G protein pathway suppressor 2 (GPS2) links inflammation and cholesterol efflux by controlling lipopolysaccharide-induced ATP-binding cassette transporter A1 expression in macrophages

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ABSTRACT: Macrophages play important roles in linking alterations of cholesterol metabolism and inflammation to the development of atherosclerosis. Previous studies have identified several positive and negative crosstalk mechanisms that connect cholesterol efflux and inflammation at the transcriptional level. Of particular relevance is that the expression of ATP-binding cassette transporter A1 (Abca1), a main regulator of cholesterol efflux, can be induced by oxysterol receptor LXR agonists but also by bacterial endotoxins, such as LPS, that activate TLR4 signaling. However, the extent to which these pathways influence each other has remained incompletely understood. We investigated the possible role of the transcriptional coregulator G protein pathway suppressor 2 (GPS2) in LPS-induced Abca1 expression and cholesterol efflux in mouse and human macrophages. To activate Abca1, GPS2 cooperates with the LPS-inducible NF-κB subunit p65, but not with LXRs nor with corepressor complex subunits that otherwise cooperate with GPS2 to repress proinflammatory gene expression. Overall, our work identifies a regulatory chromatin component of crosstalk mechanisms between cholesterol efflux and inflammation that specifically affects ABCA1. Because GPS2 expression is down-regulated in some humans with obese and type 2 diabetes, the macrophage GPS-2/ABC-A1 pathway could be altered and contribute to atherogenesis.—Huang, Z., Liang, N., Damdimopoulos, A., Fan, R., Treuter, E. G protein pathway suppressor 2 (GPS2) links inflammation and cholesterol efflux by controlling lipopolysaccharide-induced ATP-binding cassette transporter A1 expression in macrophages. FASEB J. 33, 1631–1643 (2019). www.fasebj.org

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Macrophage cholesterol overload is a hallmark of the atherosclerosis process (1). Accumulation of cholesterol triggers signaling pathways that promote cholesterol efflux, a process referred to as reverse cholesterol transport. Failure of cholesterol removal from macrophages induces foam cell conversion, which promotes the formation of atherogenic lesions by boosting local inflammatory cytokine secretion and by recruiting immune cells (2, 3). Macrophage cholesterol efflux is mediated mainly by the cholesterol transporters ATP-binding cassette transporter (ABC)-A1 and ABCG1 (4). The expression of these transporters is tightly regulated by transcription factors (TFs), such as liver X receptors (LXRs) and peroxisome proliferator activated receptor γ (PPARγ) (5–8); by coregulators, such as nuclear receptor corepressor (NCOR1) and nuclear receptor coactivator 5 (NCOA5) (9, 10); and by long, noncoding RNAs, such as macrophage-expressed LXR-induced sequeletor, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. doi: 10.1096/fj.201801123R
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changes in the membrane lipid composition trigger inflammatory signaling (15).

There is equally strong evidence that inflammation affects cholesterol efflux. Clinical reports have revealed a possible association of viral infections with atherosclerosis in humans, which was subsequently confirmed in mice (16). It has been proposed that infection modulates atherosclerosis progression by inducing substantial pathologic changes, such as oxidative stress and ER stress, in local endothelial cells, in smooth muscle cells and in immune (mainly T) cells (17, 18). In addition, genetic variants of TLR4, the major receptor of G protein pathway suppressor 2 (GPS2) is an epigenome modulator involved in inflammatory gene regulation that acts in many contexts as a core subunit of a fundamental chromatin-modifying corepressor complex containing histone deacetylase 3 (HDAC3), NCOR, and silencing mediator of retinoid and thyroid receptors (SMRT, also NCO2) (25). GPS2 interplays with TFs, such as c-Jun (26), and several of retinoid and thyroid receptors (SMRT, also NCOR2) (25).

To atherogenesis has remained controversial. Earlier in vitro studies in mouse and human macrophages have demonstrated that LPS and TNF-α induce Abca1 expression by macrophages and promote cholesterol efflux that is apparently independent of LXRs (20, 21). However, other studies have reported that crosstalk of TLR4 and LXR signaling decreases Abca1 expression and cholesterol efflux in mouse macrophages (22–24). These in part contradictory findings urge the further dissection of mechanisms underlying the regulation of Abca1 expression by inflammatory signals.

