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CUTTING EDGE

Cutting Edge: Involvement of the Type I IFN Production and Signaling Pathway in Lipopolysaccharide-Induced IL-10 Production[1]

Elmer Y. Chang,[2]†‡ Beichu Guo,[2]* Sean E. Doyle,* and Genhong Cheng[3]*§*

***Macrophages respond to LPS by the rapid activation of proinflammatory cytokines that serve to initiate host defense against microbial invasion. To prevent injury to the host from excess production of these cytokines, IL-10 is up-regulated to feedback inhibit the proinflammatory response. However, the molecular events responsible for LPS-induced up-regulation of IL-10 remain to be elucidated. In this study, we provide evidence that production of and signaling by type I IFN is required for LPS-induced IL-10 up-regulation. In addition, we demonstrate that defect in type I IFN production and signaling results in a trend toward LPS-mediated superinduction of proinflammatory genes and cytokines in bone marrow-derived macrophages. Our findings suggest a novel anti-inflammatory role for the type I IFN production and signaling pathway in regulating LPS response in bone marrow-derived macrophages. The Journal of Immunology,* 2007, 178: 6705–6709.**

Toll-like receptor 4-mediated recognition of LPS initiates MyD88-dependent signaling pathway that orchestrates the production of proinflammatory cytokines by innate immune cells such as macrophages (1). Even though these cytokines are crucial for host defense, excess proinflammatory cytokines give rise to systemic metabolic and hemodynamic disturbances that are harmful to the host. To avert these deleterious effects, IL-10 is also produced by LPS-stimulated macrophages (2–4), leading to Stat3 phosphorylation that has been correlated with the dampening of inflammatory response (5, 6).

In addition to the MyD88-dependent pathway, TLR4 triggers Toll/IL-1R homology domain-containing adaptor-inducing IFN-β (TRIF)-dependent[4] signaling events, including the activation of IFN regulatory factor 3 (IRF3) (7–10). This leads to the production of type I IFNs (IFN-$\alpha\beta$), which bind to the type I IFN receptor (IFNAR) to activate the JAK-STAT signaling pathway, resulting in the up-regulation of antiviral genes (11). In addition to their antiviral activities, type I IFNs appear to possess potent anti-inflammatory properties and have been used to treat autoimmune disease such as multiple sclerosis (12–14). The mechanism responsible for these anti-inflammatory effects is thought to be related to type I IFN-induced IL-10 production, although this premise has remained controversial due to conflicting data (13, 15–17). In the context of LPS stimulation, the role of type I IFNs in LPS-induced IL-10 production is even less clear. Therefore, in this study, we investigated the requirement for type I IFN production and signaling in LPS-induced IL-10 up-regulation and Stat3 phosphorylation in bone marrow-derived macrophages (BMDMs).

Materials and Methods

Reagents and mice

LPS was purchased from Sigma-Aldrich. Murine IFN-α and IFN-β proteins were from PBL Biomedical Laboratories. Ab reagents included anti-STAT1, anti-STAT3 (Santa Cruz Biotechnology), anti-phospho-STAT1, and anti-phospho-STAT3 (Tyr^{705}) Abs (Cell Signaling Technology). Anti-IL-10- and anti-IL-6-blocking Abs were from BD Pharmingen.

All mice used are on a C57BL/6 genetic background. B6.129S2-$Il6^{tm1Kopf}$/J (IL-$6^{-/-}$) and B6.129P2-$Il10^{tm1Cgn}$/J (IL-$10^{-/-}$) mice were purchased from The Jackson Laboratory. IFN Alpha Ro/o 129/Sv (IFNAR$^{-/-}$) mice were from B&K Universal and were backcrossed with C57BL/6J mice (The Jackson Laboratory) for five generations. $TRIF^{Lps2/Lps2}$ mutant mice were gifts from Dr. B. Beutler (The Scripps Research Institute, San Diego, CA). TLR4$^{-/-}$, MyD88$^{-/-}$, and littermate wild-type (WT) mice were gifts from Dr. S. Akira (Osaka University, Osaka, Japan). IRF3$^{-/-}$ and littermate WT mice were gifts from Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan). All mice were maintained and bred under specific pathogen-free conditions, and experiments were conducted within the parameters of our approved protocol.

