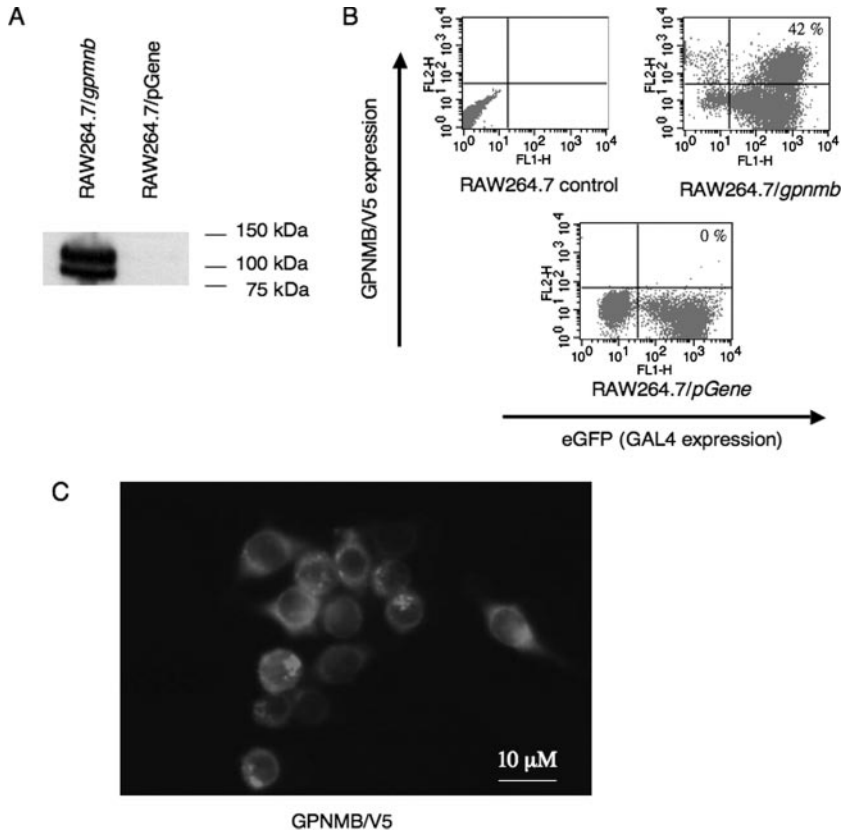

FIGURE 4. Expression of GPNMB in RAW264.7 cells. *A*, Anti-V5 Western blot of total cell lysates from stably transfected RAW264.7 cells demonstrating GPNMB expression. Cell lysates were resolved by SDS-PAGE and probed with anti-V5 Ab. Cell lysates from RAW264.7/pGene served as a negative control. *B*, Simultaneous intracellular detection of V5 epitope-tagged GPNMB and the enhanced GFP GAL4 reporter. Stably transfected cells were stained with anti-V5 and R-PE goat anti-mouse IgG conjugate secondary Ab, and their enhanced GFP expression was analyzed by flow cytometry. The percentage of double-positive cells is shown. Untransfected RAW264.7 and RAW264.7/pGene served as negative controls. Data are representative of at least two independent experiments. *C*, Detection of V5 epitope-tagged GPNMB by immunofluorescence in stably transfected RAW264.7 cells using the V5 tag Ab.

