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Dissociation of Endotoxin Tolerance and Differentiation of Alternatively Activated Macrophages

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Endotoxin tolerance is a complex phenomenon characterized primarily by decreased production of proinflammatory cytokines, chemokines, and other inflammatory mediators, whereas the expression of other genes are induced or unchanged. Endotoxin tolerance is induced by prior exposure of murine macrophages/human monocytes, experimental animals, or people to TLR ligands. Although recent studies reported a possible relationship between endotoxin tolerance and differentiation of alternatively activated macrophages (AA-MΦs or M2), we show in this study that LPS pretreatment of IL-4Rα$^{-/-}$ and STAT6$^{-/-}$ macrophages, which fail to develop into AA-MΦs, resulted in tolerance of proinflammatory cytokines, as well as molecules and chemokines previously associated with AA-MΦs (e.g., arginase-1, mannose receptor, CCL2, CCL17, and CCL22). In contrast to LPS, wild-type (WT) MΦs pretreated with IL-4, the prototype inducer of AA-MΦs, did not induce endotoxin tolerance with respect to proinflammatory cytokines, AA-MΦ–associated chemokines, negative regulators, NF-κB binding and subunit composition, and MAPKs; conversely, IL-13$^{-/-}$ macrophages were tolerized equivalently to WT MΦs by LPS pretreatment. Further, IL-4Rα deficiency did not affect the reversal of endotoxin tolerance exerted by the histone deacetylase inhibitor trichostatin A. Like WT mice, 100% of LPS-tolerized IL-4Rα–deficient mice survived LPS + D-galactosamine–induced lethal toxicity and exhibited decreased serum levels of proinflammatory cytokines and AA-MΦ–associated chemokines induced by LPS challenge compared with nontolerized mice. These data indicate that the signaling pathways leading to endotoxin tolerance and differentiation of AA-MΦs are dissociable. ***The Journal of Immunology*****, 2013, 190: 4763–4772.**

Pathogen-associated molecular patterns (PAMPs), including bacterial and viral components, are immunostimulatory and are recognized by TLRs (1, 2), a family of closely related pattern recognition receptors. Upon ligand engagement, TLR activation of intracellular signaling cascades results in robust production of proinflammatory mediators, including cytokines and chemokines (3, 4). LPS, an integral outer membrane component of Gram-negative bacteria, is the prototype PAMP recognized by the TLR4/MD2 complex and has long been associated with the strong inflammatory response associated with endotoxin shock (3, 5). However, after an initial exposure of monocytes/macrophages (MΦs), experimental animals, or people to LPS, a transient period of "endotoxin tolerance," a state of LPS hyporesponsiveness, is observed (6–8). Endotoxin tolerance has long been associated with a downregulation of proinflammatory cytokine production (e.g., TNF-α, IL-6, IL-12, and IFN-β) due to alterations in signaling cascades that affect NF-κB, MAPKs, and IRFs (9–11). In addition, endotoxin tolerance is associated with increased expression of a variety of negative regulators of TLR signaling, such as IRAK-M, ST2, SHIP-1, MyD88s, and A20 (12, 13). A similar phenomenon was observed in septic patients in whom LPS hyporesponsiveness is seen after the initial cytokine storm. Although protecting the host from the ill effects of inflammation, the diminished capacity of sepsis survivors to respond to LPS is thought to underlie increased susceptibility to secondary infection that is common is such patients (6, 14).

MΦs are heterogeneous innate immune cells that exhibit great plasticity (15). MΦs are involved in homeostasis and innate immunity and through Ag uptake, processing, and presentation, serve to initiate the adaptive immune response (16). Based on their responses to environmental stimuli, MΦs possess the ability to differentiate into functionally distinct subsets that have been termed "classically activated" MΦs (CA-MΦs or M1) or "alternatively activated" MΦs (AA-MΦs or M2). CA-MΦ polarization is typically mediated by strong inflammatory stimuli, such as IFN-γ and bacterial products, including LPS, and is characterized by increased production of proinflammatory cytokines, NO, and reactive oxygen species that mediate microbicidal activities and induce cellular immunity (16–19). AA-MΦ polarization is mediated by the type I (IL-4Rα + γc) or the type II (IL-4Rα + IL-13Rα1) receptor complexes by IL-4 or by IL-4 or IL-13 engagement, respectively (20). Upon ligand binding, both the type I and type II IL-4Rs activate the STAT6-signaling pathway (17, 18, 21). The AA-MΦ phenotype has been characterized by expression of mannose receptor (MR) (22), intracellular expression

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Abbreviations used in this article: AA-MΦ, alternatively activated macrophage; Arg-1, arginase-1; CA-MΦ, classically activated macrophage; IL-4/L, IL-4 treatment/LPS challenge; IL-4/M, IL-4 treatment/medium challenge; L/L, LPS treatment/LPS challenge; L/M, LPS treatment/medium challenge; MΦ, macrophage; M/IL-4, medium treatment/IL-4 challenge; M/L, medium treatment/LPS challenge; M/M, medium treatment/medium challenge; MR, mannose receptor; PAMP, pathogen-associated molecular pattern; qRT-PCR, quantitative real time-PCR; TSA, trichostatin A; WT, wild-type.

of arginase-1 (Arg-1), secretion of chitinases (e.g., Ym1) (23), and anti-inflammatory cytokines (21). AA-MΦs are strongly associated with helminthic infections and tissue repair (17).

Recent studies revealed a possible relationship between endotoxin tolerance and AA-MΦ polarization (24, 25). For example, Porta et al. (25) reported that CCL2, CCL17, and CCL22, chemokines that attract Th2 cells and were associated previously with the AA-MΦ phenotype (26), were upregulated in endotoxin-tolerized MΦs in an NF-κB p50–dependent manner. However, because both LPS and IL-4, prototype inducers of CA-MΦ and AA-MΦ phenotypes, respectively, induce the expression of these chemokines (27), it is difficult to know whether they are truly AA-MΦ–differentiation markers. Therefore, for the remainder of this article, we refer to these chemokines as AA-MΦ associated.

To determine the fate of these chemokines, as well as other well-known AA-MΦ markers, during endotoxin tolerance, we used IL-4Rα– and STAT6-deficient MΦs to determine whether AA-MΦ differentiation plays a role in LPS-tolerized MΦs. We show that, although both LPS and IL-4 induce the expression of AA-MΦ–associated chemokine mRNA and protein, the IL-4Rα–STAT6–signaling pathway is not required for induction of endotoxin tolerance, but it is necessary for induction of AA-MΦs by IL-4. Mechanistically, these two states of MΦ differentiation were distinguishable by NF-κB and MAPK activation, NF-κB subunit composition, modulation of negative inhibitors, and sensitivity to a histone deacetylase inhibitor. Our findings further indicate that LPS-tolerized, IL-4Rα–deficient mice, like wild-type (WT) mice, were comparably refractory in vivo to challenge with LPS/D-galactosamine and produced decreased cytokines/chemokines. Taken together, these findings dissociate the induction of endotoxin tolerance and the differentiation pathway that leads to AA-MΦs.

Materials and Methods

Reagents and mice

Protein-free *Escherichia coli* K235 LPS (<0.008% protein) was prepared as described previously (28). TLR-grade LPS from *Salmonella abortus equi* S-form was purchased from Enzo Life Sciences. Murine rIL-4 was purchased from R&D Systems. Abs directed against phospho-ERK1/2, ERK, phospho-JNK1/2, JNK, RelB, STAT3, phospho-STAT3, STAT6, phospho-STAT6, IκBα, and β-actin were purchased from Cell Signaling Technology; anti–phospho-p38 Ab was from Promega. Trichostatin A (TSA) was obtained from Calbiochem EMD-Millipore. WT C57BL/6J and BALB/cByJ mice were purchased from The Jackson Laboratory. C57BL/6 TLR4$^{-/-}$, IL-13$^{-/-}$, BALB/c IL-4Rα$^{-/-}$, IL-4Rα$^{-/-}$/Rag2$^{-/-}$, and STAT6$^{-/-}$ mice were bred at the animal facility at the University of Maryland, School of Medicine. All animal studies were carried out with institutional approval.

