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Glucocorticoid Conditioning of Myeloid Progenitors Enhances TLR4 Signaling via Negative Regulation of the Phosphatidylinositol 3-Kinase-Akt Pathway

Tian Y. Zhang and Raymond A. Daynes

The immunomodulatory effects of glucocorticoids (GCs) have been described as bimodal, with high levels of GCs exerting immunosuppressive effects and low doses of GCs being immunopermissive. While the mechanisms used by GCs to achieve immunosuppression have been investigated intensely, the molecular mechanisms underlying the permissive effects of GCs remain uncharacterized. Herein, we demonstrate that GC conditioning during the differentiation of myeloid progenitors into macrophages (Mφs) results in their enhanced LPS responsiveness, demonstrated by an overexpression of the inflammatory cytokines TNF-α, IL-6, and IL-12. Inflammatory cytokine overexpression resulted from an increased activation of NF-κB and the MAPK signaling cascade and a reduced activation of the PI3K-Akt pathway following LPS stimulation. GC conditioning during Mφ differentiation induced an increase in the expression of SHIP1, a phosphatase that negatively regulates the PI3K signaling pathway. Small interfering RNA-mediated knockdown of SHIP1 expression increased PI3K-dependent Akt activation and subsequently decreased inflammatory cytokine expression, suggesting GC-mediated up-regulation of SHIP1 expression is responsible for the augmentation in inflammatory cytokine production following LPS stimulation. We also show that splenic Mφs purified from normal mice that were implanted with timed-release GC pellets exhibited an enhanced LPS responsiveness and increased SHIP1 expression, indicating that GCs can regulate SHIP1 expression in vivo.

Our results suggest that minor fluctuations in physiological levels of endogenous GCs can program endotoxin-responsive hematopoietic cells during their differentiation by regulating their sensitivity to stimulation. The Journal of Immunology, 2007, 178: 2517–2526.

The Nobel Prize in Medicine was awarded to Kendall, Reichstein, and Hench in 1950 for their seminal work in elucidating the chemical structure of glucocorticoids (GCs) and their use as effective therapy to reduce the pathological consequences of rheumatoid arthritis (www.noble.org). Thirty years earlier, however, the noted physiologist Hans Selye put forth the theory that adrenal GCs can enhance and mediate the cellular stress response (1–5). These physiologic influences of GCs, termed their permissive effects, were largely forgotten subsequent to the high profile finding that high doses of GCs are anti-inflammatory. While the suppressive influences of GCs on the synthesis and effector functions of numerous proinflammatory mediators are quite well characterized (6–8), the permissive effects of GCs remain mechanistically unresolved.

Reports exist in the literature that describe permissive roles played by GCs in enhancing innate immune responsiveness. For example, when cortisol is administered i.v. to normal volunteers followed by an endotoxin challenge, their blood levels of TNF-α, IL-6, and IL-12 become significantly elevated compared with an endotoxin-only control population (9). These studies were later recapitulated in a rat model of endotoxemia, and a similar conclusion was reached (10).

LPS responsiveness is conferred by the TLR4/CD14 complex predominantly expressed on the cell surface of macrophages (Mφs), dendritic cells, and B cells (11, 12). Activation of TLR4/CD14 leads to the recruitment of Myd88, an adaptor molecule which subsequently facilitates the activation of downstream signaling pathways, including the NF-κB and MAPK pathways, both of which are essential for the expression of activation-induced inflammatory mediators, including chemokines and cytokines (13).

Controlling the magnitude of the inflammatory response is essential for minimizing bystander tissue damage incurred upon the host. The PI3K-Akt pathway has recently emerged as an important component in the negative regulation of TLR4 signaling. Additionally, inducible molecules such as suppressor of cytokine signaling-1 (SOCS-1) and IL-1R-associated kinase myeloid specific (IRAK-M) have also been demonstrated to be important in dampening the cytokine response to inflammatory insults and in establishing the condition of endotoxin tolerance (14, 15).

The studies presented herein have examined the molecular mechanisms responsible for the augmenting effects of GCs on endotoxin responsiveness of primary macrophages (Mφs). We
found that low-dose GC conditioning during the differentiation of murine bone marrow-derived M\(\delta\)s (BMM\(\delta\))s leads to a hyperresponsiveness to endotoxin stimulation compared with non-GC-conditioned BMM\(\delta\)s. We demonstrate that the capacity of GC-conditioned BMM\(\delta\)s to overexpress inflammatory cytokines results from an enhanced activation of NF-\(\kappa\)B and MAPK signaling cascades in response to LPS stimulation. Concomitantly, a depression in PI3K-Akt activation was also observed in GC-conditioned BMM\(\delta\)s. The increase in inflammatory cytokine expression was directly related to GC-induced overexpression of SHIP1, a negative regulator of PI3K-dependent pro-inflammatory responses. Our results have delineated a novel mechanism by which small increases in endogenous GCs at critical periods during myeloid cell differentiation are able to enhance innate immune responsiveness to endotoxin.

Materials and Methods

Animals

C57BL/6 mice were originally purchased from Charles River Laboratories and bred in-house. All mice were maintained in a temperature-controlled sterile environment on a 12-h light-dark cycle (lights on at 0700 h) with access to water and mouse chow ad libitum. The University of Utah Animal Resource Center guarantees strict compliance with the regulations established by the Animal Welfare Act. At the time of sacrifice, all animals were euthanized by inhalation of halothane followed by cervical dislocation.