G protein pathway suppressor 2 (GPS2) is an epigenome modulator involved in inflammatory gene regulation that acts in many contexts as a core subunit of a fundamental chromatin-modifying corepressor complex containing histone deacetylase 3 (HDAC3), NCOR, and silencing mediator of retinoid and thyroid receptors (SMRT, also NCOR2) (25). GPS2 interplays with TFs, such as c-Jun (26), and several lipid-sensing nuclear receptors (27–31). GPS2 expression is down-regulated, and its function in the complex is altered in adipocytes and macrophages of obese humans and mice and in humans with type 2 diabetes (26, 32). Macrophage-specific Gps2 knockout (MKO) mice, compared with wild-type (WT) mice, display a sensitized proinflammatory response to high-fat diet, accompanied by increased insulin resistance, suggesting that down-regulation of GPS2 contributes to the development of type 2 diabetes (26).

In this study, we discovered the requirement of GPS2 for the LPS/TLR-4 signaling-induced Abca1 expression in mouse and human macrophages. Our work identified a previously unknown regulatory role of GPS2 in mediating transcriptional crosstalk between inflammation and cholesterol efflux, which could help better our understanding of the macrophage-mediated pathways linking obesity, type 2 diabetes, and atherosclerosis.

MATERIALS AND METHODS

Cell cultures

We used the mouse macrophage RAW264.7 [TIB-71; American Type Culture Collection (ATCC), Manassas, VA, USA] cell line, hereafter referred to as RAW cells, as a relevant model to monitor Gps2-dependent transcriptional pathways for in vitro studies. The Gps2 KO RAW cell line was generated by using clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein 9 (Cas9) editing, as described in one of our studies (26). The human THP-1 monocyte cell line (TIB-202; ATCC) was maintained in RPMI 1640 medium with 10% fetal bovine serum and 1% penicillin-streptomycin and treated with phorbol 12-myristate 13-acetate (100 ng/ml) for differentiation. Cell lines were maintained in humidified incubators at 37°C with 5% CO₂, seeded into 24-well plates (2 × 10^5 cell/well), and treated with 10 ng/ml LPS (L4516), 2 µM LXR agonist GW3965, or 10 ng/ml IL-4 (I4269; MilliporeSigma, Burlington, MA, USA) as indicated.

Mouse models and primary macrophages

Gps2 MKO mice were characterized and genotyped as described in our previous study (26). Animal experiments were approved by the national ethics board (ID-907; Swedish Board of Agriculture, Jönköping, Sweden). Mouse primary bone marrow–derived macrophages (BMDMs) were isolated from femur or tibia. Cells were differentiated in L929-conditioned medium for 7 d. Mouse primary thioglycollate-elicited peritoneal macrophages (TEPMs) were isolated from the abdominal cavity 4 d after injecting 3 ml 3% thioglycollate (70157; MilliporeSigma). TEMPs were collected and cultured with 10% FBS and 1% penicillin-streptomycin. Both BMDMs and TEMPs were maintained in humidified incubators at 37°C with 5% CO₂, seeded into 24-well plates (2 × 10^5 cell/well), and treated with 10 ng/ml LPS, 2 µM LXR agonist GW3965, or 10 ng/ml IL-4.

Plasmids and shRNA transfection

Lentivirus shRNA plasmids were transfected into RAW macrophages, and stable cell lines were selected with 5 µg/ml puromycin for 7 d. For the lentivirus-mediated shRNA knockdown experiments, GFP shRNA lentivirus (targeting sequence: 5′-GCAACGTGACCCTGGTTCA3′), Gps2 shRNA lentivirus (targeting sequence: 5′-GAgAGATTGTTGACTT-3′), Smrt shRNA lentivirus (targeting sequence: 5′-CCAGTGTAAGACTCTACTT3′), and Hdac3 shRNA lentivirus (targeting sequence: 5′-CGTGGCTCTTGAAACCTTAAA3′) were constructed and packaged in 293A cells, Nor2 shRNA lentivirus (targeting sequence: 5′-CCGCGATTCTTGGAAACCTTAAA3′), Lxra shRNA lentivirus (targeting sequence: 5′-CGTGGACTGATGCTTACACTTT3′), and Lxrb shRNA lentivirus (targeting sequence: 5′-CGTGCAGACACAAAGGATCTTT3′) were constructed and packaged in 293FT cells by using PLKO.1-TRC (10878), psPAX2 (12260), and pMD2.G (12259) (all from Addgene, Cambridge, MA, USA). RAW macrophages were transduced in 6-well plates with lentivirus in serum-free DMEM at a multiplicity of infection of 5 or 6 h and then in full culture medium for 24 h before puromycin selection. Stable shRNA-expressing RAW cells were selected with 5 µg/ml puromycin for 7 d.