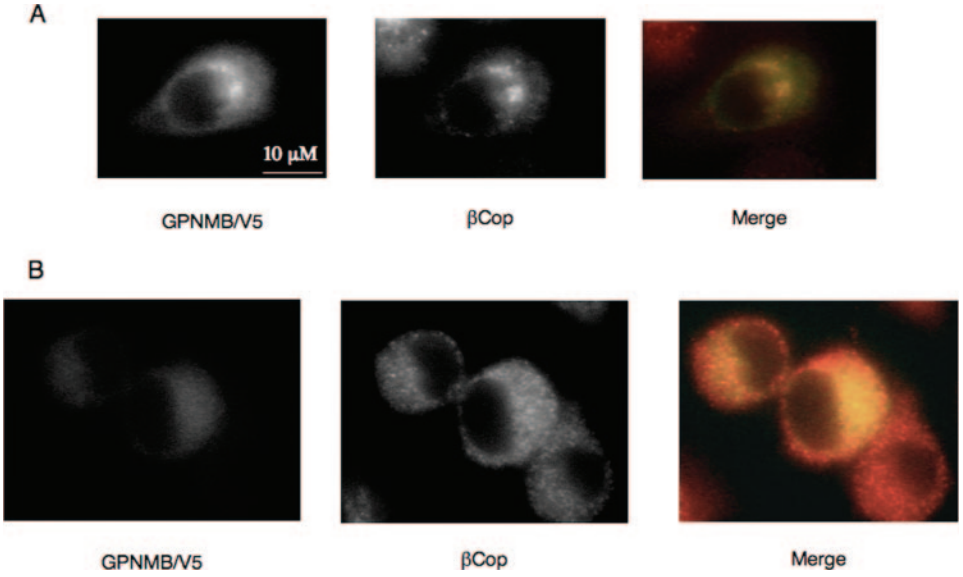

FIGURE 5. GPNMB colocalizes with the Golgi marker β-COP. RAW264.7/*gpnmb* cells were fixed and stained for the V5 tag and β-COP (*A*). Before fixation, cells were treated with 5 μg/ml BFA for 15 min and stained for V5 tag and β-COP (*B*).

but repressed in humans (Fig. 2, *A* and *B*). We also examined the regulation of *gpnmb* in the THP-1 monocyte-like cells undergoing differentiation in response to PMA. Fig. 2*C* demonstrates that, in this system, *gpnmb* was also highly up-regulated during macrophage maturation. The use of CAGE technology has enabled fine mapping of transcription start sites on a genome-wide scale (20). In keeping with macrophage-restricted gene expression in both species, the CAGE-mapped transcription start site is conserved between mice and humans and the CAGE tag frequency is highest in macrophage cDNA libraries (data can be accessed via www.macrophage.com).

Gpnmb *mRNA regulation during macrophage activation*

We also determined whether *gpnmb* mRNA expression was regulated in macrophages, by assessing the effects of pathogen components (LPS) and host cytokines that can prime macrophage responses (IFN-γ). Many studies on macrophage biology exclude the macrophage growth factor, CSF-1, from culture conditions (23). However, CSF-1 is constitutively present in vivo (24), and we recently showed that bacterial products such as LPS induce expression of a substantial set of CSF-1-repressed genes in macrophages by blocking CSF-1 signaling (25). Such genes would not be recognized as LPS-regulated unless CSF-1 was present, and we therefore assessed LPS- and IFN-γ-regulated *Gpnmb* expression in the presence and absence of CSF-1.

By contrast to the acute induction by CSF-1 in progenitor cells, Northern blot analysis showed that *gpnmb* mRNA was actually repressed by CSF-1 (or induced following growth factor deprivation) in BMM, and induced by LPS only when CSF-1 was present. *Gpnmb* expression in CSF-1-starved BMM was actually repressed by LPS (Fig. 3). Hence, *gpnmb* is a member of the class of genes that is induced by LPS via the antagonism of CSF-1 action (25). Similar to LPS, CpG DNA induced the expression of *gpnmb* by antagonizing CSF-1 action (data not shown). IFN-γ also repressed *gpnmb* mRNA in the absence of CSF-1, but modestly induced expression in the presence of CSF-1 (Fig. 3). In HMDM, *gpnmb* mRNA was not substantially LPS inducible, possibly because it is constitutively expressed at high levels in these cells (data not shown).

Two distinct *gpnmb* transcripts were detected in macrophages by Northern blotting using full-length cDNA as probe (Fig. 3). This observation is consistent with data arising from the FANTOM3 polling of full-length mouse cDNAs (20, 26), which supports the existence of 3′ truncated forms of *gpnmb* mRNA that would most likely encode a C-terminally truncated protein lacking the membrane-spanning and intracellular domains (www.macrophages.com). The two transcripts appear to be regulated in parallel. We have not explored the function of the predicted truncated protein isoform.