Reagents and cell culture

Single-cell suspensions of BMM\(\delta\)s, derived from the bone marrow of C57BL/6 animals, were prepared and maintained in GC-free complete medium (1% complete medium: RPMI 1640 (CellGro) supplemented with 1% FBS (HyClone), 2 mM l-glutamine, 100 U/ml gentamicin, and 50 \(\mu\)M 2-ME). Corticosterone, LPS (Escherichia coli strain O111:B4), and the PI3K inhibitor, wortmannin, were purchased from Sigma-Aldrich. In some experiments, LPS was repurified using chloroform/phenol extraction to eliminate any lipoprotein contamination. Abs specific for murine phosphorylated (p)Ser473-Akt, p-p38, and iIkB\(\alpha\) were obtained from Cell Signaling Technology. Abs specific for total Akt were purchased from eBioscience. Abs specific for SOCS-1 was purchased from Abcam. The monoclonal anti-murine SHIP1 Ab and SHIP1-specific small interfering RNA (siRNA) were purchased from Santa Cruz Biotechnology. Non specific oligonucleotides were purchased from Dharmacon. The IBAK-M Ab and rabbit-anti-monoclonal Abs were obtained from Sigma-Aldrich. Goat anti-rabbit-HRP Ab was obtained from Bio-Rad. Akt Inhibitor IV was purchased from Calbiochem. Murine rIFN-\(\gamma\) and all purified and biotinylated Abs specific for various murine cytokines were purchased from BD Pharmingen. Murine rm-CSF was purchased from R&D Systems. The kinase dead (DN)- and wild type (WT)-Akt plasmids were provided by Dr. D. Alessi (Medical Research Council Protein Phosphorylation Unit, University of Dundee, Dundee, Scotland, U.K.).

ELISA

The quantitative analysis of cytokines were performed using rat-anti-murine mAbs (purified and biotinylated anti-murine TNF-\(\alpha\), IL-6, IL-12, and IL-10 Abs) for capture and detection, followed by development with HRP-conjugated streptavidin and ABTS as substrate. The detection limit for all ELISAs was <16 pg/ml.

BMM\(\delta\)s

To generate murine BMM\(\delta\)s, total bone marrow cells were flushed out from the tibias and femurs of C57BL/6 animals. Single-cell suspensions of total bone marrow (0.5 \(\times\) 10\(^6\) ml) were cultured in vitro in BMM\(\delta\) medium (RPMI 1640 supplemented with 20% L929-conditioned medium (as a source of M-CSF), 30% equine serum (HyClone), 2 mM l-glutamine, 100 U/ml gentamicin, and 50 \(\mu\)M 2-ME). Medium was changed on day 4 of culture unless otherwise indicated. After 6 days in culture, the BMM\(\delta\) medium was aspirated, and adherent M\(\delta\)s were detached from bacteriologic petri plates, washed three times, and resuspended at a concentration of 0.5 \(\times\) 10\(^6\) cells/ml in 1% complete medium. GC-BMM\(\delta\)s were generated under identical conditions in the presence of added corticosterone (5–40 nM). For stimulations, 200 \(\mu\)l of cells was plated in triplicate in 96-well plates, rested for \(\approx\)5 h, and 10 ng/ml LPS was added. Viability of cells was determined at the time of plating using the trypan blue exclusion method and was consistently >99%.

Western blot analysis

BMM\(\delta\)s were generated as described above, rested overnight (0.5 \(\times\) 10\(^6\) cells/ml) in 1% complete medium, and simultaneously stimulated by adding 10 ng/ml LPS. At the indicated time points, cells were pelleted, lysed (2 mM Tris-HCl, 1 mM NaCl, 1 mM KCl, 0.3 mM MgCl\(\text{2}\) plus Complete protease inhibitor mixture tablets (Roche) supplemented with 20 mM Na\(\text{2}\)VO\(\text{4}\), 25 mM NaF, 5 mM Na\(\text{3}\)PO\(\text{4}\), and 1% Triton X-100), and the protein concentration was determined by the bicinchoninic acid (Pierce BCA kit) method. An equal amount of protein (15–20 \(\mu\)g) was mixed with SDS sample buffer and boiled for 5 min followed by SDS-PAGE. Proteins were then transferred onto polyvinylidene difluoride membranes, blocked (1% nonfat dry milk, 1% BSA in TBS-0.1% Tween 20), and probed for various proteins with the indicated specific Abs. Following probing with the primary Ab specific for the protein of interest, blots were washed and subsequently probed with the appropriate HRP-conjugated secondary Abs. Proteins of interest were detected using the ECL method (Amerks Bio- sciences), followed by exposure to x-ray film at the appropriate exposure times. Western blots were quantitated using ImageJ software (version 1.37, WS Rasband; National Institute of Health (http://rsb.info.nih.gov/ij/)).