Cell culture

Peritoneal exudate cells were obtained by peritoneal lavage from 6–8-wk-old mice 4 d after i.p. injection with sterile thioglycollate (Remel), as described previously (29). MΦs were enriched by adherence and extensive washing and cultured in RPMI 1640 supplemented with 2% FBS, 2 mM glutamine, 1% penicillin, and streptomycin, as described previously (29).

The murine MΦ cell line RAW 264.7 (American Type Culture Collection) was maintained in DMEM supplemented with 10% FBS, 2 mM glutamine, 1% penicillin, and streptomycin, as described previously (30).

In vitro endotoxin tolerance

Primary MΦs and RAW 264.7 cells were cultured at 1–1.5 × 10^6 cells/well in 12-well plates for gene expression and ELISA experiments. For signaling experiments, 3.0 × 10^6 cells/well were used in six-well plates. Cells were initially stimulated with medium only or with LPS (100 ng/ml) for 20–24 h, washed, and then "challenged" by treating the MΦs with medium only or with LPS (100 ng/ml) for the times indicated in the figures. MΦs that were treated first with medium and then challenged with medium are designated M/M, cells stimulated with one dose of LPS or IL-4 after medium pretreatment are designated M/L and medium treatment/IL-4 challenge (M/IL-4), respectively, cells stimulated with LPS for 24 h and then with medium are designated L/M, and cells stimulated with LPS for 24 h and then challenged with LPS or IL-4 are designated L/L or L/IL-4, respectively. Similarly, in some studies, cells were pretreated for 24 h with IL-4 (40 ng/ml) and challenged with medium (IL-4/M) or LPS (IL-4/L). For cytokine ELISAs, culture supernatants were collected after pretreating the cells with medium or LPS for 24 h and challenging with medium or LPS for an additional 16–18 h. For TSA experiments, MΦ cultures were pretreated with medium or LPS in the absence or presence of TSA (50 nM) for 16 h and then challenged with medium or LPS in the absence or presence of TSA for 3 h. For detection of activated MAPKs and other signaling proteins by Western blot analysis (below), cell lysates were prepared from MΦs stimulated with medium, LPS, or IL-4 for 24 h and challenged with medium or LPS for 30 min using cell lysis buffer (Cell Signaling Technology).

RNA and cDNA

Total RNA was isolated using a High Pure RNA Isolation Kit from Roche (Indianapolis, IN), according to the manufacturer's instructions. The RNA concentration was determined using a NanoDrop spectrophotometer. One microgram of total RNA was reverse transcribed using an iScript cDNA synthesis kit, according to the manufacturer's instructions (Bio-Rad).

Quantitative real-time PCR

Differential gene expression of cytokines and chemokines was analyzed by quantitative real-time PCR (qRT-PCR) using SYBR Green, per the manufacturer's guidelines, in the 7000HT Fast Real-time PCR system (Applied Biosystems). Briefly, PCR was conducted in a 25-μl reaction volume containing 20 ng cDNA template and 3 μM murine genes–specific primer mix. Primers for detection of IL-1β, TNF-α, IL-6, IL-12 p40, IL-4, IL-13, IFN-β, Arg-1, MR, FIZZ1, YM1, and HPRT were designed using the Primer Express 2.0 program (Applied Biosystems) and were published elsewhere (21). Primer sequences for CCL2, CCL17, and CCL22 were obtained from Porta et al. (25). Relative gene expression was calculated by normalizing to HPRT as a housekeeping gene.

Western analysis

Protein estimation from cell lysates was carried out using BCA Protein assay reagents (Thermo Scientific/Pierce). The proteins (25–40 μg) in the lysate were boiled in Laemmli buffer for 5 min, resolved by 10% SDS-PAGE in Tris/glycine/SDS buffer (Bio-Rad), and transferred onto a polyvinylidene difluoride membrane at constant voltage (100 V) for 2 h in the cold room. After blocking for 2 h in TBST containing 5% fat-free milk, membranes were probed overnight at 4°C with the respective Abs, according to the manufacturer's instructions. Following extensive washing (four or five times) in TBST, membranes were incubated with secondary HRP-conjugated anti-rabbit IgG from Jackson ImmunoResearch (1:10,000) for 1 h at room temperature. Membranes were washed four or five times in TBST, and bands were detected using ECL plus reagents (Amersham Pharmacia Biotech).

Preparation of nuclear extracts and EMSA

Nuclear extracts were prepared using a nuclear extraction kit (Active Motif), according to the manufacturer's instructions. NF-κB consensus sequence was [^{32}P]-end-labeled with T4 polynucleotide kinase (Invitrogen Life Technologies), as recommended by the manufacturer, and EMSA was carried out as described previously (31). Supershift assays were performed using anti-p65 and anti-p50 Abs, as described previously (31). The polyacrylamide gels were dried at 80°C for 2 h and exposed to a phosphor screen overnight, and the images were visualized using a Storm 680 scanner (Molecular Dynamics).

ELISAs

Cytokine/chemokine levels in the control and tolerized culture supernatants were analyzed by ELISA in the Cytokine Core Facility (University of Maryland, School of Medicine).

In vivo endotoxin tolerance

For mouse-survival studies, WT or IL-4Rα$^{-/-}$ mice (n = 5–6 mice/group) received PBS or LPS i.p. (25 μg/mouse). After 24–30 h, mice were challenged i.p. with LPS (1 μg) + D-galactosamine (16 mg/mouse). Mouse survival was monitored every 6 h for 3–4 d.

For analysis of serum cytokines and chemokines, WT and IL-4Rα$^{-/-}$ mice (n = 5–6/group) were treated i.p. with PBS or LPS (25 μg) and then challenged 3 d later with LPS i.p. (25 μg) are designated P/L or L/L, respectively. Two hours after challenge, mice were bled, and the sera were prepared. Cytokine and chemokine levels in sera were determined using

Multiplex beads at the Cytokine Core Lab (University of Maryland, Baltimore, MD).

Statistical analysis

One-way ANOVA with the Student Newman–Keuls post hoc test was performed to assess statistical significance (p values < 0.05) using GraphPad Prism 4.0 (GraphPad Software).

