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IL-6 Trans-Signaling Modulates TLR4-Dependent Inflammatory Responses via STAT3

Claire J. Greenhill,* Stefan Rose-John,† Rami Lissilaa,‡ Walter Ferlin,§ Matthias Ernst,§ Paul J. Hertzog,* Ashley Mansell,* and Brendan J. Jenkins,*

Innate immune responses triggered by the prototypical inflammatory stimulus LPS are mediated by TLR4 and involve the coordinated production of a multitude of inflammatory mediators, especially IL-6, which signals via the shared IL-6 cytokine family receptor subunit gp130. However, the exact role of IL-6, which can elicit either proinflammatory or anti-inflammatory responses, in the pathogenesis of TLR4-driven inflammatory disorders, as well as the identity of signaling pathways activated by IL-6 in a proinflammatory state, remain unclear. To define the contribution of gp130 signaling events to TLR4-driven inflammatory responses, we combined genetic and therapeutic approaches based on a series of gp130+/− knock-in mutant mice displaying hyperactivated IL-6–dependent JAK/STAT signaling in an experimental model of LPS/TLR4-mediated septic shock. The gp130+/− mice were markedly hypersensitive to LPS, which was associated with the specific upregulated production of IL-6, but not TNF-α. In gp130+/− mice, either genetic ablation of IL-6, Ab-mediated inhibition of IL-6R signaling or therapeutic blockade of IL-6 trans-signaling completely protected mice from LPS hypersensitivity. Furthermore, genetic reduction of STAT3 activity in gp130+/−:Stat3+/− mice alleviated LPS hypersensitivity and reduced LPS-induced IL-6 production. Additional genetic approaches demonstrated that the TLR4/Mal pathway contributed to LPS hypersensitivity and increased IL-6 production in gp130+/− mice. Collectively, these data demonstrate for the first time, to our knowledge, that IL-6 trans-signaling via STAT3 is a critical modulator of LPS-driven proinflammatory responses through cross-talk regulation of the TLR4/Mal signaling pathway, and potentially implicate cross-talk between JAK/STAT and TLR pathways as a broader mechanism that regulates the severity of the host inflammatory response. The Journal of Immunology, 2011, 186: 1199–1208.

Sepsis is a chronic, systemic inflammatory disorder that places a large burden on the public health system worldwide, with ~215,000 deaths and 750,000 cases of sepsis each year in the United States alone (1). In 45–60% of cases, sepsis is triggered by Gram-negative bacterial infections, and its most lethal form (endotoxic shock) is caused by LPS, the major component of the outer membrane of Gram-negative bacteria (2). To date, clinical trials in septic patients that tested the efficacy of potential immunotherapeutic strategies aimed at suppressing the inflammatory response, for instance, Ab-based anti–TNF-α therapy, have revealed only marginal benefits in reducing mortality (3). Such observations highlight the urgent need to identify other key inflammatory mediators driving the pathogenesis of sepsis that ultimately can be targeted for potential clinical benefit against this disorder.

TLR4 is a pathogen recognition receptor that plays a key role in the recognition of the prototypical inflammatory stimulus LPS and triggering the subsequent inflammatory response (4). LPS engagement of TLR4 initiates a cascade of signaling events via intracellular Toll/IL-1R signaling domains, which involves the primary recruitment of the Mal adaptor protein and its subsequent association with MyD88 to ultimately promote the activation of the NF-κB transcriptional complex and induction of many proinflammatory cytokine genes (i.e., MyD88-dependent pathway), such as IL-6, TNFα, and IL-1β (4–6). Host immune responses triggered by TLR4 also involve the recruitment of other intracellular signaling adaptors, in particular, TRIF and TRIF-related adaptor molecule, which also facilitate activation of NF-κB and IFN regulatory factor 3 (i.e., TRIF-dependent pathway), the latter of which promotes the transcription of proinflammatory type I IFN genes (7). After this initial wave of proinflammatory cytokine production, anti-inflammatory cytokines such as TGF-β and IL-10 are produced to dampen the TLR4-driven production of proinflammatory cytokines (8, 9).

Increased IL-6 production is a hallmark of many human chronic inflammatory states, including sepsis (10), rheumatoid arthritis (RA) (11), and inflammatory bowel disease (IBD)/colitis (12). In mice, experimentally induced RA and IBD are attenuated by aborting IL-6 signaling with a neutralizing Ab against the IL-6R (12, 13), and IL-6–deficient mice (IL-6−−) are resistant to experimentally induced colitis and arthritis (14–16). Although these observations support the notion that IL-6 is a critical cytokine contributing to these inflammatory symptoms, by contrast, the use of IL-6−− mice to investigate the role of IL-6 in response to local and systemic LPS-induced inflammatory responses has been controversial, with IL-6 having either a nonessential (17) or an anti-inflammatory (18) role during LPS-induced endotoxic shock.

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Abbreviations used in this article: CHO, Chinese hamster ovary; CLP, cecal ligation and puncture; IBD, inflammatory bowel disease; qPCR, quantitative real-time PCR; RA, rheumatoid arthritis; sIL-6Rα, soluble IL-6Rα; SOCS3, suppressor of cytokine signaling 3.