Transient transfections

BMM\(\delta\)s were generated as described above. At the end of the 6-day culture period, BMM\(\delta\)s were detached, washed, and resuspended in Nucleofector Solution at a density of 2 \(\times\) 10\(^6\)/100 \(\mu\)l containing non-specific siRNA (0.5 \(\mu\)g/10\(^6\) cells), SHIP1-specific siRNA (0.5 \(\mu\)g/10\(^6\) cells), WT-Akt (1 \(\mu\)g/10\(^6\) cells), or DN-Akt (2 \(\mu\)g/10\(^6\) cells). Transfections were conducted using the Amaxa Nucleofector (program Y-001). Following transfections, cells were immediately placed in 10% complete medium (RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml gentamicin, and 50 \(\mu\)M 2-ME) (prewarmed to 37°C) at a density of 0.5 \(\times\) 10\(^6\) and rested for the predetermined optimal time (30 h for SHIP1-specific siRNA; 6–12 h for WT- and DN-Akt). Following the resting period, transfected cells were either pelleted, and lysates were prepared for Western blot analysis or resuspended in 1% complete medium (0.5 \(\times\) 10\(^6\) ml) and used for LPS stimulations. Transfection efficiency was determined using a pmaxGFP expression construct supplied by Amaxa. The percentage of GFP-\(^{-}\)BMM\(\delta\)s was determined using flow cytometric analysis 16 h following transfection and was consistently \(\approx\)85%.

Implantation of corticosterone pellets

An incision of \(\approx\)0.2 cm was made along the midline of the dorsal side of anesthetized C57BL/6 mice. Time-release corticosterone pellets (Innovative Research of America) (1.25 mg released over 21 days) were inserted into position in between the scapula of the animals with sterilized forceps. Incisions were closed, and the animals were allowed to acclimate for \(\approx\)7 days. Sham-operated animals underwent identical surgical manipulations with the exception of the implantation of corticosterone pellets. Blood was drawn from sham-operated and pellet-implanted animals at 0900 h. Serum was separated to determine corticosterone levels using an enzyme immunoassay (Cayman Chemicals).

Purification of cell populations

Sham-operated and pellet-implanted animals were sacrificed at 7–21 days following the surgical procedure. M\(\delta\)s were purified from the spleens of animals by negative or positive selection using magnetic bead sorting kits specific for CD11b (Miltenyi Biotec). Purity of the selected cell population was determined using flow cytometric analysis and was routinely shown to be \(\approx\)95%. CD11b\(^{+}\) M\(\delta\)s were either lysed and total cellular protein extracted for Western blot analysis or resuspended in 1% complete medium (10\(^6\)/ml) for LPS stimulation.

Stimulation of peritoneal M\(\delta\)s and M\(\delta\)s purified from the bone marrow

Peritoneal resident cells were collected via injection and aspiration of 4°C PBS (5 ml) directly into the peritoneal cavity of mice. CD11b\(^{+}\) M\(\delta\)s were purified from peritoneal washes or total bone marrow cells using immunomagnetic positive selection from the pooled peritoneal washes. Purity was consistently \(\approx\)95%. Purified CD11b\(^{+}\) M\(\delta\)s were washed and resuspended (10\(^6\)/ml) in 1% complete medium. For LPS stimulations, 200 \(\mu\)l of cells was plated in triplicate in 96-well plates, and the appropriate amount of LPS was added following a \(\approx\)5 h resting period.

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Statistical analysis

Statistical analysis was performed with the EZAnalyze Freeware. The p values were determined by Student’s t test (unpaired).

Results

GC conditioning leads to the development of BMMΦs possessing an augmented capacity to produce inflammatory cytokines

The capacity of GCs to enhance innate immune responses has been demonstrated in both clinical studies and animal models (9, 10). More recently, it was shown that RAW264.7 cells (a murine MΦ-like cell line) that were pretreated with GCs possessed an augmented capacity to respond to endotoxin stimulation (16). To determine whether the validity of this observation extended to primary MΦs, we generated BMMΦs in vitro using L929 supernatant as a source of M-CSF in the absence (normally derived, ND-BMMΦ) or presence of increasing concentrations of the endogenous GC corticosterone (5–40 nM) (GC-conditioned, GC-BMMΦ). At the end of the 6-day culture period, both ND- and GC-BMMΦs were washed, rested, and stimulated with LPS. Shown in Fig. 1, A–C, BMMΦs conditioned with 10–20 nM corticosterone produced ≥2-fold more inflammatory cytokines (TNF-α, IL-6, and IL-12) in response to LPS compared with ND-BMMΦs. BMMΦs that had been conditioned with 30 nM corticosterone produced more IL-6 and IL-12 but not TNF-α. Increased inflammatory cytokine production did not result from decreased IL-10 production by GC-BMMΦs because both populations produced identical levels of IL-10. Consistent with higher levels of GCs having a suppressive influence, BMMΦs conditioned with 30 nM GC produced lower levels of TNF-α and IL-6 following LPS stimulation (Fig. 1, A–C). Similar results were obtained when we used chloroform/phenol repurified LPS, eliminating the possibility that contaminating lipoproteins in the commercial LPS contributed to the induction of cytokines in these BMMΦs (data not shown). Conditioning of BMMΦs with 10 nM corticosterone consistently programmed BMMΦs for hyperresponsiveness to LPS stimulation and is within the normal physiological range for circulating GC levels in vivo. Therefore, we used BMMΦs that were conditioned with 10 nM corticosterone for all subsequent experiments performed with GC-BMMΦs.

To examine the timing and duration required to achieve the programming effects on BMMΦs by GC conditioning, GCs were added or removed at various times following the initiation of culture. When these BMMΦs were stimulated with LPS, we found that GCs must be added to the cultures between 0 and 48 h and remain for ≥48 h for the observed programming effects to take
place (Fig. 1, D–F). To determine whether the GC-conditioning effects extended beyond myeloid progenitors, differentiated CD11b+ Mφs were purified from the bone marrow and conditioned with corticosterone (10–20 nM) for 24–48 h. Results shown in Fig. 1, G–I, indicate that differentiated Mφs were refractory to the programming effects of GCs. Similar GC conditioning of Mφs purified from the peritoneal cavity also did not result in their hyperresponsiveness to LPS stimulation (data not shown).