Generation of stably transfected RAW264.7 cells expressing GPNMB

To investigate the localization and function of *gpnmb* in mouse macrophages, and the likely consequences of elevated expression

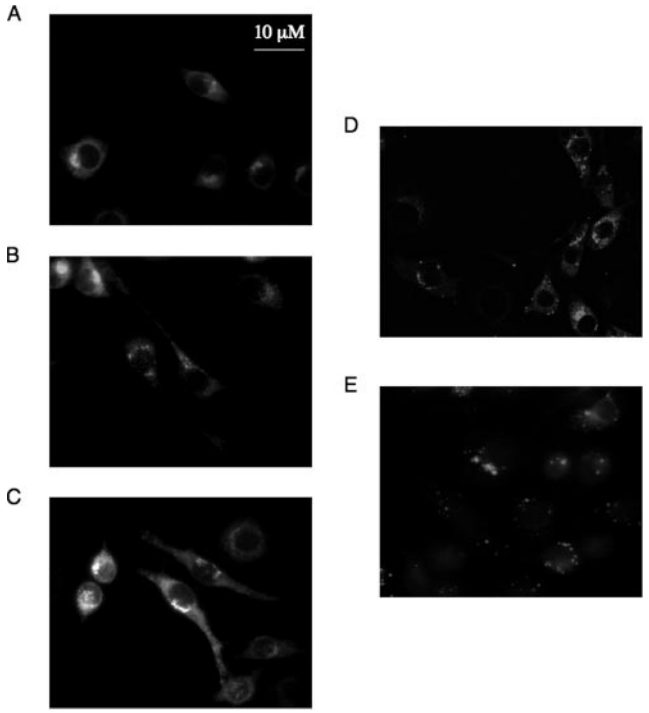

FIGURE 6. LPS and IFN-γ synergistically induced changes in the localization of GPNMB. RAW264.7/*gpnmb* were left unstimulated (*A*), or were treated for 24 h with 10 ng/ml LPS (*B*) or 500 pg/ml IFN-γ (*C*), or were primed overnight with 500 pg/ml IFN-γ and treated with 10 ng/ml LPS for 8 h (*D*) or 24 h (*E*). After indicated treatments, cells were fixed and stained for the V5 tag.

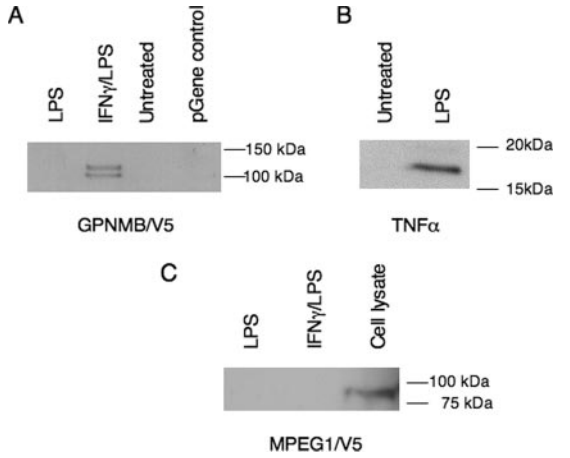

FIGURE 7. GPNMB was detected in culture medium in response to IFN-γ and LPS. RAW264.7/*gpnmb* cells or RAW264.7 cells stably transfected with an unrelated V5-tagged protein were grown in serum-free medium, primed overnight with IFN-γ (500 pg/ml), and treated with LPS (10 ng/ml) for 24 h or were treated with LPS alone. Medium was collected, and acetone precipitated. Protein extracts were analyzed by Western blotting using anti-V5 (*A* and *C*) and anti-TNF-α (*B*) Abs. *C*, Corresponds to a V5-tagged intracellular overexpressed protein control.

in inflammatory cells, the mouse full-length cDNA was overexpressed in the macrophage-like cell line RAW264.7. A C-terminally V5 epitope-tagged version of GPNMB was stably expressed in RAW264.7 cells using the binary UAS/GAL4 system (27). GPNMB expression was analyzed by flow cytometry, Western blotting, and immunofluorescence (Fig. 4). Immunoblotting with the mAb to the V5 tag detected two bands consistent with the size predicted for glycosylated GPNMB (28) (Fig. 4*A*). Intracellular staining for the V5 tag and flow cytometry revealed that ~42% of the cells expressed GPNMB ectopically using the inducible binary system (Fig. 4*B*). This percentage might be increased by selecting clones in which the UAS-GPNMB construct was favorably integrated for inducible expression. Finally, indirect immunofluorescence using the V5 tag Ab confirmed that V5-tagged GPNMB was expressed and localized to intracellular compartments (Fig. 4*C*).