Adenovirus shRNAs were constructed and packaged by using the Block-it Adenoviral RNAi expression system according to the manufacturer’s instructions (K49410; Thermo Fisher Scientific, Wal thaw, MA, USA). In brief, shRNA oligos were synthesized and cloned into the entry vector and recombined in vitro. The recombinant adenoviral vector was linearized and packaged in 293A cells, and recombinant adenovirus particles were collected by a repeated freeze–thaw procedure. Adenovirus was used to infect THP-1 macrophages for 48 h at a multiplicity of infection of 10, followed by treatments with LPS or GW3965, as indicated. The shRNA targeting sequences were as follows: sh-Luciferase (forward) 5′-CACCGCG-TACCCGGAATCTTGGACGAGATCCGCGTACG-3′ and sh-Gps2 (forward) 5′-CACCGAGAGACCAAAAGGATCTTT3′.

RNA isolation and sequencing

Total RNA was extracted with the E.Z.N.A. Total RNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer’s
instructions. One microgram total RNA was reverse transcribed into complementary DNA with the Superscript II reverse transcriptase kit (Thermo Fisher Scientific). GAPDH was used for normalization to quantify relative mRNA expression levels (expressed in arbitrary units). Relative changes in mRNA expression were calculated with the comparative cycle method (2^(-ΔΔCt)). The mouse quantitative (q)PCR primers were as follows: *Abca1* forward (F): 5’-CCAGACGAAAAAGGGCCTC-3’, *Abca1* reverse (R): 5’-CTGCTTCACGTCATTGTGGAC-3’; *Abcg1* F: 5’-GAATATGGTCCGGATTGC-3; Abcg1 R: 5’-TGTCCAGTCCAGTAATGGTTCTGT-3’; *Arg1* reverse (R): 5’-AGTTC-3; Ggcct T: 5’-GCCCTTAGTCCTAGGTGTGG-3’. 

**Results**

After cell lysis, the nuclei were sonicated during 30 cycles in the UCDO Biopurifier (Diagenode; Liege, Belgium), to generate DNA fragment sizes of 0.2–0.5 kb. ChiP primers were as follows: *Abca1* forward (F): 5’-TGCGCGCCATAGTTCCCTTTT-3’, *Abca1* R: 5’-ACCTGCTTCTGCTGTAGTGA-3’ (Promoter primer); *Abca1 2F*: 5’-GGCTCTGATGAACAAAGGGCGGAC-3’, *Abca1 2R*: 5’-TTAGGCTACCGGTCCAAGC-3’ (Enhancer primer); *Abca1c F*: 5’-CCGCTACCCACATCTGCGGATC-3’, *Abca1c R*: 5’-TATCTGCTGACCCGACATTCTCT-3’ (control primer). ChiP was performed in RAW cells, for the histone modification H3K27ac (ab4729; Abcam). ChiP-seq samples were purified with the ChiP DNA Clean & Concentrator Capped Zymo-Spin I kit (Zymo Research, Irvine, CA, USA). For library preparation and sequencing, 5–10 ng of DNA was processed at the European Molecular Biology Laboratory Genomics Core Facility (Heidelberg, Germany), according to standard protocols, and 50 SE reads were obtained with the HiSeq 2000 (Illumina).

**ChiP-seq data analysis**

BMDM ChiP-seq data were obtained from public Gene Expression Omnibus (National Institutes of Health, Bethesda, MD, USA) datasets for *NCOR* (GSM69529) (33), *SMRT* (GSM69525) (33), *HDAC3* (GSM83471) (34), *GPS2* (GSM163866) (26), H3K27ac (GSM163858) (26), LXR (GSM2095084) (35), p65 (GSM2095112, GSM2095114) (35), and signal transducer and activator of transcription 6 (STAT6) (GSM1023200, GSM1023205) (36). RAW cell ChiP-seq data were obtained from the public GEO dataset for *GPS2* (GSM1631869) (26) and generated in this study for H3K27ac. The ChiP-seq binding profiles at the *Abca1* gene locus were visualized with the University of California Santa Cruz (UCSC) gene browser and Integrative Genomics Viewer (IGV) 3.0 software (Broad Institute, Massachusetts Institute of Technology, Cambridge, MA, USA).

**Cell-based cholesterol efflux assay**

RAW cells were treated with 100 ng/ml LPS at various time points, and cells and medium were collected to test cholesterol levels according to the manufacturer’s instructions (ab196985; Abcam). In brief, GPS2 CRISPR KO cells were seeded in 96-well plates (1 x 10^5 cells per well) and maintained at 37°C for 2 h. The cells were washed with cold PBS and incubated with labeling reagent overnight. Then they were washed with cold PBS and incubated in DMEM containing ± 2 μM GW3965 or ±100 ng/ml LPS in the presence of human serum for additional 1, 3, and 6 h. After incubation, fluorescence (Ex/Em = 482/515) was measured (Ex/Em = 482/515) was measured in medium (cell supernatant) and cell lysates, respectively. Cholesterol efflux was calculated using the formula C = 100× (fluorescence intensity of the medium)/(fluorescence intensity of the medium and cell lysate).