GC-conditioning effects were achieved when ND- and GC-BMMs were generated using rM-CSF, eliminating the possibility that additional factors present in the L929-conditioned medium and/or equine serum were partially responsible for the programming effects (Table I). We also asked whether GC-conditioning effects required the presence of lineage-committed cells by conditioning bone marrow cells that have been depleted of CD4$,CD8$, B220$, and MHCII$ cells. The remainder of the bone marrow cells were conditioned with corticosterone (10 nM), washed, rested, and stimulated with LPS (10 ng/ml). Cytokine levels in the supernatants were quantitated by ELISA.

Table I. The hyperresponsiveness of GC-conditioned BMMφs is independent of factors present in L929-conditioned medium and the presence of lineage-committed immune cells during Mφ differentiation

<table>
<thead>
<tr>
<th>Fold Increases in</th>
<th>TNF-α</th>
<th>IL-6</th>
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<tr>
<td>rM-CSF$^a$</td>
<td>2.3$^c$</td>
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<tr>
<td>Lineage depletion$^b$</td>
<td>2.4</td>
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$^a$ BMMφs were GC conditioned using murine rM-CSF as the source of growth factors for the 6-day culture period. Following the conditioning period, BMMφs were washed rested and stimulated with LPS (10 ng/ml). Cytokine levels in the supernatants collected at 24 h were quantitated by ELISA.

$^b$ Total bone marrow cells were depleted of CD4$, CD8$, B220$, and MHCII$ cells. The remainder of the bone marrow cells were conditioned with corticosterone (10 nM), washed, rested, and stimulated with LPS (10 ng/ml). Cytokine levels in the supernatants collected at 24 h were quantitated by ELISA.

$^c$ Results are presented as fold differences in cytokine production and are representative of at least three independent experiments.

The induction of inflammatory cytokines in BMMφs in response to LPS requires the activation of at least two signaling pathways, the NF-κB and the MAPK cascade (13, 17). Our analysis of various cell surface markers, including the LPS receptors CD14 and TLR4, did not reveal phenotypic differences on ND- and GC-BMMφs (Fig. 2). We next asked whether GC conditioning somehow altered the activation pattern of the above-mentioned signaling pathways. ND- and GC-BMMφs were generated as described previously. At various times following LPS stimulation, cells were collected, and total cellular extracts were prepared for Western blot analysis.

The phosphorylation and subsequent degradation of IkBα leads to NF-κB nuclear translocation and transcription of genes for inflammatory mediators (13). As indicated by the decrease in IkBα protein level from the total cell lysates of ND-BMMφs, NF-κB activation occurred by 60 min following LPS exposure (Fig. 3A).
In contrast, in GC-BMMφs that were exposed to an identical dose of LPS, ξB receptor levels dropped below the detection limit of Western blot analysis by 60 min, indicating increased NF-κB nuclear translocation. Similarly generated cellular lysates were used to characterize MAPK activation using Abs specific for the phosphorylated forms of p38 and ERK44/42. As shown in Fig. 3, B and C, p-p38 and p-ERK44/42 levels were increased in GC-BMMφs compared with ND-BMMφs at 45 min following the addition of LPS and remained enhanced throughout the time course.

**GC conditioning of BMMφs leads to selective alterations in the negative regulation of TLR4 signaling**

An altered negative regulation of TLR4 signaling in GC-BMMφs may contribute to the observed augmentation in the molecular signaling pathways that lead to inflammatory cytokine expression. Therefore, we evaluated the expression of IRAK-M and SOCS-1, negative regulators of LPS and LPS-induced cytokine signaling, respectively (14, 15). Fig. 4, A and B, shows the results of Western blot analysis of IRAK-M and SOCS-1 expression in cell lysates prepared from ND- and GC-BMMφs following LPS stimulation. Although SOCS-1 expression was slightly higher in GC-BMMφs at earlier time points, by 24 h following LPS stimulation, SOCS-1 expression levels were induced to similar levels in ND- and GC-BMMφs. IRAK-M expression in GC-BMMφs was induced to peak levels at 1 h following LPS stimulation and subsequently decreased to below the level of detection by 24 h. This is in contrast to the gradual increase in IRAK-M expression in ND-BMMφs throughout the 24-h time course.

![Western blot analysis](Image1)

**FIGURE 4.** GC conditioning of BMMφs leads to selective alterations in the negative regulation of TLR4-signaling ND- and GC-BMMφs were stimulated with LPS (10 ng/ml). Cells were pelleted at various time points followed by immediate lysis. Total cellular proteins were prepared for Western blot analysis using Abs specific for murine SOCS-1 (A), IRAK-M (B), and pSer473-Akt (C). All blots were stripped and reprobed with the appropriate Abs to determine equal protein loading. Results are representative of three or more experiments.