Preparation of BMDMs

Murine BMDMs were generated by flushing bone marrow cells from femurs and tibias of mice. These cells were cultured for 7 days in DMEM (Mediatech) containing 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% conditioned medium (CM) from L929 cells overexpressing M-CSF.

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[4] Abbreviations used in this paper: TRIF, Toll/IL-1R homology domain-containing adaptor-inducing IFN-β; BMDM, bone marrow-derived macrophage; CHX, cycloheximide; CM, conditioned medium; IRF3, IFN regulatory factor 3; Q-PCR, quantitative PCR; WT, wild type.

Immunoblotting and ELISA

For Western blot analyses, protein samples extracted from stimulated cells in modified radioimmunoprecipitation assay buffer were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted using standard methods. For ELISA, cytokines in supernatant of stimulated cells were assayed using mouse ELISA kits from eBioscience, according to the manufacturer's instructions.

Real-time quantitative PCR (Q-PCR)

RNA was harvested from stimulated cells using the TRIzol reagent (Invitrogen Life Technologies), and cDNA templates were made using iScript (Bio-Rad) per the manufacturer's protocol. Q-PCR analyses were done using the iCycler thermocycler (Bio-Rad) as described previously (9). All sample values are normalized to their average β-actin values and are presented as relative expression units.

Results

IL-10 plays an important role in sustained LPS-induced Stat3 phosphorylation in BMDMs

Previous studies have demonstrated that IL-6 and IL-10 can induce Stat3 phosphorylation in macrophages (6, 18). Since LPS is known to up-regulate both IL-6 and IL-10, we investigated the relative contribution of these cytokines to LPS-induced Stat3 phosphorylation. Our examination of the kinetics of LPS-induced activation of Stat3 revealed maximal Stat3 phosphorylation 3 h after BMDMs were stimulated with LPS (Fig. 1*A*). Cycloheximide (CHX) experiments showed that LPS-induced Stat3 phosphorylation at this time point requires de novo protein synthesis (Fig. 1*B*).

To assess whether IL-6 or IL-10 is the newly synthesized protein that is required for LPS-induced Stat3 phosphorylation, BMDMs were stimulated with LPS for 3 h in the presence of excess blocking Abs against IL-6 or IL-10. We also used cell-free CM of BMDMs treated with LPS for 3 h to stimulate fresh BMDMs in the presence of excess blocking Abs against IL-6 or IL-10. Notably, LPS-induced or CM-induced Stat3 phosphorylation was completely suppressed by anti-IL-10 Ab but was only partially inhibited by anti-IL-6 Ab (Fig. 1*C*).

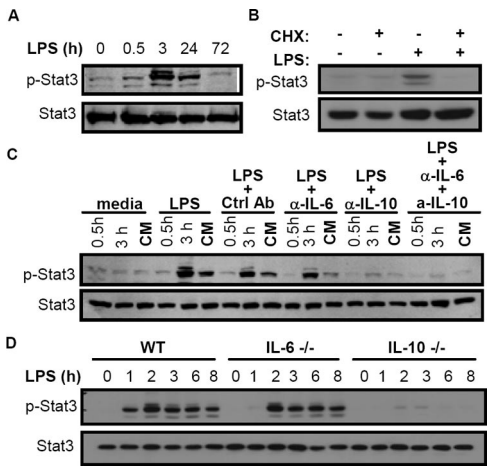

FIGURE 1. Sustained LPS-induced Stat3 phosphorylation is dependent on IL-10 in BMDMs. *A*, BMDMs from WT mice were stimulated with medium or 100 ng/ml LPS for the indicated time points. *B*, BMDMs were pretreated with or without CHX for 10 min, followed by stimulation with 100 ng/ml LPS for 3 h. *C*, BMDMs were stimulated with medium or 100 ng/ml LPS for 30 min or 3 h in the presence or absence of control Ab (Ctrl Ab), IL-6-blocking Ab (α-IL-6), IL-10-blocking Ab (α-IL-10), or both IL-6- and IL-10-blocking Abs. CM from BMDMs stimulated with medium or LPS for 3 h in the presence or absence of the aforementioned Abs was also used to stimulate fresh BMDMs for 30 min. *D*, WT, IL-$6^{-/-}$, and IL-$10^{-/-}$ BMDMs were stimulated with 100 ng/ml LPS for indicated time points. Western blot analysis was done to determine phospho-Stat3 (Tyr^{705}) and total Stat3 expression levels. Data shown are representative of three independent experiments.