GPNMB colocalized with the Golgi marker β-COP

Previous reports on the intracellular location of *gpnmb* in different cell types suggested some form of membrane association and proximity to the nucleus (28). GPNMB subcellular distribution resembled that of a Golgi protein (Fig. 4*C*), and this observation was supported by colocalization with the Golgi-endoplasmic reticulum (ER) protein β-COP (29) (Fig. 5*A*). The association of GPNMB with the Golgi apparatus was further confirmed using BFA, which induces the fusion of Golgi membranes with the ER or endosomal compartments (30). BFA treatment altered the localization of both β-COP and GPNMB from the single, large perinuclear vesicle to small speckled vesicles dispersed throughout the cytosol (Fig. 5*B*).

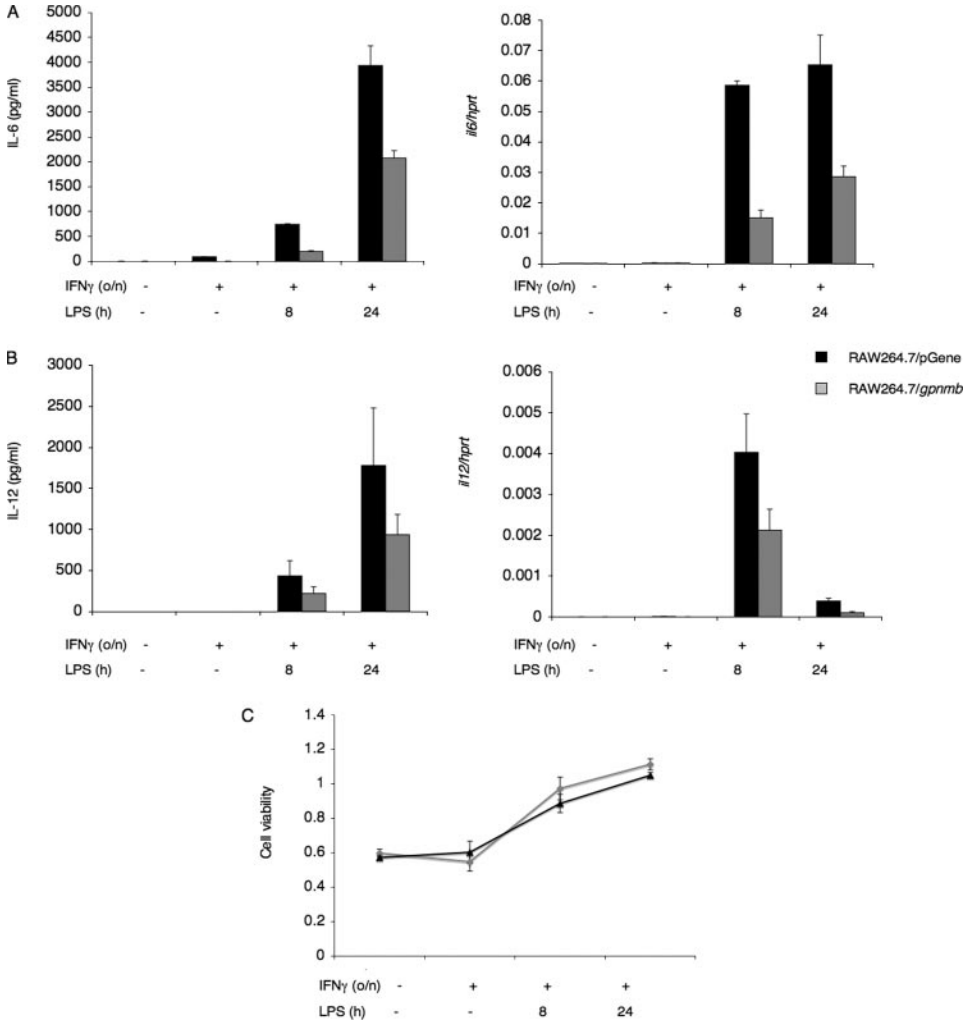

FIGURE 8. *Gpnmb* overexpression reduced LPS/IFN-γ-induced cytokine secretion in RAW264.7 macrophages. Equal numbers of RAW264.7/pGene and RAW264.7/*gpnmb* cells were primed overnight with IFN-γ (500 pg/ml) and treated with LPS (10 ng/ml) for 8 and 24 h. IL-6 and IL-12p40 ELISAs were performed on supernatants (*A* and *B*). Total RNA was collected at indicated time points, and levels of *il-6* and *il-12p40* mRNA relative to *hprt* were estimated by real-time PCR using SYBR Green amplification (*A* and *B*). In parallel, MTT proliferation assays were performed for the cells used in the experiment (*C*). Data points represent the mean of triplicates, and SDs are displayed. These data are representative of at least three independent experiments.