The altered activation kinetics of NF-κB and MAP suggested that GC conditioning of BMMφs compromised TLR4-negative regulation at early time points following LPS stimulation. To examine this possibility, we characterized the PI3K-Akt pathway, which has been established to be an immediate-early negative regulatory pathway for TLR4 signaling in myeloid cells (18–21). Phosphorylation of Akt was examined in total cellular extracts prepared from ND- and GC-BMMφs at various time points following LPS stimulation using an Ab specific for the pSer473-Akt. We found that pSer473-Akt levels were depressed in GC-BMMφs compared with ND-BMMφs between 0 and 90 min following stimulation with identical doses of LPS (Fig. 4C), suggesting that GC conditioning of BMMφs somehow altered PI3K-Akt signaling.

**LPS signaling in BMMφs is negatively regulated by the PI3K-Akt pathway**

Both proinflammatory and anti-inflammatory effects have been reported to involve the PI3K pathway following TLR4 activation (19, 20, 22). To ascertain the roles played by PI3K-Akt activation following TLR4 engagement in BMMφs, we initially used wortmannin (a PI3K specific inhibitor) and Akt Inhibitor IV (an Akt phosphorylation inhibitor). BMMφs that were pretreated with wortmannin or Akt Inhibitor IV and control BMMφs (not pre-exposed to either inhibitor) were stimulated with LPS (1–100 ng/ml), and supernatants were collected at 24 h to assess cytokine levels. As shown in Fig. 5, A–C, wortmannin pretreated BMMφs produced more TNF-α, IL-6, and IL-12 compared with BMMφs that had not been pretreated with the PI3K inhibitor at all doses of LPS (1–100 ng/ml) used for stimulation. Similarly, pretreatment of BMMφs with the Akt phosphorylation inhibitor also increased their capacity to produce TNF-α, IL-6, and IL-12 in response to LPS.

To overcome any nonspecific effects associated with the usage of chemical inhibitors, we expressed hemagglutinin (HA)-tagged WT- and DN-Akt plasmids in primary BMMφs as an additional approach to clarify the role played by Akt in LPS signaling. Plasmids encoding HA-WT-Akt or HA-DN-Akt were transfected into BMMφs. Fig. 5D shows the overexpression of Akt in BMMφs that had been transfected with HA-WT- or HA-DN-Akt as early as 6 h. BMMφs transiently expressing WT- or DN-Akt were stimulated with LPS to determine the effects of WT-Akt overexpression or competitive inhibition of endogenous Akt kinase activity by DN-Akt on inflammatory cytokine expression. As shown in Fig. 5, E–G, stimulation of BMMφs overexpressing WT-Akt in BMMφs decreased IL-6 production by 3.6-fold and diminished TNF-α and IL-12 production almost completely. In contrast, expression of the DN-Akt correlated with increased TNF-α (5.0-fold), IL-6 (3.2-fold), and IL-12 (4.8-fold) production. These observations are consistent with Akt activation playing a negative regulatory role in TLR4 signaling in BMMφs.

**SHIP1 levels are augmented in BMMφs conditioned with corticosterone and positively regulate LPS-induced inflammatory cytokine expression**

Upon LPS-binding and tyrosine phosphorylation of TLR4, hematopoietic cell-specific Src-homology-2 containing 5'-inositol phosphatase-1 (SHIP1) is rapidly up-regulated and targeted to the TLR4/MyD88 signaling complex via its SH2-domain (23, 24). Targeted SHIP1 depletes phosphatidylinositol-3,4,5-trisphosphate (PIP₃) generated by PI3K via its 5'-phosphatase activity, thus suppressing PIP₃-facilitated activation of Akt and other PIP₃-dependent processes (24). Our observation that
p-Akt levels are depressed in GC-BMM\(^\text{\textregistered}\)s following TLR4 signaling led us to question whether modifications in basal or inducible SHIP1 expression could be involved. SHIP1 protein levels were examined in cellular extracts of LPS-stimulated ND- and GC-BMM\(^\text{\textregistered}\)s by Western blot analysis, and the results are shown in Fig. 6A. SHIP1 expression was up-regulated by LPS in both populations of BMM\(^\text{\textregistered}\)s. Moreover, consistent with it playing a role in the down-regulation of PI3K-Akt activation following LPS stimulation, both basal (2-fold difference at 0 h) and inducible SHIP1 expression (4-fold difference at 1–3 h) in GC-BMM\(^\text{\textregistered}\)s was elevated in comparison to ND-BMM\(^\text{\textregistered}\)s at all the time points analyzed.

The regulatory role played by SHIP1 in LPS-induced cytokine production in BMM\(^\text{\textregistered}\)s was further examined using siRNA-mediated knockdown of SHIP1 protein expression. BMM\(^\text{\textregistered}\)s were transfected with SHIP1-specific siRNA oligonucleotides and SHIP1 knockdown was verified at 30 h following transfection of the siRNA oligonucleotides by western blot analysis (Fig. 6B). We next established the effect of SHIP1 knockdown on the status of PI3K-dependent Akt activation. BMM\(^\text{\textregistered}\)s transfected with nonspecific siRNA or SHIP1-specific siRNA were stimulated with LPS, and cell lysates were prepared to determine pSer473-Akt levels by Western blot analysis. As shown in Fig. 6C, concomitant to the inhibition of LPS induced SHIP1 up-regulation, pSer473-Akt levels were enhanced in BMM\(^\text{\textregistered}\)s that had been transfected with SHIP1-siRNA. Furthermore, siRNA-mediated knockdown of SHIP1 in BMM\(^\text{\textregistered}\)s resulted in a 2-fold decrease in TNF-\(\alpha\), IL-6, and IL-12 production following their LPS stimulation (Fig. 6D–F), consistent with SHIP1 playing a positive regulatory role in LPS-inducible inflammatory cytokine production in primary BMM\(^\text{\textregistered}\)s.