**Statistics**

All statistical tests were performed with Prism 6.0b (GraphPad Software, San Diego, CA, USA), and all data are means ± SEM. Student’s t tests were applied for data comparisons. All experiments were repeated at least 3 times.

**Results**

GPS2 is essential for LPS-induced *Abca1* expression and cholesterol efflux in mouse macrophages

To discover GPS2-regulated genes involved in lipid metabolism in proinflammatory macrophages we treated RAW cells with 10 ng/ml LPS for 6 h. One microgram total RNA was extracted from the cells, and the polyA RNA sequencing method was used for library preparation. RNA library preparation and sequencing were performed at the BEA Core Facility (Karolinska Institutet), according to standard protocols, with a HiSeq 2000 (Illumina, San Diego, CA, USA).

**Chromatin immunoprecipitation**

The chromatin immunoprecipitation (ChiP) experiments were performed as described using GPS2 and p65 antibody (26). In brief, RAW cells were treated with 100 ng/ml LPS for 4 h and cross-linked with 2 mM disuccinimidyl glutarate (DSP) for 30 min, followed by 1% formaldehyde for 10 min. The reaction was stopped with glycerine at a final concentration of 0.125 M for 5 min.
GPS2 KO RAW cells, generated using CRISPR-Cas9 editing, and WT cells with LPS and determined genome-wide gene expression changes using RNA-seq. Although GPS2 depletion caused enhanced expression of proinflammatory genes including chemokine C-C-motif ligand 2 (Ccl2) and Ccl7, consistent with our previous data from Gps2 KO mice (26), the LPS-induced expression of Abca1 was reduced in Gps2 KO cells (Fig. 1A). We confirmed the specific requirement of GPS2 for LPS-induced Abca1 expression by real-time qRT-PCR in RAW cells, as well as in BMDMs and TEPMs, from Gps2 KO mice (Fig. 1B). These experiments also revealed that, in contrast to Abca1, the expression of Abcg1 was not induced by LPS and was not affected by GPS2 depletion. We then examined ABCA1 protein levels in WT vs. GPS2–depleted RAW cells and found that the LPS induction of ABCA1 protein was abolished in the absence of GPS2 (Fig. 1C and Supplemental Fig. S1). Finally, to prove the physiologic significance of Abca1 regulation by GPS2, we performed cholesterol efflux assays in RAW cells (Fig. 1D). In GPS2–expressing WT cells, LPS induced cholesterol efflux, whereas this induction was almost abolished in GPS2–deficient KO cells. Overall, the data suggest that GPS2 is necessary for LPS-induced Abca1 expression and cholesterol efflux in mouse macrophages.

**Function of GPS2–associated corepressor complex subunits in Abca1 regulation**

Our previous work had demonstrated that GPS2 cooperates with other subunits of the HDAC3 corepressor complex to repress transcription of inflammatory genes (25), but whether the complex is involved in GPS2–dependent
activation of Abca1 remained to be clarified. We therefore used published ChIP-seq data to analyze the corepressor complex cistrome (i.e., genome-wide chromatin-binding patterns of individual subunits) at the Abca1 gene locus in BMDMs (26). We also performed ChIP-seq for GPS2 and the histone modification H3K27ac (a marker for active enhancers and promoters) in RAW cells. The GPS2 and H3K27ac profiles at the Abca1 locus were nearly identical between BMDMs and RAW cells (Fig. 2A). The analysis revealed that the core subunits GPS2 NCOR, SMRT, and HDAC3 co-occupy both the promoter (<2 kb from the transcription start site) and several potential distal enhancers up-stream and down-stream of the transcription start site (i.e., active chromatin regions having high levels of H3K27ac).

Next, to test whether co-occupancy results in functional co-operation of the subunits in Abca1 transcription, we individually depleted them in RAW cells using lentivirus-shRNAs (Supplemental Fig. S2A–D) and analyzed the LPS induction of Abca1 (Fig. 2B). The following results were obtained: 1) shRNA-knockdown of GPS2 or HDAC3 reduced LPS-induced Abca1 expression, thereby confirming the results obtained with Gps2 KO macrophages (see Fig. 1) and identifying an additional role of HDAC3; 2) in contrast, shRNA-knockdown of NCOR enhanced Abca1 expression both upon LPS treatment and in basal conditions, consistent with previous data from BMDMs of Ncor1 MKO mice (9); 3) shRNA-knockdown of SMRT had no effect on LPS-mediated Abca1 induction, revealing a clear difference from the documented functional cooperation of SMRT with HDAC3 in repressing LPS-induced proinflammatory genes such as Ccl2 (26).