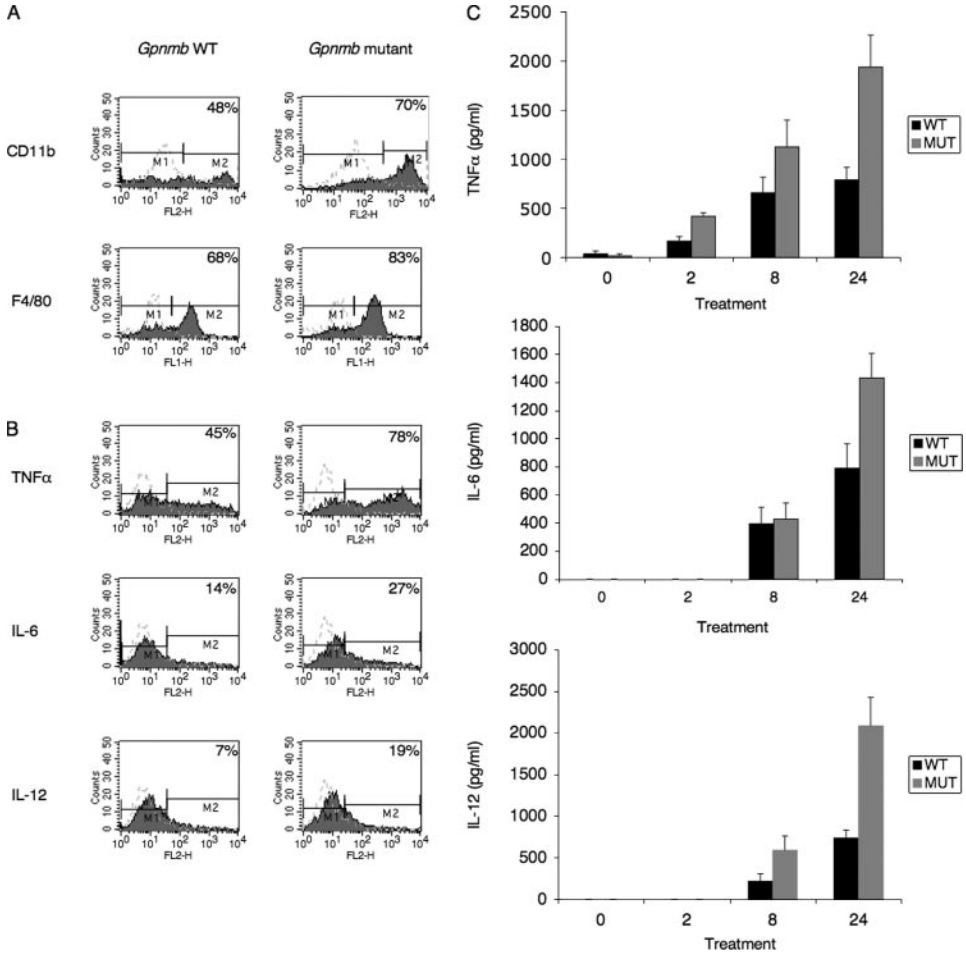

FIGURE 9. TEPM inflammatory responses were increased in *gpnmb* mutant mice. TEPM from *gpnmb* mutant and wild-type mice were prepared and stained for the macrophage surface markers CD11b and F4/80 (*A*). Equal numbers of TEPM from *gpnmb* mutant and wild-type mice were treated for 12 h with 10 ng/ml LPS in the presence of 5 μg/ml BFA. Intracellular staining was performed using PE-conjugated mAbs against the indicated cytokines (*B*). Histogram overlays show staining produced by isotype control Abs. The percentage of positively stained cells determined by the marker M2 is shown. Data are representative of two independent experiments. Equal numbers of TEPM from *gpnmb* mutant and wild-type mice were treated with 10 ng/ml LPS for 2, 8, and 24 h, and culture supernatants were tested for production of TNF-α, IL-6, and IL-12p40 by ELISA (*C*). Data points represent the mean of triplicates, and SDs are displayed. Bars indicate the SD between triplicates.