Results

Effect of endotoxin tolerance on classically activated (M1) and alternatively activated (M2) MΦ-specific gene-expression profiles

Previous studies reported that murine and human AA-MΦ–specific genes were upregulated during endotoxin tolerance; therefore, it was concluded that endotoxin tolerance and alternative activation were, in fact, related states of MΦ differentiation (24, 25). To confirm and extend these findings, we initially sought to validate the expression of CA-MΦ– and AA-MΦ–specific genes induced during endotoxin tolerance. To accomplish this, C57BL/6J primary peritoneal MΦ cultures were stimulated for 24 h with medium, as a control, or protein-free *E. coli* K235 LPS to induce a state of "endotoxin tolerance" and then the cells were washed and challenged with medium or LPS for 3, 6, or 24 h. Fig. 1A shows that CA-MΦ proinflammatory cytokine gene mRNA (e.g., TNF-α, IL-1β, IL-6, IL-12 p40, and IFN-β) was induced by LPS stimulation of medium-pretreated MΦs but was poorly induced in LPS-pretreated (i.e., tolerized) cells. For each of these genes, maximal induction occurred at the 3-h and/or 6-h time points. Next, we examined the induction of mRNA for two well-characterized AA-MΦ markers, Arg-1 and MR, as well as three chemokine genes (CCL2, CCL17, and CCL22) that have more recently been AA-MΦ associated (25). Fig. 1B shows that, in contrast to LPS-inducible CA-MΦ–associated genes, the protype AA-MΦ genes, Arg-1 and MR, were poorly induced (<10-fold) by LPS and only after 24 h of stimulation (M/L); nonetheless, expression of both genes was downregulated upon subsequent LPS stimulation (i.e., these genes are also "tolerizable"). Finally, like the proinflammatory CA-MΦ genes, the AA-MΦ–associated genes encoding the chemokines CCL2, CCL17, and CCL22 were detectable early (at 3 and 6 h after LPS stimulation [M/L]), and CCL2 expression remained elevated as late as 24 h after LPS stimulation (Fig. 1C). The genes that encode these chemokines were also tolerizable, as evidenced by the failure of LPS restimulation (L/L) to increase their expression much above baseline (M/M). Thus, two well-characterized AA-MΦ markers (16), as well as chemokine genes previously associated with AA-MΦ differentiation (25), are downregulated by LPS pretreatment that results in tolerance of CA-MΦ genes. Similar experiments using *S. abortus equi* S-form LPS, as used by Porta et al. (25), were also performed, and the results mirrored those obtained using *E. coli* LPS as the stimulant (Supplemental Fig. 1A). Both LPS preparations were highly purified, as evidenced by their failure to induce gene expression in TLR4$^{-/-}$ MΦs (Supplemental Fig. 1A).

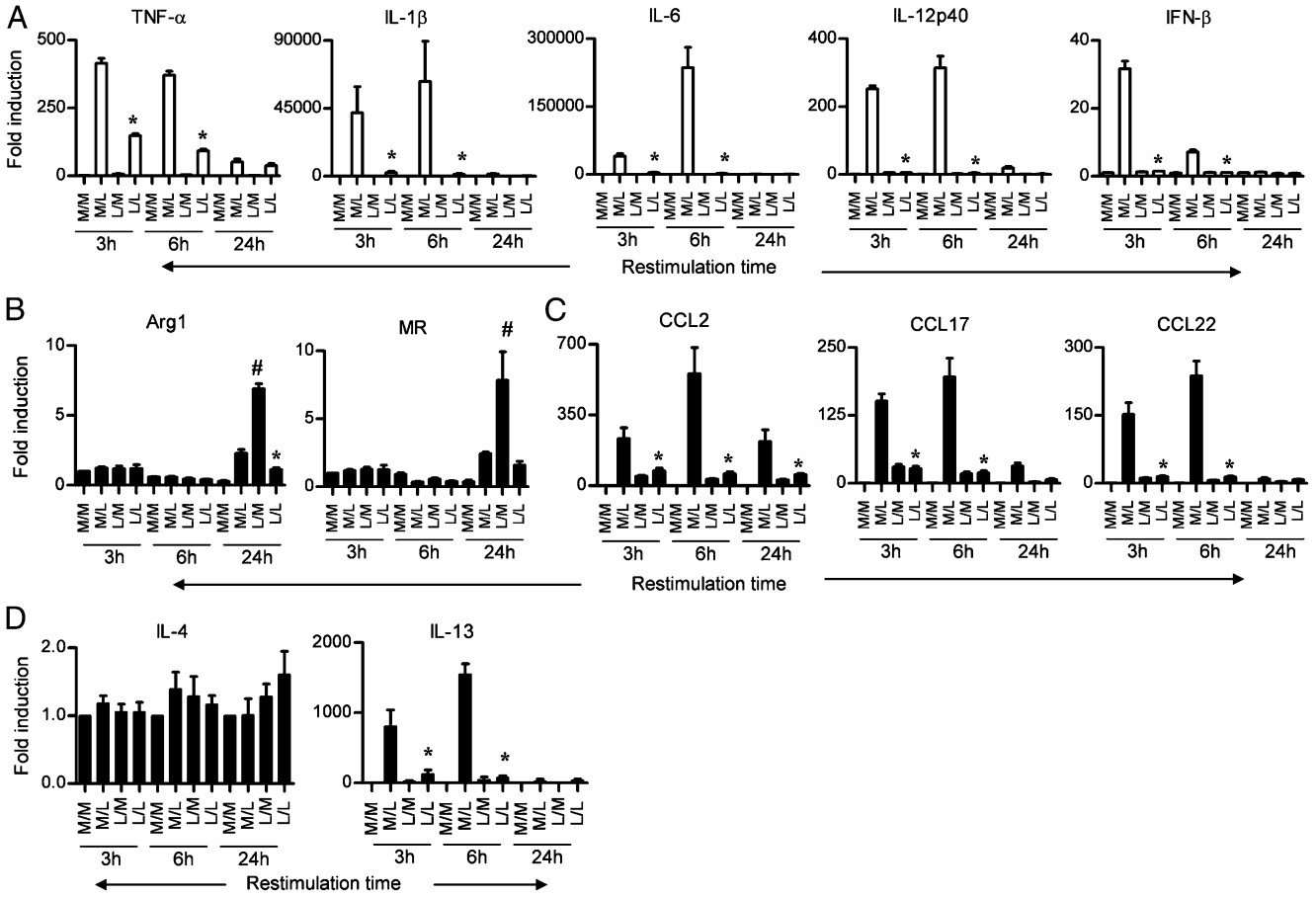

FIGURE 1. CA-MΦ and AA-MΦ marker profiles in control and endotoxin-tolerized murine MΦs. Gene-expression profiles for CA-MΦ cytokines (**A**), AA-MΦ markers (**B**), AA-MΦ–associated chemokines (**C**), and IL-4 and IL-13 (**D**) were analyzed in C57BL/6J thioglycollate-elicited MΦ cultures pretreated for 24 h with medium (M) or LPS (L; 100 ng/ml), washed, and then challenged with medium (M) or LPS (L; 100 ng/ml) for 3, 6, or 24 h. The x-axis indicates the primary/challenge treatments. Results represent the mean ± SEM from three independent experiments. $^{*}p < 0.05$, nontolerized (M/L) versus tolerized (L/L) groups, $^{\#}p < 0.05$, nontolerized (M/L) versus tolerized (L/M) groups.

Because IL-4 and IL-13 play a key role in AA-MΦ polarization, we also examined the expression of these genes during endotoxin tolerance. Interestingly, LPS failed to modulate the expression of IL-4 mRNA (Fig. 1D). However, IL-13 mRNA expression was strongly upregulated in medium-pretreated MΦs by primary LPS stimulation (M/L) at 3 and 6 h; however, it was inhibited in LPS-pretreated cells (Fig. 1D), indicating that it too is a "tolerizable" gene. However, in supernatants derived from MΦ cultures following a prolonged (24-h) posttreatment period, neither IL-4 nor IL-13 protein levels were detectably modulated above levels seen in supernatants from medium-treated MΦs (data not shown).