**Elevation of circulating GC levels in vivo augments endotoxin responsiveness of host immune cells through increased expression of SHIP1**

To establish whether an elevation of circulating GCs in vivo can promote a more proinflammatory phenotype, we implanted timed-release corticosterone pellets (equivalent to the release of \(~62.5\ \mu\text{g corticosterone/day}\) into normal mice. Blood was drawn from sham-operated and pellet-implanted animals after 7 days to quantitate serum GC levels. Following sacrifice, M\(^\text{\textregistered}\)s

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**FIGURE 5.** Negative regulation of TLR4 signaling by the PI3K-Akt pathway. A–C, ND- and GC-BMM\(^\text{\textregistered}\)s that were pretreated with wortmannin (100 nM) or the Akt-Inhibitor IV (10 nM) or not for 1 h were stimulated with LPS (1–100 ng/ml). Supernatants were collected at 24 h to determine TNF-\(\alpha\), IL-6, and IL-12 levels by ELISA. D, ND-BMM\(^\text{\textregistered}\)s were transfected with plasmids expressing HA-WT-Akt, HA-DN-Akt, or the empty vector 6 h before analysis. Transfected cells were pelleted and lysed, and total cellular proteins were prepared for Western blot analysis using Abs specific for the HA-tag and total cellular Akt to determine the expression level of the plasmids. E–G, At 6 h following the transfections, cells were stimulated with LPS (10 ng/ml). Supernatants were collected at 24 h to determine TNF-\(\alpha\), IL-6, and IL-12 levels by ELISA. Results are representative of three or more experiments. Error bars represent mean \(\pm\) SD. *, \(p < 0.05\); **, \(p < 0.001\).
were purified from the spleens of sham-operated and pellet-implanted animals and stimulated with LPS. Cytokine levels were determined in the supernatants collected at 24 h by ELISA. In addition, parallel sets of cells were lysed for analysis of SHIP1 expression by Western blot analysis. C, ND-BMMs were transfected with SHIP1-specific or -nonspecific siRNA 30 h previously. Cells were then stimulated with LPS (10 ng/ml) 30 h previously. At the indicated time points, cells were pelleted and lysed, and total cellular proteins were prepared to determine SHIP1 and pSer473-Akt levels by Western blot analysis. D–F, ND-BMMs were transfected with SHIP1-specific or -nonspecific siRNA 30 h previously, followed by LPS (10 ng/ml) stimulation. Supernatants were collected at 24 h to determine TNF-α, IL-6, and IL-12 levels by ELISA. Results are representative of three or more experiments. Error bars represent mean ± SD. *, p < 0.05.

Shown in Fig. 7B, Western blot analysis showed increased SHIP1 protein expression in splenic Mφs from the pellet-implanted animals compared with the levels of SHIP1 found in the Mφs of sham-operated animals, both previous to and following LPS stimulation. Thus, elevated circulating GCs augmented SHIP1 expression in host Mφs in vivo. Consistent with our in vitro observations, splenic Mφs from pellet-implanted animals produced significantly more TNF-α, IL-6, and IL-12 following endotoxin stimulation compared with Mφs from sham-operated animals (Fig. 7, C–E).

FIGURE 7. GCs modify SHIP1 expression and LPS responsiveness in vivo. A, Normal mice were implanted with timed-release (~62.5 μg/day released) corticosterone pellets ≥7 days previously. Blood was drawn at 0900 h to determine serum corticosterone levels by ELISA. B, CD11b⁺ Mφs were purified from the spleens of pellet-implanted animals and stimulated with LPS (10 ng/ml). At the indicated time points, splenic Mφs were pelleted and lysed, and total cellular proteins were extracted to determine SHIP1 expression by Western blot analysis. C–E, At 24 h following LPS stimulation, supernatants were collected to determine TNF-α, IL-6, and IL-12 levels by ELISA. Results are representative of three or more experiments. Error bars represent mean ± SD. *, p < 0.05; **, p < 0.001.
Discussion

The therapeutic use of supraphysiological doses of GCs for immunosuppression, have led to the conceptualization of GCs as having unidirectional suppressive effects on immune function (4, 25). What is less often appreciated is the belief that GCs may function to coordinate the magnitude and duration of host immune responses. Indeed, the capacity of GCs to enhance immune function are backed by several experimental studies as well as clinical outcomes of sepsis in the setting of adenocortical insufficiency (4, 5).

Herein, we report that conditioning of murine BMMDefs during their differentiation with physiological doses of corticosterone consistently resulted in their overexpression of TNF-α, IL-6, and IL-12 in response to LPS. We demonstrated that the programming effects of GCs occurred within 48 h of culture initiation, and the effect was independent of lineage-committed cells.