LPS and IFN-γ synergistically induced changes in the localization of GPNMB

LPS and IFN-γ, which both regulate *gpnmb* mRNA, also cause profound changes in post-Golgi trafficking (31, 32). We therefore examined whether treatment of RAW264.7/*gpnmb* cells with these proinflammatory agents might provoke changes in GPNMB localization. Neither LPS nor IFN-γ alone generated any apparent shift in the perinuclear distribution of GPNMB (Fig. 6, *B* and *C*). However, macrophage activation by sequential treatment with both stimuli, which induces an activated cytocidal phenotype (33), caused a relocation of the protein, with translocation from the Golgi to small vesicular compartments scattered toward the cell periphery, most prominently after long-term treatment (Fig. 6, *D* and *E*). This finding suggested that GPNMB might be transported to the plasma membrane or secreted during macrophage activation. Indeed, upon combined treatment with IFN-γ and LPS, GPNMB was detected in culture supernatants (Fig. 7*A*), whereas LPS alone was sufficient to induce TNF-α secretion (Fig. 7*B*). The effect of IFN-γ and LPS on GPNMB secretion was selective; the same stimuli did not induce release of another V5-tagged protein, although it was clearly detected in cell lysates (Fig. 7*C*). The epitope tag on GPNMB is on the C terminus of this membrane-anchored protein, so the secreted GPNMB detected by the Ab must be membrane bound and is most likely associated with exosomes (34).

GPNMB *overexpression in RAW264.7 reduced the production of inflammatory mediators*

Because IFN-γ plus LPS induced GPNMB translocation and secretion, the effect of GPNMB overexpression on cytokine production in response to both stimuli was examined. IFN-γ/LPS-induced production of IL-6 and IL-12p40, and their respective transcripts, was reduced at least 2-fold in RAW264.7/*gpnmb* cells as compared with cells expressing empty vector (Fig. 8, *A* and *B*). Similarly, *gpnmb* overexpression also resulted in a 2-fold reduction of NO release (data not shown). These differences were not due to differences in proliferation or LPS-mediated growth inhibition because cell viability between the two populations was not significantly different over a 24-h LPS time course (Fig. 8*B*). These results suggest that GPNMB acts as a feedback regulator of macrophage activation.

The Gpnmb *mutant mouse displays subtle changes in myeloid differentiation*

A natural mutation of *gpnmb* in the DBA mouse genetic background was originally identified because of a glaucoma phenotype. Subsequent evidence indicated that there is a bone marrow-dependent contribution to eye-associated inflammation (35, 36). We confirmed that DBA/2J mice have a point mutation resulting in a premature truncated GPNMB protein (data not shown).

Gpnmb mRNA levels were regulated by CSF-1 during the process of macrophage differentiation from marrow precursors (Fig. 2*A*). To determine whether GPNMB contributes to macrophage maturation or differentiation, the number and morphology of BMM colonies from *gpnmb* mutant and wild-type mice generated by colony-forming cell assays were compared. No differences were observed in either macrophage numbers or morphology between *gpnmb* mutant and wild-type mice (data not shown). To

investigate the myeloid compartment in vivo, bone marrow, peripheral blood, and spleen macrophages were immunostained for the F4/80, CD11b, and Ly-6G myeloid markers and analyzed using flow cytometry. In bone marrow and spleen, only minor differences in the proportion of CD11b- and Ly-6G-positive cells were apparent between *gpnmb* mutant and wild-type mice. In blood, *gpnmb* mutant mice had ~30% fewer CD11b-positive and Ly-6G-positive cells than the wild type (data not shown).