Endotoxin-tolerant MΦs and AA-MΦs exhibit marked differences in CA-MΦ and AA-MΦ gene expression

Although it was reported that the expression of AA-MΦ–associated chemokines was not "tolerizable" by LPS (24, 25), these studies did not directly compare endotoxin-tolerant and alternatively activated MΦ populations with regard to gene expression. IL-4 or IL-13 is necessary and sufficient for MΦ polarization toward an AA-MΦ phenotype (17, 32, 33). To directly compare the responses of cells rendered endotoxin tolerant by LPS pretreatment versus alternatively activated by IL-4 pretreatment, peritoneal exudate MΦ cultures were initially treated with medium, LPS, or IL-4 for 24 h and then washed and restimulated with medium, LPS, or IL-4 for 3 h. Consistent with Fig. 1, all CA-MΦ cytokine genes (Fig. 2A) and AA-MΦ–associated chemokine genes (Fig. 2B) were upregulated in medium-pretreated MΦs 3 h after LPS stimulation (M/L) and were tolerized by a 24-h pretreatment with LPS, with or without LPS challenge (L/L or L/M). In contrast, IL-4 failed to induce any of the CA-MΦ genes, and IL-4 pretreatment of MΦs did not tolerize against LPS challenge for expression of these genes (Fig. 2A). IL-4 treatment for 3 or 24 h failed to induce CCL2. However, IL-4 only induced CCL17 (Fig. 2B) and CCL22 (data not shown) when MΦs were treated for 24 h. Similar to CA-MΦ genes, IL-4 failed to induce tolerance to LPS for each of these AA-MΦ–associated chemokine genes (Fig. 2B). In contrast, Arg-1 and MR (data not shown) mRNA were poorly induced by LPS treatment for 3 or 24 h, whereas IL-4 pretreatment for 24 h resulted in strong Arg-1 and MR expression that was refractory to LPS stimulation (IL-4/L versus IL-4/L) (Fig. 2B).

Endotoxin-tolerant MΦs and AA-MΦs exhibit marked differences in NF-κB binding and MAPK activation

NF-κB is the key transcription factor involved in the induction of inflammatory mediator gene expression during LPS signaling. A switch in the preponderance of p65/p50 heterodimers to p50/p50 homodimers in endotoxin-tolerized murine and human cells has been well established (34). Accordingly, we next confirmed by EMSA with supershifts that formation of p50/p50 homodimers was increased in endotoxin-tolerized MΦs (Fig. 2C). In contrast, IL-4–pretreated (IL-4/L and IL-4/M) cells failed to upregulate the expression of p50/p50 homodimers and failed to alter the pattern of NF-κB p65 or p50 binding induced by LPS (compare M/L with IL-4/L) (Fig. 2C). This indicates that IL-4 does not induce a state of LPS tolerance with respect to altered expression of the relative composition of p65 and p50 proteins. Hence, induction of AA-MΦ differentiation by IL-4 does not render MΦs endotoxin tolerant by increasing the expression of negative regulators, including p50/p50 homodimers.

The data provided in Fig. 2D further support the conclusion that induction of LPS tolerance does not affect IL-4 signaling and that differentiation of AA-MΦs with IL-4 does not interfere with LPS signaling. Specifically, medium-pretreated cells show robust phosphorylation of MAPKs in response to a 30-min exposure to LPS (M/L), whereas inhibition of MAPK activation was observed in MΦs pretreated with LPS, as evidenced by decreased phosphorylation of MAPKs in response to a 30-min LPS stimulation (L/L; Fig. 2D). LPS-induced MAPK activity was not affected in IL-4–pretreated AA-MΦs (IL-4/L). IL-4 (M/IL-4) did not induce MAPK activity at 30 min, and overnight exposure of MΦs to IL-4 failed to tolerize against LPS-induced MAPK (IL-4/L).

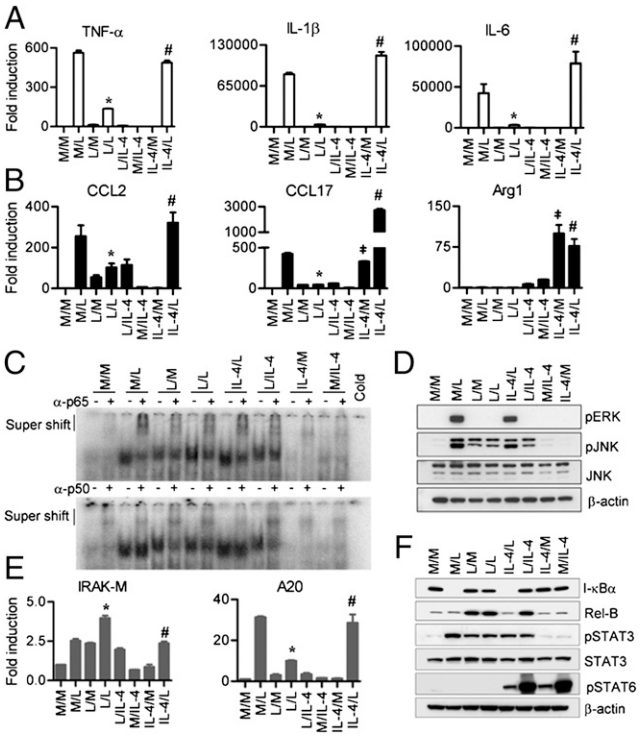

FIGURE 2. IL-4 pretreatment of MΦs failed to induce LPS tolerance, modulate NF-κB and MAPK activation, or induce negative regulators associated with tolerance. Gene-expression profiles of CA-MΦ proinflammatory cytokines (**A**) and AA-MΦ–associated chemokines and AA-MΦ marker Arg-1 (**B**) were analyzed in C57BL/6J thioglycollate-elicited MΦ cultures pretreated for 24 h with medium (M), LPS (L; 100 ng/ml) or IL-4 (40 ng/ml); washed; and then challenged with medium (M), LPS (L; 100 ng/ml), or IL-4 (40 ng/ml) for 3 h. (**C**, **D**, and **F**) Whole-cell lysates and nuclear extracts were prepared from peritoneal exudate cells pretreated for 24 h with medium, LPS, or IL-4 and then challenged with medium, LPS, or IL-4 for 30 min and 1 h, respectively. (C) Nuclear extracts were preincubated with p65 and p50 antisera and analyzed for supershift assay by EMSA using an NF-κB consensus sequence. (D) Whole-cell lysates were subjected to Western blot analysis for MAPK activity. (**E**) Gene-expression profile of negative regulators of LPS signaling, IRAK-M and SHIP1, was analyzed as in (A) and (B). (F) I-κBα, RelB, p-STAT3, STAT3, and p-STAT6 protein expression was analyzed in whole-cell lysates as in (D). Results represent the mean ± SEM from two or three independent experiments. $^{*}p < 0.05$, nontolerized (M/L) versus tolerized (L/L) groups, $^{\#}p < 0.05$, L/L versus IL-4/L groups, $^{\ddagger}p < 0.05$, M/M versus IL-4/M groups.

Endotoxin-tolerant MΦs and AA-MΦs exhibit marked differences in expression of negative regulators of LPS signaling

Endotoxin tolerance has been associated with the production of many negative regulators (12). For example, IRAK-M was implicated as a mediator of tolerance, as evidenced by the resistance of IRAK-M$^{-/-}$ mice to LPS-induced tolerance (35). Endotoxin-

tolerized, LPS-restimulated MΦs (L/L) exhibited a significant increase in IRAK-M gene expression compared with medium-pretreated, LPS-stimulated cells (Fig. 2E). Boone et al. (36) showed that A20 is rapidly induced by LPS stimulation and negatively regulates LPS signaling. However, this same group found that knockdown of A20 in mouse MΦs failed to affect tolerance induced by LPS (36). Similarly, we found that A20 gene expression was induced by LPS challenge (M/L) and was down-regulated in endotoxin-tolerized MΦs (L/L) (Fig. 2E). The observed gene expression of A20 also correlated with protein expression (data not shown). In the case of both IRAK-M and A20, IL-4 stimulation of MΦs for either 3 h (M/IL-4) or 24 h (IL-4/M) failed to elicit any of the changes induced by LPS pretreatment and failed to alter the response to LPS stimulation (IL-4/L) (Fig. 2E).