Our evaluation of cell surface markers on ND- and GC-BMMdfs did not reveal differences in the expression of class II MHC and the costimulatory molecules, CD80 and CD86. In addition, the expression of TLR4 and CD14 were not modified by GC conditioning, despite tlr4 being reported to be a GC-responsive gene (8). Instead, the hyperresponsiveness of GC-BMMdfs resulted from augmentations in the activation of NF-κB and MAPKs, as indicated by increased IκBα degradation, and increased levels of p-p38 and p-ERK44/42 in the lysates of GC-BMMdfs following LPS stimulation. IκBα gene expression is up-regulated in response to pharmacological doses of synthetic GCs (26). In our in vitro BMMdf culture system, however, the level of IκBα protein in ND-BMMdfs and GC-BMMdfs were found to be similar, suggesting cellular IκBα expression is unaffected by physiological levels of GCs. The increased activation of these two major signaling pathways could explain, at least in part, the overexpression of inflammatory cytokines observed in the LPS stimulated GC-BMMdfs. Our observations also offer a mechanistic explanation for the ability of GCs to augment innate immune responsiveness to LPS.

An overproduction of inflammatory cytokines is the underlying cause of endotoxin shock (27). To maintain effective control over the magnitude of inflammatory responses, multiple negative regulatory mechanisms have evolved to minimize tissue damage (28). The expression of IRAK-M and SOCS-1 are induced in monocytes following TLR stimulation and function to control the production of inflammatory cytokines and the signaling cascades initiated following engagement of these cytokines to their receptors, respectively (15, 29). Although we did not find significant differences in SOCS-1 induction, IRAK-M was differentially expressed between the two populations of BMMDefs. GC-BMMdfs expressed significantly more IRAK-M, the levels of which decreased throughout the 24-h period following LPS stimulation. It is known that IRAK-M down-regulates TLR4 signaling via its affinity for TNFR-associated factor 6, thereby preventing IRAK1 degradation and the propagation of TLR4 signaling downstream (30). Higher basal IRAK-M expression should therefore enhance negative regulation of TLR4 signaling in the GC-BMMdfs. GC-BMMdfs exhibited hyperresponsiveness to LPS stimulation despite increased basal IRAK-M expression, suggesting that GC-BMMdfs may be refractory to the inhibitory effects of IRAK-M during the earlier time points following LPS stimulation. In support of this hypothesis, analysis of IRAK1 degradation by Western blot showed equal IRAK1 degradation in both ND- and GC-BMMdfs subsequent to LPS stimulation (data not shown). Alternatively, the down-regulation of IRAK-M by GC-BMMdfs following their LPS stimulation may have effectively abrogated the inhibitory effect of IRAK-M on IRAK/TNFR-associated factor 6 signaling. How GC conditioning leads to increased basal IRAK-M expression in BMMdfs and its degradation in response to LPS remains to be established.

Following TLR4 activation, PI3K is rapidly recruited to MyD88 via its Src homology 2 domain. This allows the generation of phosphatidylinositol 3,4,5-triphosphate from membrane associated phosphatidylinositol 4,5-biphosphate to facilitate the docking of PH domain-containing kinases phosphatidylinositol-dependent kinase 1 and 2, which are required for full activation of Akt kinase activity (31, 32). Activated Akt is known to inhibit the activity of crucial kinases (MEK1, Raf-1, and MAPK kinase kinase 3/4) in MAPK signaling by the inhibitory phosphorylation of regulatory amino acid residues and to decrease the nuclear translocation of NF-κB (20, 21, 33, 34).

The augmentation in the activation of both the NF-κB and the MAPK pathways following endotoxin challenge suggested that GC-BMMdfs failed to sufficiently dampen the activation of these two early signaling cascades, possibly due to modifications in PI3K-dependent Akt activation. Our finding that Akt activation was depressed in GC-BMMdfs suggests that a decreased negative regulation provided by PI3K-dependent Akt activation could be responsible for the enhanced activation of NF-κB and MAPK signaling cascades.

In a murine model of cecal ligation and puncture, the inhibition of PI3K activities by wortmannin increased serum levels of proinflammatory cytokines and enhanced early mortality in septic mice (18). Moreover, bone marrow-derived dendritic cells from PI3Kγ−/− mice have been reported to produce increased levels of IL-12 following LPS stimulation (21). While the above reports clearly support a suppressive role for PI3K-mediated activities in TLR signaling, other groups have found that the inhibition of PI3K activity can lead to a decrease in proinflammatory cytokine production following LPS stimulation (23). These disparate observations imply the possibility that cross-talk between the MAPK and PI3K-Akt pathway can operate at different levels and depends on the specific condition, the cell types studied, and the strength of the stimulus.

A negative regulatory role for Akt in TLR4 signaling in primary BMMDefs is supported by the significant increase in proinflammatory cytokine production when the PI3K-Akt pathway was inhibited either chemically (using wortmannin or the Akt Phosphorylation Inhibitor IV) or by the expression of DN-Akt. Our observations are also supported by published studies where RAW264.7 cells expressing DN-Akt produced increased levels of TNF-α and IL-6 upon LPS stimulation (19). Additionally, transient overexpression of WT-Akt suppressed IL-6 production and almost completely abolished TNF-α and IL-12 expression in response to LPS stimulation. Although the transfection of plasmids induced an IL-6 response, as indicated by the presence of low levels of IL-6 in the supernatant of BMMDefs transfected with the empty vector, the expression of IL-6 was significantly higher following LPS stimulation.