TEPM inflammatory responses were increased in gpnmb *mutant mice*

The detailed analysis of *gpnmb* was initiated because of the primary observation that the mRNA level was 20-fold higher in TEPM compared with BMM. We therefore examined the effect of the mutation on the recruitment and function of these cells. Immunostaining using F4/80 and CD11b demonstrated that *gpnmb* mutant mice had ~20% more F4/80- and CD11b-positive cells in the thioglycolate-elicited inflammatory exudate than the wild-type mice (Fig. 9*A*). Consistent with a role for GPNMB as a negative regulator of macrophage activation, production of TNF-α, IL-6, and IL-12p40 in response to LPS was ~2-fold higher in the mutant TEPM, as assessed by intracellular cytokine staining (Fig. 9*B*) or ELISA (Fig. 9*C*).

Discussion

This study commenced with a structured comparison of gene expression in mouse macrophage populations vs other cell types. The simple logic of searching for this gene set is that genes expressed specifically in macrophages are likely to have a function in innate immunity and inflammation. This view was supported by our analysis of *Gpnmb* function in macrophages.

Gpnmb mRNA was expressed in a macrophage-enriched fashion (Fig. 1, *A–C*). Prior studies suggested that *gpnmb* expression in mice, rats, and humans was enriched in osteoblasts (37–39), the DC line XS52 (28), retinal pigment epithelial cells, and melanocytes (40, 41), and, more recently, in osteoclast-like cells (42). From some of theses studies, *gpnmb* was previously named osteoactivin and DC heparan sulfate proteoglycan dependent integrin ligand (*dc.hil*). However, we have found that conventional primary mouse osteoblast cultures are contaminated with macrophages and that this is responsible for the apparent osteoblast-enriched expression profile (data not shown). Our findings indicate that *gpnmb* expression is not restricted to DC. The macrophage-enriched expression was consistent with our informatic analysis of the promoter and putative transcription binding sites, which share many features with known macrophage-specific genes such as *csf-1r*. The shared expression in melanocytes, retinal pigment epithelial cells, and osteoclasts is most likely a consequence of the perfectly conserved binding site for microphthalmia transcription factor, which functions in all of these cell types (43). The specific function of this site is being investigated in our laboratory.

The perinuclear localization of GPNMB in macrophages revealed its possible connection with the Golgi apparatus. This hypothesis was supported by several observations, as follows: first, the substantial colocalization of GPNMB with β-COP, a protein that associates with membranes of the Golgi complex (29); second, GPNMB staining was dispersed into the ER and endosomal compartments when the Golgi network was disrupted by BFA treatment. GPNMB localization to the Golgi apparatus is also consistent with evidence that GPNMB is a highly glycosylated protein (28, 37) and that it contains a polycystic kidney diseases domain that is shared with the polycystin family of vesicle-trafficking mediators (44). Differences in the distribution of GPNMB- and *trans*-Golgi-derived vesicles containing the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor proteins Stx6 and Vti1b (data not shown) suggested that GPNMB may be predominantly associated with *cis*-Golgi membranes. Indeed, β-COP is thought to play a role in trafficking from the intermediate compartment of the ER to the Golgi (29).

Because the Golgi apparatus is the site of protein sorting within the cell, it is most likely that GPNMB accumulates in the Golgi and is transported to the plasma membrane or is secreted upon specific signals. Indeed, GPNMB translocated to small vesicular compartments scattered toward the cell periphery upon activation with IFN-γ plus LPS (Fig. 6, *D* and *E*). This change in GPNMB localization at late time points is consistent with the up-regulation of *gpnmb* mRNA by LPS at 21 h (Fig. 3), and suggests that the epitope tag does not cause inappropriate cellular location or sorting. The delayed regulation of GPNMB localization suggests the involvement of other IFN-γ/LPS-induced molecules such as NO (45) or type I IFNs (46) in this process.

GPNMB, epitope tagged at the C terminus, was also secreted into the medium of activated macrophages. Because it is a type I transmembrane protein, secreted GPNMB presumably exists in a membrane-associated form. The secretion of such membrane vesicles (exosomes) by various cell types, including activated macrophages, has been reported previously, and they are known to contain many plasma membrane proteins (34, 47). There is no direct evidence that GPNMB is specifically associated with, or involved in the formation of, such vesicles. These studies do not eliminate the possibility that some GPNMB is released from the cells or medium as soluble protein following proteolytic cleavage, and secretion of the putative soluble form of the protein encoded by the truncated mRNA confirmed in this study awaits verification.