IκBα, the cytosolic inhibitor of NF-κB, is degraded upon LPS stimulation, thereby allowing NF-κB p65/p50 to translocate to the nucleus where it can induce many proinflammatory genes (37). As expected, IκBα was degraded in response to LPS in medium-pretreated cells (Fig. 2F), and it was not degraded in LPS-tolerized cells, as previously reported (38). RelB protein, an NF-κB family member previously associated with tolerance (39), was upregulated in LPS-tolerized cells (Fig. 2F). IL-4 pretreatment of MΦs also failed to modulate the expression of RelB.

STAT3 was shown to regulate LPS-induced SOCS3 via IL-10 production, and increased LPS-induced cytokine secretion was observed in STAT3-deficient MΦs (40, 41). Although phospho-STAT3 was induced by a 30-min LPS treatment, it was not significantly modulated by LPS pretreatment. IL-4 pre- or posttreatment failed to phosphorylate STAT3 and had no effect on STAT3 phosphorylation induced by LPS (Fig. 2F).

IL-4R engagement is known to induce STAT6 (42) and, indeed, IL-4 strongly induced phospho-STAT6; however, phospho-STAT6 was not activated by LPS challenge in either control (M/L) or LPS-tolerized (L/L) MΦs, nor did LPS stimulation alter the response induced by IL-4 (Fig. 2F).

A summary of the gene-expression profile of cytokines and chemokines, M2 markers, and negative regulators induced by LPS in naive or endotoxin-tolerized MΦs, in the absence or presence of IL-4, is presented in Table I.

IL-4Rα, STAT6, and IL-13 are not required to establish endotoxin tolerance in MΦs

The IL-4Rα–mediated STAT6-signaling pathway plays an essential role in AA-MΦ polarization in vitro and in vivo (16, 17, 42). Because a 24-h pretreatment of MΦs with IL-4 failed to induce endotoxin tolerance (Fig. 2), we next used IL-4Rα$^{-/-}$, STAT6$^{-/-}$, and IL-13$^{-/-}$ MΦs to confirm whether this pathway plays a role in LPS-induced tolerance. IL-4Rα is necessary for signaling by both IL-4 and IL-13 through the shared type II IL-4R (42), and STAT6 is activated downstream of IL-4/IL-13–mediated signaling through this receptor complex (20, 43). Arg-1 (Fig. 3A) and MR (data not shown) mRNA levels were measured as positive controls for the AA-MΦ phenotype and were strongly induced by IL-4 in WT BALB/cByJ MΦs but not in IL-4Rα$^{-/-}$ MΦs (Fig. 3A). Consistent with the findings in C57BL/6 MΦs, both Arg-1 and MR gene expression was increased only ~5-fold in endotoxin-tolerized WT BALB/cByJ MΦs (Fig. 3B; L/M and L/L), whereas LPS-induced Arg-1 and MR mRNA expression in IL-4Rα$^{-/-}$ MΦs was no greater than in cells treated with medium (Fig. 3B). This indicates that the weak induction of Arg-1 and MR mRNA detected in endotoxin-tolerized MΦs requires IL-4Rα signaling. However, we did not observe any difference in AA-MΦ–associated chemokine mRNA (Fig. 3C) or protein (Fig. 3D) levels during endotoxin tolerance. Like WT MΦs, IL-4Rα$^{-/-}$ MΦs become tolerant to LPS following a 24-h exposure to endotoxin (L/M and L/L). As an additional positive control, LPS-induced tolerance was shown to be induced both at the level of mRNA (Fig. 3E) and protein (Fig. 3F) for the proinflammatory CA-MΦ markers analyzed. A nearly identical pattern of endotoxin tolerance was observed in LPS-tolerized STAT6$^{-/-}$ and IL-13$^{-/-}$ MΦs (Supplemental Fig. 2). Collectively, these data indicate that AA-MΦ polarization and endotoxin tolerance are dissociable pathways and that the LPS-induced AA-MΦ–associated chemokines are not affected by the lack of IL-4Rα–mediated signaling or IL-13.

Because attenuation of TLR4-dependent signaling is a hallmark of endotoxin tolerance, we sought to determine whether such a loss of TLR4 signaling was observed in MΦs that cannot be alternatively activated because of a lack of IL-4Rα or STAT6. Lack of IL-4Rα (Fig. 4A) or STAT6 (Fig. 4B) did not alter the ability of

Table I. Induction of cytokines, chemokines, M2 markers, and negative regulators by LPS in naive versus tolerized MΦs and the effect of IL-4 pretreatment on LPS signaling

	M/M	M/L	L/M	L/L	IL-4/L	L/IL-4[b]	M/IL-4	IL-4/M
Cytokines								
TNF-α	–	+++	+/−	+	+++	–	–	–
IL-1β	–	+++	+/−	+	+++	–	–	–
IL-6	–	+++	+/−	+	+++	–	–	–
Chemokines								
CCL2	–	+++	+/−	+	+++	+	–	–
CCL17	–	+++	+/−	+/−	++++	+	–	++
CCL22	–	+++	+/−	+/−	++++	+	–	++
M2 markers								
Arg-1	–	–	–	–	+++	+	+	+++
MR	–	–	–	–	+++	+	+	+++
Negative regulators								
IRAK-M	–	++	++	+++	++	++	–	–
A20	–	+++	+/−	+	+++	–	–	–
I-κBα[a]	+++	–	+++	+++	–	+++	+++	+++
RelB[a]	–	–	+++	+++	–	+++	–	–

Pretreatment = 24 h; challenge = 3 h.
[a]Protein expression.
[b]L/IL-4, LPS treatment/IL-4 challenge.
–, No induction; +/−, mild/no induction; +, mild induction; ++, moderate induction; +++, high induction; ++++, very high induction.