To evaluate the temporal requirement for IL-6 and IL-10 in LPS-induced Stat3 phosphorylation, we stimulated WT, IL-$6^{-/-}$, and IL-$10^{-/-}$ BMDMs with LPS in a kinetic experiment. Interestingly, LPS-induced Stat3 phosphorylation in IL-$6^{-/-}$ BMDMs was defective only at 1 h but appeared to be intact thereafter. On the other hand, Stat3 phosphorylation was significantly abrogated in LPS-stimulated IL-$10^{-/-}$ BMDMs, regardless of stimulation time (Fig. 1*D*).

LPS-induced IL-10 production in BMDMs requires the type I IFN production and signaling pathway

Given that IL-10 is required for sustained LPS-induced Stat3 phosphorylation at later time points, we next examined the signaling pathway involved in LPS-stimulated IL-10 production in BMDMs. As anticipated, LPS-stimulated TLR4$^{-/-}$ BMDMs failed to up-regulate IL-10 mRNA (Fig. 2*A*) and protein (Fig. 2*B*). To examine whether MyD88 or TRIF is involved in LPS-induced IL-10 production, we measured IL-10 mRNA and protein in LPS-stimulated MyD88$^{-/-}$ and TRIF$^{Lps2/Lps2}$ BMDMs. Expectedly, LPS-treated MyD88$^{-/-}$ BMDMs were defective in IL-10 production (data not shown). However, unexpectedly, TRIF$^{Lps2/Lps2}$ BMDMs also demonstrated severe defect in LPS-induced IL-10 up-regulation compared with WT cells (Fig. 2, *C* and *D*).

To investigate whether the TRIF-mediated type I IFN production and signaling pathway is required for LPS-induced IL-10 production, we stimulated BMDMs from mice deficient of IRF3 or IFNAR with LPS. We found that the induction of both IL-10 mRNA (Fig. 2*C*) and protein (Fig. 2*D*) was markedly diminished in the knockout BMDMs compared with WT cells. Furthermore, the defect in IL-10 cytokine production lasted throughout the 24 h following LPS stimulation (Fig. 2*E*).

IFN-β is involved in LPS-induced IL-10 production in BMDMs

Previous studies have shown that the TRIF-dependent pathway is responsible for LPS-mediated up-regulation of type I IFNs (8–10). Since we demonstrated that BMDMs defective of critical signaling components in the TRIF-dependent pathway produced markedly lower levels of IL-10 in response to LPS, we next investigated whether a defect in type I IFN production in these BMDMs correlates with these findings. As anticipated, LPS-stimulated TRIF$^{Lps2/Lps2}$ and IRF3$^{-/-}$ BMDMs failed to produce any IFN-β, while it is strongly induced in the WT and IFNAR$^{-/-}$ BMDMs (Fig. 2*F*). To determine whether IFN-β per se plays an important role in LPS-induced IL-10 production, we treated LPS-stimulated BMDMs with incremental doses of blocking Ab against IFN-β. IL-10 mRNA was measured and found to be dose-dependently inhibited by the anti-IFN-β Ab (Fig. 2*G*).

IFN-β induces IL-10 production and IL-10-dependent Stat3 phosphorylation in BMDMs

Since we showed that IFN-β is required for LPS-induced IL-10 production, we next investigated whether IFN-β per se can stimulate IL-10 production and subsequent Stat3 phosphorylation through IFNAR in BMDMs. Strikingly, WT BMDMs