The only prior insight into the function of GPNMB came from studies of its extracellular domain expressed as an Fc fusion protein (28). However, such functional studies can be complicated because Fc fusion proteins are likely to exist as dimers, which in many cases does not represent the true biological setting. A function in cellular adhesion to endothelium was demonstrated in this original study (28). We did not assess this directly; however, we found no supportive evidence for this phenotype in our study of the mutant mice, which actually showed greater macrophage recruitment in response to thioglycolate (Fig. 9). Our data on GPNMB overexpression in macrophages (Fig. 8), as well as phenotypic analysis of DBA/2J mice that express a truncated form of GPNMB (Fig. 9), indicate that *gpnmb* falls into the class of genes referred to elsewhere as inflammation suppressor genes, inducible feedback regulators that act at every level of the inflammatory cascade (48).

At least two potential mechanisms may be involved in the anti-inflammatory actions of GPNMB on macrophages. First, LPS enhances the trafficking and release of inflammatory cytokines in macrophages by regulating SNARE protein expression and function (49). The association of GPNMB with macrophage secretory pathways could provide one explanation for its effect on cytokine secretion. Although it is unlikely that GPNMB directly controls secretion, because the levels of intracellular and extracellular cytokines were similarly affected (Fig. 9), GPNMB might regulate the cellular distribution of receptors such as TLR4. Another possibility is that GPNMB is an anti-inflammatory secreted product analogous to factors such as IL-10, TGF-β, or ST2 (50–52), which act in autocrine or paracrine fashion to suppress the production of proinflammatory mediators in macrophages. The extracellular region of GPNMB has two domains that could potentially signal via a cell surface receptor. First, it contains a polycystic kidney disease 1 domain, which is thought to mediate protein-protein interactions.

The extracellular region also contains an inhibin-βB domain. Inhibins are members of the TGF-β family, which have well-characterized roles in suppressing macrophage activation. We are now generating a recombinant form of the GPNMB extracellular domain to determine whether it regulates TGF-β signaling pathways in macrophages.

The proinflammatory macrophage phenotype of *gpnmb* mutant mice described in this study also suggests a possible role for macrophages in the pathogenesis of pigmentary glaucoma apparent in this strain (53). Macrophages are normal resident cells in the eye, particularly in the retina and the choroid region (54, 55). This raises the possibility that the *gpnmb* may be involved in ocular immune abnormalities that promote iris diseases. This hypothesis is supported by the finding that transferring bone marrow expressing wild-type *gpnmb* into *gpnmb* mutant mice largely improved the pigment dispersion (35, 56). Additionally, histological studies from human pigmentary glaucoma patients revealed the presence of a large number of pigment-containing macrophages (57). Finally, a recent report by Zhou et al. (58) demonstrated that *gpnmb* mutant mice produced increased levels of IL-18 in the iris/ciliary, via a mechanism involving elevated activated NF-κB and phosphorylated MAPKs. IL-18 is an IL-1 family member secreted from activated macrophages that synergizes with IL-12 in the promotion of Th1 responses (59). It is likely that resident macrophages of the retina and iris of *gpnmb* mutant mice produce increased IL-18, mediating the immune dysfunction and chronic inflammation in these mice. IL-18 promotes the secretion of TNF-α and IL-6 and up-regulates inducible NO synthase (60). Hence, it is possible that GPNMB acts to limit IL-18 production and consequently other proinflammatory molecules, including TNF-α, IL-6, and NO.

Genes that act as suppressors of inflammation are commonly polymorphic, and such variation controls the extent and nature of inflammatory responses (48). Based upon our findings, variation in *gpnmb* function could regulate other inflammatory disease susceptibilities. The published mouse cDNA sequences of the gene differ at the amino acid level, and several different nonsynonymous single nucleotide polymorphism in the protein-coding region are recorded in both mouse and human data sets (National Center for Biotechnology Information snp database). Susceptibilities to osteoporosis (61), multiple sclerosis (62), and asthma (63) have each been mapped by genome-wide linkage analysis to the vicinity of 7p15, the location of the human *gpnmb* gene. In conclusion, data described in this study suggest that GPNMB is a feedback regulator of inflammation. Future studies will assess whether the expression or function of this gene is dysregulated in inflammatory disease processes.

Disclosures

The authors have no financial conflict of interest.

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