To further investigate the relationship of GPS2 to NCOR and SMRT, which are the direct binding partners of GPS2 within the complex (37), we studied the effect of lentivirus-shRNA knockdown of NCOR or SMRT in CRISPR-generated Gps2 KO RAW cells (Fig. 2C). The data indicate that neither knockdown of NCOR or SMRT further reduced Abca1 expression in GPS2–deficient cells. Also, the LPS-mediated down-regulation of Abcg1 was not reversed by single or double depletion of any of the subunits, suggesting even this regulation to be independent of the corepressor complex. The RAW cell data were confirmed with ex vivo knockdown of NCOR or SMRT in TEPMs from WT vs. Gps2 MKO mice (data not shown). We concluded that, although these corepressor complex subunits co-occupy promoters and enhancers of Abca1, GPS2 does not functionally cooperate with them to activate Abca1 transcription in response to LPS.

**The NF-κB subunit p65 is essential for GPS2 activation of ABCA1**

As a coregulator, the likely function of GPS2 is in modulating the activity of TFs involved in the regulation of Abca1 expression in macrophages. A study in human THP-1 cells suggested that TNF-α induces Abca1 expression through NF-κB, independent of LXRα (21). Although both TNF-α and LPS/TLR4 signaling activate NF-κB, the 2 pathways are not identical concerning Abca1 induction and LXR crosstalk in mouse macrophages (8) and thus required further investigation in our model.

We analyzed macrophage ChIP-seq data for GPS2 (26) and p65 (35) at the Abca1 locus (Fig. 3A). The analysis revealed that GPS2 colocalizes with p65 at the promoter and enhancers making a functional cooperation possible at these sites. This cooperation was first confirmed using p65 ChIP experiments in WT vs. Gps2 KO RAW cells (Fig. 3B) revealing that GPS2 depletion reduced the LPS-induced binding of p65, but not protein levels (Supplemental Fig. S2E), both at the Abca1 promoter (primers Abca1 1) and enhancer (primers Abca1 2), but not at a negative control region (primers Abca1 c).

The functional cooperation of GPS2 with p65 for the LPS-induction of Abca1 was further confirmed by lentivirus-shRNA–mediated knockdown of p65 (Supplemental Fig. S2F) combined with chromatin recruitment analysis by ChIP (Fig. 3C) and gene expression analysis by real-time qRT-PCR (Fig. 3D). Specifically, p65 depletion decreased GPS2 recruitment to the Abca1 promoter and enhancer (Fig. 3C), and the Abca1 induction was lost upon p65 depletion in LPS–expressing and –depleted cells (Fig. 3D). No significant effects of single p65 and double p65 and GPS2 knockdowns were observed on the LPS-reduced Abcg1 expression, suggesting no involvement of these factors. In summary, these data indicate that GPS2 cooperates with the p65 subunit at Abca1 promoter and enhancers to induce gene transcription in response to LPS/TLR4 signaling.

**LXR activation of Abca1 does not require GPS2, but LXR transrepression does**

Our demonstration that p65 is a likely target for GPS2 does not exclude the possibility that the LPS-induction of Abca1 requires additional TFs that interplay with GPS2. We thus specifically addressed the role of LXRs, which are the main regulators of Abca1 expression in response to LXR ligands (7, 8) and which cooperate with GPS2 at other genes and in other cellular contexts (29, 30). Analysis of recent ChIP-seq data for endogenous LXRs (35) revealed that LXR is present at the GPS2/p65-containing promoter and enhancers (Fig. 3A). To see whether LXR-dependent activation of Abca1 was modulated by GPS2 in our RAW cell model, we knocked down Gps2 by lentivirus shRNA (70% mRNA reduction) and then treated the cells with the synthetic LXR agonist GW3965 (Fig. 4A). Although agonist treatment induced Abca1 and Abcg1 expression, confirming that they were LXR functional, this induction was not affected by GPS2 removal. These data suggest that the classic Abca1 activation by LXRs does not involve GPS2.