A restricted expression of SHIP1 by hematopoietic cells has been implicated recently in the negative regulation of proinflammatory cytokine production following LPS stimulation in both human and murine myeloid cells (35). Elevated SHIP1 expression could therefore be responsible for the decreased pSer473-Akt levels seen in GC-BMMdfs following LPS stimulation. In support of our hypothesis, knockdown of SHIP1 expression in BMMDefs led to significantly increased pSer473-Akt levels concomitant to decreased TNF-α, IL-6, and IL-12 production in response to LPS. These observations are consistent with data demonstrating that SHIP1−/− splenic and resident peritoneal Mdfs exhibited increased Akt phosphorylation and decreased NFκB and MAPK activation following their stimulation (23).
The association of GC/GR homodimers to GC response elements (GREs) within the promoter region of GC-responsive genes leads to the transactivation or transrepression of gene expression (36). Analysis of the 100-kb region upstream of the transcription initiation site within the SHIP1 promoter did not reveal canonical GREs (our own observation). This does not exclude the possibility that GREs are not present further upstream or downstream from the promoter of the ship1 gene. Alternatively, it is also possible that GCs may increase SHIP1 expression indirectly. The only factor presently known to directly regulate SHIP1 expression is TGF-β, consistent with the reported Sma3d3/4 binding sites found within the promoter region of SHIP1 (36). Various cell types present in total bone marrow culture have the capacity to produce TGF-β, including stromal cells, pre- and fully differentiated adipocytes, as well as Mφs themselves (37–39). It is therefore possible that GCs augment SHIP1 expression via their ability to increase the level of bioactive TGF-β in culture (through either increased synthesis and/or increased release of preformed latent-TGF-β from intracellular pools).

To establish whether elevations in GCs can lead to increased SHIP1 expression in vivo, circulating GC levels were elevated in WT animals by implanting them with a controlled-release corticosterone pellet. Levels of proinflammatory cytokines produced by the splenic Mφs from these animals were found to increase 2- to 3-fold compared with splenic Mφs from sham-operated animals following LPS stimulation. Concomitantly, SHIP1 protein expression was also increased, both previous to and following endotoxin challenge, in Mφs from the pellet-implanted mice. Taken together, our data indicate that GCs are able to increase cellular SHIP1 expression via a direct or indirect mechanism both in vitro and in vivo.

Elevated cellular SHIP1 expression can compromise efficient dampening of TLR signaling by negative regulation of the PI3K-Akt signaling pathway. Consequently, the NF-κB and MAPK signaling cascades would become overactivated following stimulation, resulting in augmented inflammatory cytokine production. Thus, influences by GCs seem to program the "set point" of Mφ responsiveness to endotoxin via their capacity to regulate SHIP1 expression.

The capacity of GCs to enhance LPS-inducible inflammatory cytokine production has been reported in RAW264.7 cells (16). Consistent with our own observations, enhanced NF-κB activation was also observed in RAW264.7 cells that were pre-exposed to GCs. However, an inability to consistently duplicate the proinflammatory effects of GCs on RAW264.7 cells has prevented us from establishing whether GCs augment LPS responsiveness by increasing SHIP1 expression in this transformed Mφ cell line.

Since ligand activation of numerous distinct TLRs results in the activation of the PI3K pathway (20), it is likely that GC-induced enhancement in SHIP1 expression would affect the responsiveness of Mφs stimulated with other TLR ligands. Indeed, our preliminary results indicate that inflammatory cytokine production in response to CpG DNA also becomes augmented in GC-conditioned BMMφs (data not shown).

In contrast to the hyporesponsive phenotype observed in the SHIP1−/− Mφs and our own observations, it was reported recently that transgene-induced overexpression of SHIP1 in RAW264.7 cells negatively regulates TNF-α and IL-6 production following LPS stimulation (22). SHIP1 is a cytosolic enzyme under resting conditions and its role in the regulation of intracellular calcium levels has recently been suggested due to the finding that SHIP1 can generate IP3 (inositol-1,3,4-triphosphate) from IP2 (inositol-1,3,4,5-tetrakisphosphate) in vitro via its constitutive 5′-phosphatase activity (40). It is therefore possible that a marked overexpression of SHIP1 may disrupt the normal cellular physiology of calcium mobilization post activation, causing a generalized decrease in inflammatory cytokine production in response to LPS.

The generation of lipid-derived second messengers via the PI3K pathway following ligand/receptor interactions controls numerous cellular processes, including cellular proliferation and survival (41). The capacity of SHIP1 to balance the "on/off" state of the PI3K pathway implies a role for SHIP1 in controlling the magnitude of PI3K-mediated cellular responses. Thus, small perturbations in steroid hormone levels (such as minor increases in basal circulating GC levels) that could cause increases in the expression of SHIP1 would be predicted to have far reaching global physiologic consequences.

The increase in circulating GC levels associated with disease conditions such as rheumatoid arthritis, HIV, and many other chronic microbial infections are currently thought to represent a host-induced response in an attempt to protect against exaggerated or runaway inflammatory responses (25). Our observation that minor fluctuations in circulating GC levels can actually enhance inflammatory responsiveness through processes involving the selective up-regulation of SHIP1 expression suggests an alternative possibility that elevated GC levels may actually be contributing to, rather than suppressing, the inflammatory responses associated with these disease states. In addition, various animal models have provided evidence that optimal T cell-mediated responses require the presence of GCs in vivo (42, 43). This suggests that the immunopressressive effects of GCs may not be limited solely to effects on myeloid cells. A clearer appreciation of the molecular mechanisms used by GCs to achieve their augmenting effects on inflammatory responses should lead to better recommendations for the usage of GCs in a variety of natural and clinical conditions.

Disclosures
The authors have no financial conflict of interest.

References