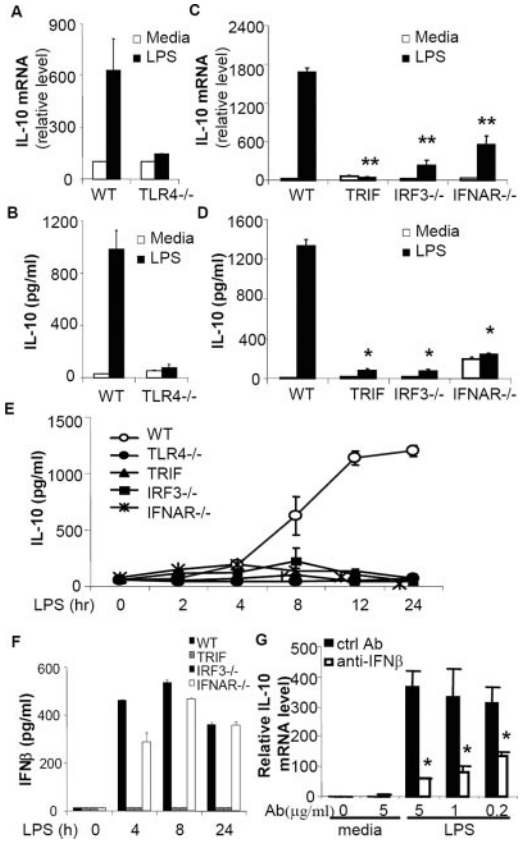

FIGURE 2. Type I IFN production and signaling pathway are required for LPS-induced IL-10 production in BMDMs. WT and TLR4$^{-/-}$ BMDMs were stimulated with medium or 100 ng/ml LPS. The amount of IL-10 mRNA transcript (*A*) and protein (*B*) was measured after 4 and 24 h of stimulation, respectively. WT, TRIF$^{Lps2/Lps2}$ (TRIF$^{-/-}$), IRF3$^{-/-}$, and IFNAR$^{-/-}$ BMDMs were stimulated with medium or LPS. The amount of IL-10 transcript (*C*) and protein (*D*) was measured after 4 and 24 h of stimulation, respectively. *E*, IL-10 protein levels in the supernatant of WT and different mutant BMDMs stimulated with medium or LPS for the indicated time points. *F*, WT, TRIF$^{Lps2/Lps2}$ (TRIF$^{-/-}$), IRF3$^{-/-}$, and IFNAR$^{-/-}$ BMDMs were stimulated with medium or LPS for 24 h. The amount of IFN-β in the supernatant was measured using ELISA. *G*, WT BMDMs were stimulated with medium or LPS for 4 h in the presence of incremental doses of Ctrl Ab or IFN-β-blocking Ab (anti-IFN-β). The amount of IL-10 transcript was measured by Q-PCR. Statistical significance of the differences in IL-10 level is indicated (*, $p < 0.05$, and **, $p < 0.01$; Student's *t* test). Data shown are representative of four independent experiments.

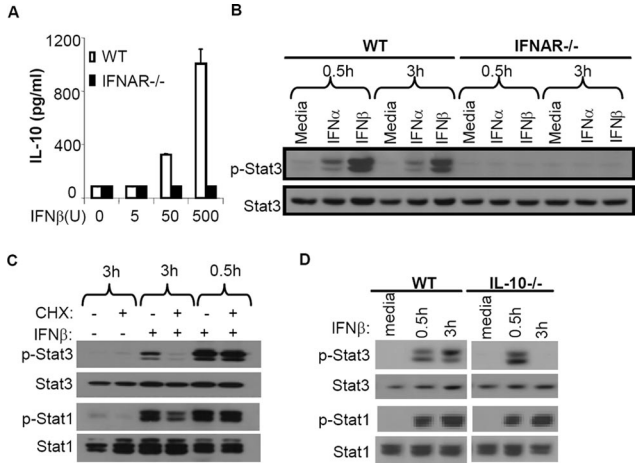

FIGURE 3. Type I IFN can induce IL-10 production and IL-10-dependent Stat3 phosphorylation in BMDMs. *A*, WT and IFNAR$^{-/-}$ BMDMs were stimulated with incremental doses of IFN-β for 24 h. The amount of IL-10 protein was measured using ELISA. *B*, WT and IFNAR$^{-/-}$ BMDMs were stimulated with 500 U/ml IFN-α or IFN-β for 30 min or 3 h. *C*, WT BMDMs were pretreated with medium or CHX for 10 min, followed by stimulation with either medium or 500 U/ml IFN-β for 30 min and 3 h. *D*, WT or IL-10$^{-/-}$ BMDMs were stimulated with medium or IFN-β for 30 min and 3 h. Phospho-Stat3, Stat3, phospho-Stat1, and Stat1 expression levels were determined by Western blot analysis. Data shown are representative of two independent experiments.

treated with IFN-β produced IL-10 in a dose-dependent manner, whereas IFNAR$^{-/-}$ BMDMs failed to do so (Fig. 3*A*). In addition, IFN-β induced Stat3 phosphorylation in BMDMs in an IFNAR-dependent manner (Fig. 3*B*). This induction was not due to LPS contamination, given that there is no difference in Stat3 phosphorylation between IFN-β-stimulated WT and TLR4$^{-/-}$ BMDMs (data not shown).