To confirm these results in a different macrophage population, we treated BMDMs derived from WT and Gps2 MKO with GW3965 (Fig. 4B). The data clearly verified the GPS2 independency of Abca1 induction by LXR agonist and suggest that possible differences concerning LXR subtype expression levels in RAW cells vs. BMDMs are not critical. In line with this finding, selective knockdown of either LXRα or -β (Supplemental Fig. S3A, B) had no effect on the induction of Abca1 by LPS (Supplemental Fig. S3C, D).
Although our analysis revealed a differential requirement of GPS2 for LPS- vs. LXR-agonist-induced Abca1 expression, the two pathways may influence each other in a proinflammatory environment linked to infections or atherogenesis. To address this question in our model, we treated RAW cells, either alone or together with LPS and GW3965 for up to 24 h (Fig. 4C). The data revealed that GW3965 and LPS act synergistically to induce Abca1,

Figure 2. GPS2 regulates LPS-induced Abca1 expression in mouse macrophages independent of the NCOR/SMRT/HDAC3 corepressor complex. A) UCSC genome browser view of ChIP-seq tracks for NCOR (GSM665926), SMRT (GSM665925), HDAC3 (GSM830471), GPS2 (GSM1631866), and H3K27ac (GSM1631858), in BMDMs, compared to ChIP-seq tracks generated in this study for GPS2 (GSM1631869) and H3K27ac in RAW cells, at the Abca1 locus. B) Real-time qRT-PCR analysis of LPS-induced gene expression upon lentivirus-mediated shRNA knockdown of the indicated corepressor complex subunits in WT RAW cells (n = 3). Top: Abca1 expression in the indicated subunit-depleted cells. Bottom: knockdown efficiency for each subunit. C) Real-time qRT-PCR analysis of LPS-modulated Abca1 and Abcg1 expression upon lentivirus-mediated shRNA knockdown of NCOR or SMRT in WT vs. Gps2 KO RAW cells (n = 3). All data are represented as means ± SEM. Comparisons between WT and KO groups were made by Student’s t test and between 6 groups were made by 1-way ANOVA followed by Tukey’s test. *P < 0.05, **P < 0.01, ***P < 0.001.
consistent with LXR and p65 to cooperate at Abca1 promoters and enhancers. Depletion of GPS2 reduced this synergistic Abca1 activation (Supplemental Fig. S4A, left 2 panels). In contrast, SMRT depletion had no effect (Supplemental Fig. S4B, left 2 panels), and NCOR depletion further enhanced the synergistic Abca1 activation (Supplemental Fig. S4C, left 2 panels). Our analysis further revealed kinetic differences in Abca1 induction by GW3965 vs. LPS, the latter peaking at ~6 h (Fig. 4C), suggesting feedback regulation of LPS/TLR4 activation. Synergism
was specific to Abca1 and not observed with Abcg1 (only induced by GW3965) or Ccl7 (only induced by LPS) (Fig. 4C). LPS/GW3965 synergism was confirmed at the ABC-A1 protein level (Fig. 4D). We also performed cholesterol efflux assays and found that LXR agonist–induced efflux was unaffected by GPS2 depletion (Fig. 4E).

Classic LXR activation and LPS activation of Abca1 may influence each other via anti-inflammatory crosstalk mechanisms termed transrepression (25)—particularly in light of the demonstration that ABCA1 seems to be necessary for LXR transrepression in mouse macrophages (15). Given the role of GPS2 in LPS-induction of Abca1, we asked whether LXR transrepression was affected by GPS2. We choose the LPS-inducible Ccl7 gene, which can undergo significant transrepression by the LXR agonist GW3965 in RAW cells (Supplemental Fig. S4, right). LPS-induced Ccl7 expression was further elevated upon depletion of either GPS2 or SMRT (Supplemental Fig. S4A, B, right), whereas NCOR depletion had the opposite effect (Supplemental Fig. S4C, right). Depletion
of GPS2 abolished the ability of LXR agonist to inhibit LPS-dependent Ccl7 expression, whereas depletion of SMRT or NCOR had no effect (Supplemental Fig. S4, right).

To further investigate the role of GPS2 in modulating signal-dependent Abca1 expression in macrophages, we examined IL-4/STAT6 signaling linked to alternative M2 macrophage activation, as it was reported to inhibit Abca1 expression in mouse macrophages (38). ChIP-seq analysis from published BMDM datasets suggests colocalization of GPS2 with the major IL-4–induced TF STAT-6 at both the Abca1 promoter and enhancer (Supplemental Fig. S5A). In RAW cells, 12 h IL-4 treatment inhibited Abca1 expression in the RAW cells (Supplemental Fig. S5B, left), whereas the major IL-4 target gene Arg1 was strongly induced. In BMDMs, Abca1 repression was observed at 2 h of IL-4 treatment (Supplemental Fig. S5B, right). GPS2 depletion had no effect on the IL-4/STAT6–mediated Abca1 repression in these 2 macrophage models (Supplemental Fig. S5C).