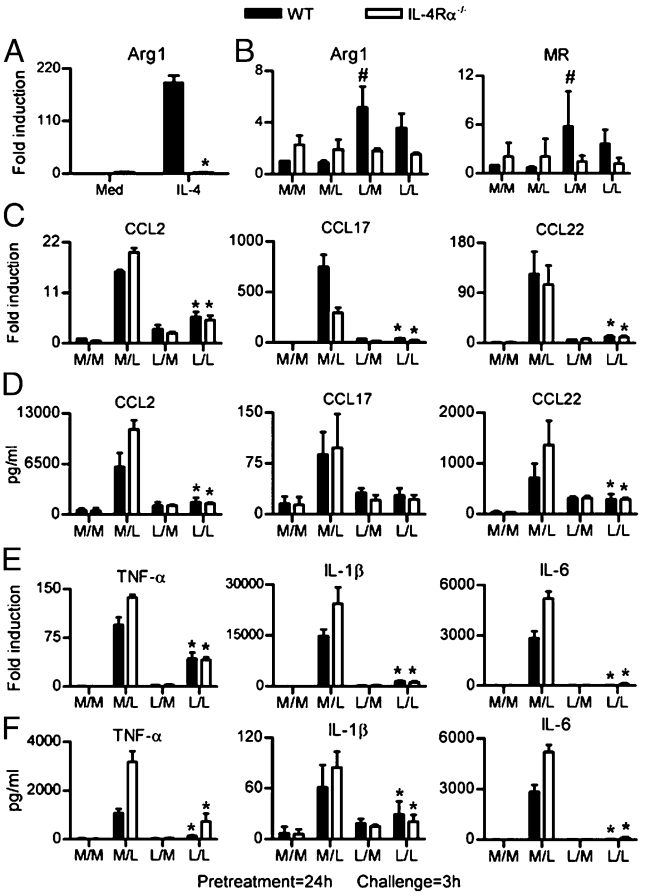

FIGURE 3. IL-4Rα signaling is not required for endotoxin tolerance. (**A**) Thioglycollate-elicited MΦ cultures from WT BALB/cByJ and IL-4Rα$^{-/-}$ mice were pretreated with medium (Med) or IL-4 (40 ng/ml) for 24 h, and mRNA was analyzed for Arg-1 mRNA. (**B**–**F**) MΦs from WT BALB/c ByJ and IL-4Rα$^{-/-}$ mice were pretreated with medium (M) or LPS (L) for 24 h, washed, and then challenged with medium (M) or LPS (L) for 3 h. Analysis of gene expression for AA-MΦ markers (B), AA-MΦ–associated chemokines (C), and CA-MΦ cytokines (E), measured by qRT-PCR. (D and F) Analysis of AA-MΦ–associated chemokine protein and CA-MΦ cytokines in supernatants derived from cultures in (C) and (E), respectively, by ELISA. Results represent the mean ± SEM from two independent experiments. $^{*}p < 0.05$, nontolerized (M/L) versus tolerized (L/L) groups, $^{\#}p < 0.05$, nontolerized (M/L) versus tolerized (L/M) groups.

LPS to activate MAPKs nor did it restore MAPK signaling in endotoxin-tolerized MΦs. These findings correlate well with gene expression and protein levels of proinflammatory cytokines during tolerance observed both in IL-4Rα$^{-/-}$ and STAT6$^{-/-}$ MΦs (Fig. 3, Supplemental Fig. 2).

Inhibition of histone deacetylation induced by endotoxin tolerance in MΦs is not affected by IL-4Rα deficiency

Histone modification by acetylation and deacetylation of chromatin is important in the regulation of transcription (44). In general, histone acetylation is a positive regulator of transcription, whereas deacetylation is a negative regulator (44). It was reported that the downregulated expression of certain "tolerizable" genes can be reversed and their expression upregulated in the presence of TSA, an inhibitor of histone deacetylase (45, 46). Therefore, MΦs were pretreated with medium or LPS (as described in Fig. 1) in the absence or presence of TSA. Proinflammatory (Fig. 5A) and M2-associated chemokine (Fig. 5B) gene expression was measured. TSA profoundly inhibited IL-6 mRNA expression and, to a lesser

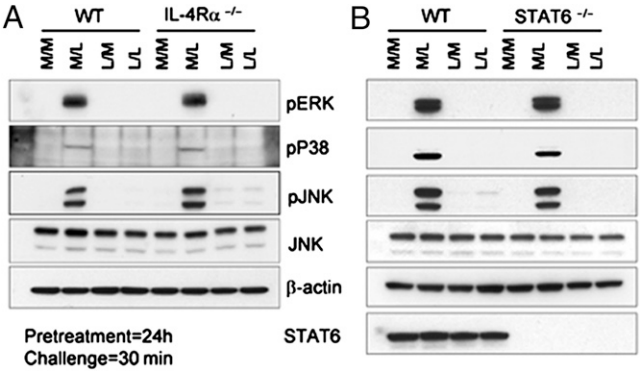

FIGURE 4. IL-4Rα or STAT6 deficiency does not affect the inhibition of LPS-driven MAPK activity seen in endotoxin-tolerized cells. Thioglycollate-elicited MΦ cultures from WT BALB/cByJ and IL-4Rα$^{-/-}$ mice (**A**) or WT BALB/cByJ and STAT6$^{-/-}$ mice (**B**) were pretreated with medium (M) or LPS (L) for 24 h, washed, and challenged with medium (M) or LPS (L) for 30 min. Whole-cell lysates from these cells were analyzed for MAPKs by Western blotting. Results of a single representative experiment of two independent experiments are shown.

extent, IL-1β and TNF-α mRNA expression in M/L-treated MΦs. In LPS-tolerized (L/M or L/L) MΦs, TSA led to a reversal of the tolerance phenotype only with respect to IL-1β gene expression. Importantly, IL-4Rα deficiency had no effect on this pattern of gene expression.

TSA also suppressed the expression of M2-associated chemokines (CCL2, CCL17, and CCL22) (Fig. 5B) induced by LPS in nontolerized MΦs (M/L). In LPS-tolerized MΦs, TSA reversed the suppression of all three M2-associated chemokines (L/L; Fig. 5B), but it failed to increase the expression of CCL2 mRNA unless the cells were restimulated with LPS. Again, IL-4Rα deficiency did not alter the effect of TSA on endotoxin tolerance, consistent with the data observed in Fig. 3.

LPS-pretreated WT and IL-4Rα–deficient animals are comparably tolerant to LPS + D-galactosamine–induced lethal shock and exhibit decreased LPS-induced cytokine levels in vivo

To extend the in vitro results that we observed in WT and IL-4Rα–deficient MΦs, WT and knockout mice were pretreated with PBS or LPS 24–30 h prior to challenge with LPS + D-galactosamine. All of the tolerized WT and IL-4Rα$^{-/-}$ mice survived an otherwise lethal challenge (Fig. 6A). Nontolerized (PBS-pretreated) control animals died within 24–30 h of LPS + D-galactosamine administration (Fig. 6A). The level of serum cytokines/chemokines in LPS-tolerized (L/L) and nontolerized (P/L) mice was also determined. IL-4Rα$^{-/-}$ mice were responsive to LPS to a comparable degree as were WT mice. Also, the levels of TNF-α, IL-6 (Fig. 6B), CCL2, and CCL22 (Fig. 6C) were equivalently downregulated in WT and IL-4Rα$^{-/-}$ mice that were pretreated with LPS, consistent with the survival data (Fig. 6A), as well as in vitro cytokines and chemokines that we observed throughout the study. Although CCL17 levels were slightly lower in the sera of LPS-tolerized mice challenged with LPS, the difference did not achieve statistical significance compared with PBS-pretreated, LPS-challenged values.

Discussion

Endotoxin tolerance has been recognized for >70 y, but it remains an enigma mechanistically. Because endotoxin tolerance is not a global shutdown of gene expression [i.e., some genes are repressed, whereas others are either upregulated or unchanged (47)],