To assess whether IFN-β activates Stat3 directly, we stimulated CHX-pretreated or untreated WT BMDMs with IFN-β. At the 30-min time point, there was no difference in the phospho-Stat3 band between CHX-pretreated or untreated BMDMs. However, after 3 h, IFN-β-induced Stat3 phosphorylation was present in untreated cells but absent in CHX-pretreated cells (Fig. 3*C*). This indicates that de novo protein synthesis is required for sustained IFN-β-induced Stat3 phosphorylation at later time points. To determine whether IL-10 is the newly synthesized protein responsible for this sustained Stat3 phosphorylation, we stimulated WT and IL-10$^{-/-}$ BMDMs with IFN-β and found that IFN-β-induced Stat3 phosphorylation at 3 h require IL-10 (Fig. 3*D*).

Type I IFN production and signaling pathway is involved in the dampening of LPS-mediated proinflammatory genes and cytokines in BMDMs

Since our data demonstrate that IL-10 may be secondarily induced through the type I IFN production and signaling pathway, we investigated how this secondary induction of IL-10 may affect delayed LPS-induced gene program. Through Q-PCR analysis, we observed the superinduction of proinflammatory genes, such as IL-1β and TNF-α, in IL-10$^{-/-}$ BMDMs stimulated with LPS (Fig. 4, *A* and *C*). This superinduction of proinflammatory genes was confirmed by DNA microarray analysis on WT BMDMs stimulated with LPS for 12 h in the presence or absence of excess IL-10-blocking Ab. Gene expression profiles revealed that inhibition of IL-10 resulted in the increased expression of an array of LPS-induced proinflammatory genes, including IL-1β, TNF-α, and IL-12 (data not shown). We also observed the superinduction of proinflammatory cytokines, including IL-12 (Fig. 4*E*) and keratinocyte-derived chemokine (data not shown), in the LPS-stimulated IL-10$^{-/-}$ BMDMs as measured by ELISA.

Based on the importance of the type I IFN production and signaling pathway in LPS-induced IL-10 up-regulation, we hypothesized that mutation or deficiency of critical signaling components within this pathway would result in superinduced proinflammatory response in LPS-stimulated BMDMs at later time points. Indeed, TRIF$^{Lps2/Lps2}$ and IFNAR$^{-/-}$ BMDMs treated with LPS appeared to produce more proinflammatory genes and cytokines (Fig. 4, *B*, *D*, and *F*) compared with WT BMDMs, especially at later time points (i.e., 8 h).