We conclude from these experiments the following: first, LXRs are not involved in GPS2/LPS-dependent induction of Abca1; second, GPS2 is not involved in the modulation of Abca1 expression by LXRs or by IL-4/STAT6; and third, GPS2 appears to participate in anti-inflammatory LXR transrepression (at least at Ccl7).

**The GPS2/ABCA1 pathway is conserved in human THP-1 macrophages**

We finally investigated whether the GPS2/ABCA1 regulatory network is conserved between mouse and human macrophage models. An earlier study had reported that LPS induces Abca1 expression independent of LXR in human THP-1 macrophage cells (20), suggesting similarities to the regulation described for RAW cells. We first studied the LPS response profiles of Abca1, Abcg1, and Ccl7 in differentiated THP-1 cells (Fig. 5A), which was comparable to RAW cells (Fig. 4C), with Abca1 induction peaking at 6 h.

We then analyzed WT vs. GPS2-deleted THP-1 cells and observed that GPS2 depletion abolished the LPS-induction of Abca1 (Fig. 5B), whereas Abcg1 expression was not affected in WT vs. GPS2–deficient cells upon LPS treatment. We finally studied the role of GPS2 in LXR-dependent induction of Abca1 and Abcg1 (Fig. 5C). We found that Abca1 induction by GW3965 was not affected by GPS2 depletion in THP-1 cells, consistent with the mouse data (Fig. 4A, B). Further, consistent with our previous work (30), the Abcg1 induction by GW3965 was reduced by GPS2 depletion in THP-1 cells, revealing potential human–mouse differences regarding the regulation of Abcg1.

We concluded from the analysis of human THP-1 cells that the regulation of Abca1 is essentially similar in human vs. mouse macrophages (see our model in Fig. 6) (i.e., GPS2 is required for LPS-induced but dispensable for LXR-induced expression of both human Abca1 and mouse Abca1).

**DISCUSSION**

Excessive macrophage cholesterol accumulation generates foam cells and contributes to atherogenic plaque formation by triggering local inflammation (39). Cholesterol efflux represents a major mechanism of macrophage cholesterol removal and recycling. ABCA1 and ABCG1 are major macrophage cholesterol transporters involved in cholesterol efflux, both are tightly regulated through TFs and coregulators (1, 4, 18, 25).

In this study, we discover a novel mechanism of endotoxin-induced Abca1 expression and cholesterol efflux by the coregulator GPS2 which seems conserved in both mouse and human macrophages. We provide evidence that GPS2 specifically modulates the NF-κB pathway to activate Abca1, independent of LXR signaling and of other corepressor complex subunits. Our work identified thereby yet another regulatory role of GPS2 in macrophages that links inflammation and cholesterol efflux at the transcriptional level.

Given that obesity and hyperlipidemia are major risk factors for development of atherosclerosis (1), it is striking that the interplay between obesity-associated inflammation and macrophage cholesterol metabolism is still poorly understood. Many studies focus on macrophage inflammation induced by abnormal intracellular cholesterol accumulation, the major process to initiate atherosclerosis. It is yet less known whether and how inflammation itself interferes with ABCA1 regulation. This interplay is potentially important for several reasons: first, obesity as a major atherosclerosis risk factor is well accepted to be associated with increased immune cell–derived systemic inflammation (40); second, metabolic disorders such as type 2 diabetes, especially at late disease stages, are susceptible to bacterial and virus infections (41); and third, a dysregulated microbiome in metabolic disorder patients has been associated with altered circulating endotoxin levels (42), a potential contributor to altered macrophage function.

Both metabolic and inflammatory signals seem to trigger overlapping NF-κB activation mechanisms (40). Although NF-κB activation modulates multiple pathways involved in macrophage inflammation and survival, inhibition of NF-κB activation by depleting IKK2 in Ldlr KO mice did not reverse, but on the contrary accelerated atherosclerosis progression (43). Similarly, although macrophage–specific NF-κB inhibition decreased atherogenic pathologic factor–induced inflammation, it also reduced foam cell formation in Ldlr KO mice (44). These data collectively suggest alternative roles of NF-κB in regulating protective factors including Abca1, supporting the conclusions of our study.