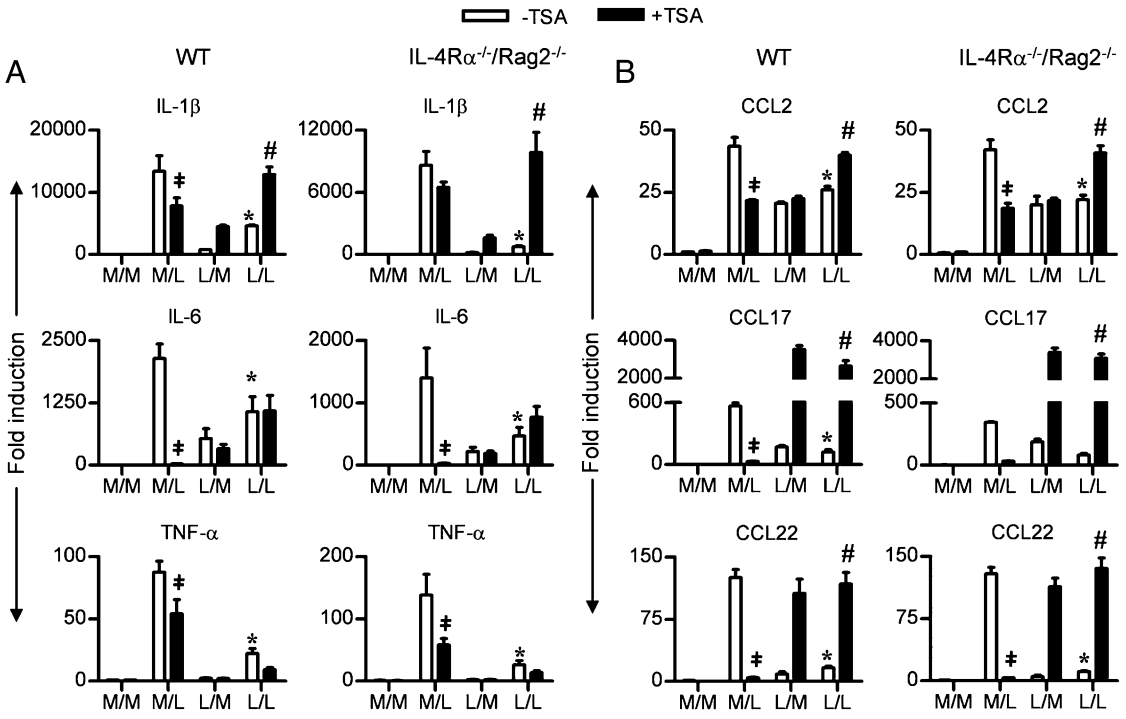

FIGURE 5. IL-4Rα deficiency does not affect the endotoxin-driven histone modification in endotoxin-tolerized MΦs. Thioglycollate-elicited MΦ cultures from WT BALB/cByJ and IL-4Rα$^{-/-}$/Rag2$^{-/-}$ mice were pretreated with medium (M) or LPS (L) in the presence or absence of TSA for 16–18 h, washed, and challenged with medium (M) or LPS (L) in the presence of absence of TSA for 3 h. Analysis of gene expression for CA-MΦ cytokines (**A**) and AA-MΦ–associated chemokines (**B**), measured by qRT-PCR. $^{‡}p < 0.05$, between nontolerized groups (M/L) in the absence or presence of TSA, $^{*}p < 0.05$, nontolerized (M/L) versus tolerized (L/L) groups, $^{\#}p < 0.05$, between tolerized (L/L) groups in the absence or presence of TSA.

the use of the word "reprogramming" was coined by Morrison and coworkers (48, 49) to describe the mixed hyporesponsive/responsive transcriptional phenotype. The mechanisms implicated in the induction of endotoxin tolerance are also complex: failure to recruit TLRs to rafts (50); failure to assemble the TLR-signaling complex or recruit downstream adapter molecules (10, 51, 52); diminished NF-κB, AP-1, MAPK, and other signaling pathways (10, 53); an increase in p50/p50 homodimers (54) and RelB (39); and chromatin remodeling (45, 55) have all been implicated in the induction of the tolerant state. During sepsis, acute and prolonged stimulation of MΦs and monocytes by LPS or other TLR ligands may lead to excessive and pathological inflammation. It was suggested that the hyporesponsiveness that develops in septic patients following the "cytokine storm" is an adaptive mechanism to protect the host against inflammatory damage; however, this TLR hyporesponsiveness may also render the host more susceptible to secondary bacterial infection (56).

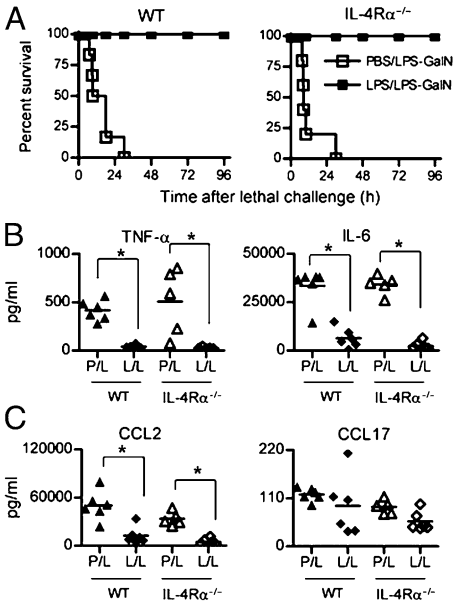

FIGURE 6. WT and IL-4Rα$^{-/-}$ mice are rendered comparably LPS-tolerant to LPS + D-galactosamine–induced lethal shock and LPS-induced cytokine production. (**A**) Survival of WT BALB/cByJ and IL-4Rα$^{-/-}$ mice pretreated with PBS or LPS (25 μg/mouse) and then challenged 1 d later with LPS + D-galactosamine (LPS-GalN). Serum cytokine (**B**) and chemokine (**C**) levels were measured by ELISA in nontolerized (PBS-pretreated) and tolerized (LPS-pretreated; 25 μg/mouse) WT and IL-4Rα$^{-/-}$ mice challenged with 25 μg LPS for 2 h. Each symbol represents an individual mouse. $^{*}p < 0.05$, between nontolerized (P/L) and tolerized (L/L) groups.

Recently, several studies found that the AA-MΦ differentiation state resembles endotoxin-tolerized MΦs phenotypically in both human monocytes and murine thioglycollate-elicited or bone marrow–derived MΦs, suggesting a common induction pathway (19). Specifically, Porta et al. (25) reported induction of tolerance by exposure of murine and human MΦs to LPS-induced gene-expression profiles that was consistent with AA-MΦ polarization (as evidenced by expression of CCL2, CCL17, and CCL22) and that the expression of these markers is associated with increased NF-κB p50, consistent with the findings of Ziegler-Heitbrock et al. (54) that p50/p50 homodimers predominate in endotoxin-tolerized MΦs. However, Porta et al. (25) also reported that LPS restimulation of LPS-pretreated MΦs resulted in sustained, rather than limited, expression of these same chemokines. In an effort to extend their findings, we first sought to replicate their protocol exactly: thioglycollate-elicited C57BL/6 MΦs were tolerized by overnight treatment in vitro with our protein-free *E. coli* K235 LPS. Tolerance induction was confirmed by the finding that expression of proinflammatory genes (e.g., TNF-α, IL-β, IL-6, IL-12 p40 and IFN-β) were, indeed, strongly induced in re-

sponse to primary exposure to LPS but clearly repressed upon secondary exposure to LPS (Fig. 1). Two markers frequently associated with the AA-MΦ phenotype, Arg-1 and MR, were also poorly induced by LPS pre- or postexposure (<10-fold; Fig. 1B); however, both were downregulated upon LPS restimulation in C57BL/6 MΦs, indicating that they, too, were subject to the effects of LPS-induced tolerance (Fig. 1). Although our data completely mirrored those of Porta et al. (25), in that the AA-MΦ–associated chemokine genes were strongly upregulated by LPS, we observed that expression of these chemokine genes was strongly inhibited in endotoxin-tolerized cells upon LPS restimulation. At this time, we cannot account for these discrepancies; however, we observed similar patterns of responsiveness in thioglycollate-elicited BALB/c MΦs, bone marrow–derived C57BL/6J MΦs (data not shown), and in the RAW 264.7 cell line, which was derived from BALB/c mice (Supplemental Fig. 1B). Thus, it is unlikely that the differences observed are due to strain differences or the source of MΦs.