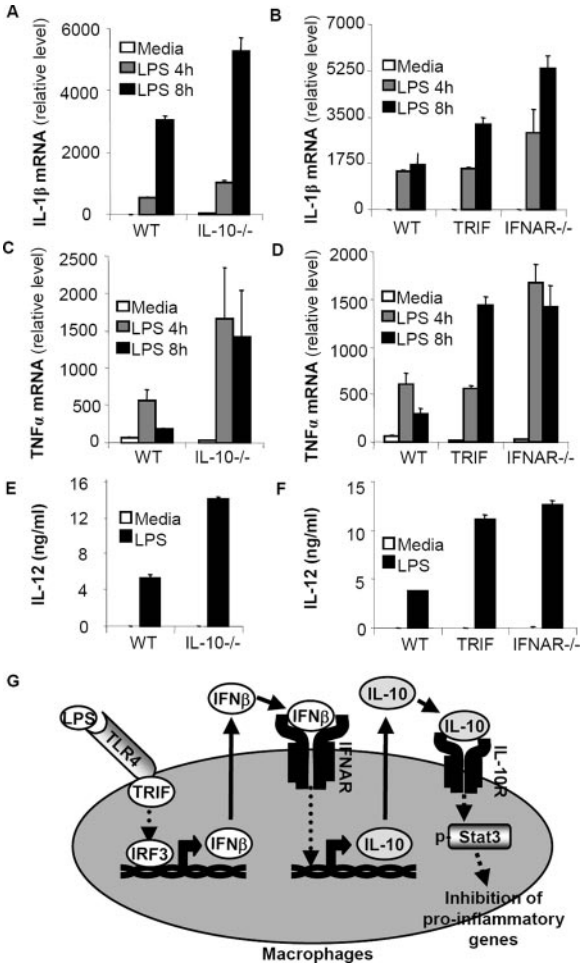

FIGURE 4. Deficiency of components of the type I IFN production and signaling pathway results in a trend toward superinduction of LPS-mediated proinflammatory response in BMDMs at late time points. *A* and *B*, Relative IL-1β mRNA expression levels from WT, IL-10$^{-/-}$, TRIF$^{Lps2/Lps2}$ (TRIF$^{-/-}$), and IFNAR$^{-/-}$ BMDMs stimulated with 100 ng/ml LPS for 4 and 8 h were measured by Q-PCR. *C* and *D*, Relative TNF-α mRNA expression levels from WT and different mutant BMDMs stimulated with LPS for 4 and 8 h. *E* and *F*, IL-12 protein levels from WT and different mutant BMDMs were assessed using ELISA after LPS stimulation for 24 h. Data shown are representative of two independent experiments. *G*, Model of the dependency of LPS-induced IL-10 production on type I IFN production and signaling pathway.

Discussion

In this study, we showed that while both IL-6 and IL-10 are important for transient early Stat3 phosphorylation in LPS-stimulated BMDMs, the IL-10-mediated autocrine/paracrine pathway is the main contributor to sustained LPS-induced activation of Stat3. This capacity to sustain Stat3 phosphorylation distinguishes the role of IL-10 from that of IL-6 in LPS signaling and may account for the anti-inflammatory property of IL-10.

Examining the role of type I IFN production and signaling pathway in LPS-induced IL-10 production in BMDMs, we found that TRIF, IRF3, and IFNAR are required. This is in agreement with unpublished as well as published data that other important components of the type I IFN production pathway, such as TANK-binding kinase-1 (data not shown) and TNFR-associated factor 3 (19), are also involved in LPS-induced up-regulation of IL-10. The central role of IFN-β in LPS-induced IL-10 production is demonstrated by the ability of IFN-β-blocking Ab to dose-dependently inhibit IL-10 mRNA up-regulation. Moreover, IFN-β per se was able to induce IL-10 production and Stat3 phosphorylation via IFNAR. Consistent with its requirement for sustained LPS-induced activation of Stat3, IL-10 is also essential for sustaining IFN-β-induced Stat3 phosphorylation at later time points. These data, together with the IL-10 production defect in LPS-stimulated TRIF$^{Lps2/Lps2}$, IRF3$^{-/-}$, and IFNAR$^{-/-}$ BMDMs, suggest that IL-10 may be secondarily up-regulated through LPS-induced IFN-β production. Given these observations, it would be interesting to uncover how IFN-β modulates IL-10 transcriptional events following LPS stimulation. Several candidate transcription factors that may be involved in LPS-induced IL-10 production in macrophages have been described previously (5, 20–23). Our findings in this study should serve as the basis for future investigations into the connection between IFN-β and these candidate transcription factors.

Finally, the functional role of the type I IFN production and signaling pathway in LPS-induced inflammatory response is demonstrated by the trend toward superinduction of several key proinflammatory genes and cytokines in LPS-stimulated TRIF$^{Lps2/Lps2}$ and IFNAR$^{-/-}$ BMDMs at later time points. These findings highlight the importance of this pathway in the delayed IL-10-orchestrated down-regulation of LPS-induced proinflammatory response in BMDMs. The biological implications of the type I IFN-mediated immunomodulatory effects remain controversial and could well be cell type specific. Further studies will be necessary to help characterize the in vivo significance of our findings.

In conclusion, the results from this study support a model in which the type I IFN production and signaling pathway is involved in LPS-induced IL-10 production and sustained Stat3 phosphorylation (Fig. 4*G*). The requirement of this pathway for IL-10 induction suggests that, in addition to its antiviral functions, the type I IFN pathway may serve a novel anti-inflammatory role in TLR4-mediated signaling in BMDMs.

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Disclosures

The authors have no financial conflict of interest.

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