The involvement of GPS2 in LPS-stimulated ABCA1 regulation appears rather NF-κB–specific and seems independent of LXR signaling but conserved in mouse and human macrophages. In contrast, our earlier work had identified an LXR-dependent role of GPS2 in regulating Abcg1 expression in human THP-1 cells (30). The data presented herein suggest that this Abcg1 pathway is not
conserved in mouse macrophages, pointing at a mechanistic difference in the coregulator-chromatin-mediated control of LXR-dependent cholesterol efflux. Concluding from all present results, a likely possibility is that the chromatin landscape, including promoter and enhancers, substantially differs between mouse and human macrophages at the ABCG1 locus, while being similar at the ABCA1 locus.

In addition to possible chromatin differences, the GPS2–interacting set of TFs that define gene-specific effects remain yet to be clarified. Notably, GPS2 was first identified as an inhibitor of TNF-α activation (27), consistent with subsequently demonstrated nongenomic GPS2 actions in the cytoplasm (45, 46). As we show here ChIP-seq data clearly demonstrate nuclear chromatin-binding of GPS2 and p65 (e.g., at the Abca1 locus). Moreover, Gps2 KO macrophages reduced NF-κB p65 recruitment to Abca1 promoter and enhancer upon LPS activation. Both findings clearly support a direct function of GPS2 as a p65 coregulator in the nucleus.

The interplay of GPS2 with p65 in activating Abca1 expression contrasts the interplay of GPS2 with AP-1/c-Jun in repressing the expression of inflammatory genes such as Ccl2, as we earlier reported (26). Because GPS2, p65 and c-Jun co-occupy the regulatory regions of most LPS-inducible genes including Abca1 and Ccl2, the difference may be due to other mechanisms. In addition to the above-mentioned chromatin differences a possible explanation could be that GPS2 works at some macrophage genes independent of the corepressor complex as a pioneering coactivator (as suggested in refs. 30 and 31), whereas cooperating with the SMRT corepressor complex subunit at most of the repressed genes, as shown in our previous work (26).

In this study that GPS2 depletion did not influence IL-4/STAT6–dependent repression of Abca1, further supporting the LPS/p65 selectivity of the GPS2/ABCA1 pathway. However, given the genomic colocalization of STAT6 and GPS2 at many IL-4–regulated gene loci, the potential interplay of these 2 factors remain to be explored (36, 38). Of interest is further that depletion of HDAC3 in RAW cells reduced Abca1 expression, the opposite of what would be expected if HDAC3 solely functions as a key component of the corepressor complex. This coactivating function is consistent with independent reports demonstrating that HDAC3 can positively modulate gene expression in different contexts by deacetylation of TFs such as p65 (47) or coactivators such as PGC-1 (48). With regard to the specific regulation of Abca1, it remains possible that GPS2 functionally cooperates with HDAC3 to activate p65.

Figure 5. GPS2 is involved in LPS-induced Abca1 expression in human THP-1 macrophages. A) Kinetic analysis of LPS-dependent gene expression of ABCA1, ABCG1, and CCL7 by real-time qRT-PCR (n = 3). B, C) Real-time qRT-PCR analysis of GPS2, ABCA1, and ABCG1 expression in WT (shLuc) vs. GPS2-depleted (shGPS2) THP-1 cells upon treatment with (B) LPS vs. vehicle and (C) GW3965 vs. vehicle (n = 3). All data are represented as means ± SEM. Comparisons between WT and KO groups were made with Student’s t test. **P < 0.01; ***P < 0.001.
Dissecting these complicated intrinsic connections between gene-specific chromatin structure, TF and coregulator composition remains a challenging task and goes beyond the resolution of today’s applied experimental approaches. [For further discussion, see our review (25).] Last, the expression of GPS2 appeared to be downregulated in adipose tissue macrophages and primary blood monocytes of some obese humans and those with type 2 diabetes (25, 26, 32). This provides a potential link of the herein identified macrophage GPS2–NF-κB–ABCA1 axis to the epidemiologically supported increased cardiovascular risk associated with obesity and diabetes (1). Given that ABCA1 reduction enhances macrophage inflammation (15), the down-regulation of GPS2 may induce a vicious cycle to pronounce metabolic inflammation partly by lowering ABCA1 (this study) and partly by directly inducing proinflammatory gene expression (26). Therefore, a deep understanding of these GPS2–dependent proinflammatory pathways may help to further decipher the macrophage-controlled pathologic processes that contribute to the progression of cardiovascular diseases, an ultimate requirement for the development of novel anti-inflammatory interventions.

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AUTHOR CONTRIBUTIONS

Z. Huang and R. Fan designed the experiments and analyzed the data; Z. Huang performed most of the experiments and wrote the manuscript with input from R. Fan; N. Liang and A. Damdimopoulos contributed to the analysis of sequencing data; and E. Treuter supervised the work with R. Fan, contributed to study design and analysis, and edited the manuscript.

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