Importantly, pretreatment of MΦs with IL-4 to induce an AA-MΦ state of MΦ differentiation (as evidenced by robust induction of Arg-1 mRNA; Fig. 2B and MR mRNA; data not shown) failed to diminish the LPS-induced expression of proinflammatory genes (e.g., TNF-α, IL-1β, and IL-6) (Fig. 2A); the AA-MΦ–associated chemokines CCL2, CCL17, and CCL22 (Fig. 2B); NF-κB binding or relative expression of p65 and p50, as observed in EMSA and supershift assays (Fig. 2C) or MAPK activation (Fig. 2D); and negative regulators of LPS signaling (Fig. 2E, 2F), in response to LPS challenge, in contrast to MΦs pretreated with LPS. Collectively, these data suggest that IL-4 stimulation of MΦs engagement does not induce the well-characterized signaling changes previously associated with LPS-induced tolerance. Because IL-4/IL-13–induced STAT6 activation is critical for the establishment of AA-MΦs (23), we reasoned that examining the requirement for IL-4Rα (which is used by both IL-4 and IL-13 in the type II IL-4R) and/or STAT6 (which is downstream of both IL-4/IL-13 signaling) might shed light on a possible relationship between these the AA-MΦ polarization and endotoxin tolerance. We confirmed that induction of both Arg-1 and MR mRNA by LPS was entirely dependent on both the IL-4Rα-chain and STAT6 signaling (Fig. 3B, Supplemental Fig. 2B). Although LPS induced IL-13, but not IL-4, mRNA, we were unable to detect a measurable increase in IL-13 protein in supernatants from LPS-stimulated MΦs (data not shown). However, the finding that IL-13$^{-/-}$ MΦs are fully tolerizable by LPS pretreatment (Supplemental Fig. 2G), but not by IL-4 pretreatment, further supports the hypothesis that neither LPS-induced IL-4 nor IL-13 mediates tolerance. Interestingly, the lack of IL-13 does not preclude the expression of CCL17 mRNA induced by LPS, suggesting that an alternate pathway for the induction of AA-MΦ–associated markers exists. The low level of Arg-1 mRNA induced by LPS was IL-4Rα and STAT6 dependent. Qualls et al. (57) showed that, in *Mycobacterium tuberculosis*–infected MΦs, Arg-1 was induced in the absence of STAT6 by autocrine stimulation by cytokines (IL-6, IL-10, and G-CSF) in a manner that was STAT3 and partially C/EBPβ dependent. We observed a low level of phospho-STAT3 in response to LPS, although this did not differ in WT and IL-4Rα$^{-/-}$ MΦs (data not shown). Moreover, STAT3 activation was slightly increased in response to LPS, but not IL-4 (Fig. 2F), in WT MΦs. Thus, it seems unlikely that the low level of Arg-1 mRNA detected in LPS-stimulated MΦs is due to the STAT3-dependent mechanism described by Qualls et al. (57). However, lack of IL-4R, STAT6, or IL-13 failed to affect the response to LPS for the AA-MΦ–associated chemokine or traditional proinflammatory gene and protein expression (Fig. 3C–F, Supplemental Fig. 2C–G). This was also the case for MAPK activation (Fig. 4). These data further support our central conclusion that the ability of MΦs to differentiate into AA-MΦs is not a prerequisite for endotoxin tolerance. Moreover, this same pattern of responsiveness to LPS in naive versus tolerized MΦs was observed in both C57BL/6 and BALB/c backgrounds for proinflammatory and chemokine genes.

A significant issue highlighted in our work concerns the relative plasticity and responsiveness of the AA-MΦ and endotoxin-tolerant phenotypes. AA-MΦs induced by IL-4 remained competent to respond to TLR ligands and could robustly induce the full array of classical proinflammatory cytokines. As such, this AA-MΦ phenotype can be readily reversed by TLR4 signaling. The signaling and epigenetic changes conferred in endotoxin tolerance appear to be far more durable, because exposure to subsequent TLR stimulation or to IL-4 does not restore either the M1 or M2 polarization, respectively. It is also important to note that the molecular differences that we describe in the regulation of TLR4 signaling between AA-MΦs and endotoxin-tolerant MΦs may reflect the different biological contexts in which these populations accumulate in vivo. As elegantly described in a recent publication by Jenkins et al. (58), CA-MΦs and, subsequently, endotoxin-tolerant MΦs rapidly accumulate at sites of microbial infection through the induced recruitment of Ly6C^{hi} "inflammatory" monocytes from the blood. In contrast, AA-MΦs appear to be generated in situ via IL-4/IL-13–dependent cell division in response to chronic "sterile" inflammation, such as generated during nematode infection or in cases of wound repair. Additionally, our data indicate that these AA-MΦs retain the ability to respond to microbial PAMPs via TLR signaling.

At the core of the current study, as well as those of other investigators, is the molecular definition of the AA-MΦ phenotype. There are relatively few studies that address why there are so few AA-MΦ markers in common between murine and human cells. Perhaps this is attributable to differences in the way in which the MΦs are prepared (unelicited, elicited, or bone marrow derived in the case of mouse versus differentiated overnight or longer and treated with growth-promoting agents or not in the case of human monocytes/MΦs) prior to treatment with AA-MΦ–inducing agents. There is a clear need for systematic examination of different cell types for their responsiveness to IL-4 or IL-13 over time to determine whether there is a truly common set of "markers" that defines the AA-MΦ phenotype in both species. For example, there does not seem to be consensus in the literature about the suitability of CC chemokines as MΦ alternative activation markers. We observed that these previously AA-MΦ–associated CC chemokines were also strongly induced by LPS, with kinetics that strongly resemble proinflammatory CA-MΦ genes, and that the LPS-dependent induction of the CC chemokines was blunted in LPS-tolerized MΦs. This same observation was reported by Foster et al. (45), who used microarray analysis to show that CCL22 was categorized as an endotoxin-tolerizable gene, and Carmody et al. (59), who showed that CCL2 gene expression was also repressed during tolerance. However, the fate of CCL17 gene expression during endotoxin tolerance has not been reported previously, although the induction of its gene expression by LPS was reported (27, 60).

IL-4 and IL-13 are considered anti-inflammatory cytokines that downmodulate the expression of proinflammatory cytokines, including TNF-α, IL-6, IL-8, and other inflammatory mediators (e.g., inducible NO synthase and cyclooxygenase-2), using IL-4Rα as a common receptor chain (61, 62). Of note, we observed slightly increased mRNA and protein levels of proinflammatory cytokines upon LPS stimulation in IL-4Rα$^{-/-}$ MΦs, indicating that the IL-4–signaling pathway plays an important role in keeping the

inflammatory response induced by LPS in check. Interestingly, we did not observe increased proinflammatory cytokine gene or protein expression in STAT6$^{-/-}$ MΦs, consistent with published reports showing that suppression of proinflammatory cytokines mediated by IL-4 via both STAT6-dependent and STAT6-independent mechanisms (63). Other signaling pathways downstream of the IL-4R complex, such as IRS2 and Shc, may suppress proinflammatory gene expression (64).

Additionally, IL-10 is another potential mediator of tolerance that we did not examine in the context of IL-4Rα$^{-/-}$. Previous studies showed that IL-10 suppressed the proinflammatory response induced by LPS (65, 66). However, Berg et al. (67) showed that IL-10 deficiency did not alter the endotoxin tolerance and that tolerized IL-10$^{-/-}$ mice nonetheless survived LPS-induced lethal shock. Finally, our molecular observations demonstrating a dissociation between endotoxin tolerance and alternative activation are directly supported by the in vivo LPS-induced lethality experiment in which both WT and IL-4Rα$^{-/-}$ mice survived a lethal exposure to LPS following the establishment of endotoxin tolerance. Based on the observed results, we believe that CCL2, CCL17, and CCL22 are not pure AA-MΦ markers (25), rather they are inducible by both LPS and IL-4 and may help to attract Th2 cells in vivo (26). Overall, our data indicate that endotoxin tolerance and AA-MΦ polarization are distinct pathways that are differentially regulated.

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Disclosures

The authors have no financial conflicts of interest.

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