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The diverse portfolio and often opposing roles of IL-6 during the inflammatory response might be explained, at least in part, by its ability to initiate two modes of signaling: “classical” signaling via the interaction of IL-6 with its membrane-bound IL-6Rα subunit (19), and “trans-signaling” via a naturally occurring soluble IL-6Rα (sIL-6Rα) that is proteolytically cleaved from the cell surface (20). In both scenarios, intracellular signaling by IL-6 is dependent on the ubiquitously expressed common gp130 receptor subunit and is triggered by gp130-associated JAK kinases, which tyrosine phosphorylate the gp130 cytoplasmic domain to enable activation of STAT3 and, to a lesser extent, STAT1, as well as SH2 domain-containing protein tyrosine phosphatase-2-mediated MAPK and PI3K pathways (19). Conversely, tyrosine phosphorylation of gp130 at position 757 (759 in human gp130) plays a crucial role in the negative regulation of gp130 signaling by recruiting suppressor of cytokine signaling 3 (SOCS3) (21). However, the differential roles, if any, of IL-6 classical versus trans-signaling during sepsis and LPS/TLR4-driven inflammatory responses remain to be explored.

Although STAT3 is predominantly activated by IL-6 and other gp130-activating cytokines (e.g., IL-11, LIF, oncostatin-M), numerous innate immune cytokines including the anti-inflammatory IL-10 and proinflammatory type I and II IFNs also activate STAT3, which raises the question of the mechanistic basis by which STAT3 can mediate both opposing anti-inflammatory and proinflammatory responses. In light of the embryonic lethality displayed by Stat3−/− mice (22), mouse strains with a conditional deletion of Stat3 in macrophages/neutrophils or endothelial cells display an increased susceptibility to LPS associated with increased production of proinflammatory cytokines that has been attributed to defective IL-10–induced STAT3 activation (23, 24). Although these studies imply a key role for STAT3 in mediating the potent anti-inflammatory effects of IL-10, the role of STAT3 in facilitating proinflammatory responses of IL-6 and other STAT3-activating cytokines remains ill-defined with current Stat3 gene knock-out mouse models. Moreover, considering persistent STAT3 activation is a feature of numerous human inflammatory diseases (e.g., human ulcerative colitis, RA) (25, 26), there is a growing need for genetically defined mouse models displaying hyperactivated levels of endogenous STAT3 to investigate the mechanisms by which STAT3 promotes the pathogenesis of inflammatory diseases.

To define the regulatory role of IL-6–mediated STAT3 signaling during LPS/TLR4-driven inflammation, we present studies that use gp130F/F knockout mice harboring gp130-dependent endogenous STAT3 hyperactivation as a consequence of an impaired negative feedback loop by SOCS3 to downmodulate gp130/STAT3 signaling (27). Our studies demonstrate that gp130F/F mice are hyperresponsive to LPS/TLR4-driven local and systemic inflammatory responses, which is associated with the selective upregulation of IL-6. Using a combination of genetic and therapeutic approaches, we reveal that the targeted disruption of IL-6 trans-signaling via STAT3 in gp130F/F mice protects against LPS hypersensitivity. Furthermore, we identify a causal role for the TLR4/MyD88-dependent pathway, but not the TLR4/TRIF-dependent pathway, in the LPS hypersensitivity of gp130F/F mice, because gp130F/F mice lacking Mal, but not the type I IFN IFNAR2, displayed reduced sensitivity to LPS. Collectively, our observations suggest a mechanism whereby IL-6 trans-signaling involving STAT3 modulates LPS-driven inflammatory responses via the TLR4/Mal signaling axis.

Materials and Methods

Mice

The generation of gp130F/F and compound gp130F/F mutant mice heterozygous for Stat3 (gp130F/F;Stat3+/−) or homozygous null for IL-6 (gp130F/F; IL-6−/−) has been previously described (27–29). Mice homozygous null for the Ifnar2 gene have previously been generated (30) and were crossed with gp130F/F mice to generate the compound mutant gp130F/F;Ifnar2−/− mice. Mice homozygous null for the Mal gene (Mal−/−) were generated from mice in which exons 2 and 3 of the Mal gene were flanked with LoxP sites and then crossed with EIIA-Cre mice to generate null mice (D. Truman, L. O’Neill, P. Hertzog, manuscript in preparation). Mal−/− mice were then crossed with gp130F/F mice to generate compound mutant gp130F/F;Mal−/− mice. All experiments were performed following Animal Ethics approval from the Monash Medical Centre “A” Committee, and included gp130F/F (wild-type) littermate controls that were genetically matched. All mice were maintained under specific pathogen-free conditions and were age matched for each experiment.

LPS purification and quantification

Purification was performed to avoid contaminating proteins in the LPS preparations (lyophilized powder from Escherichia coli K-235; Sigma-Aldrich, St. Louis, MO) that would stimulate TLR pathways other than TLR4. This involved the preparation of a 10 mg/ml solution in 0.2% triethylamine and washes in sodium deoxycholate and water-saturated phenol with the top aqueous layer being collected. This purification was repeated on the phenol phase, after which aqueous phases were combined and ethanol precipitated and resuspended in 0.2% triethylamine to give a final LPS concentration of 10 mg/ml. RAW-Elam cells expressing an NF-κB reporter were stimulated and luciferase assays performed, to quantify LPS bioactivity.

Administration of LPS to mice

Mice were subjected to i.p. injection of repurified LPS at 4 mg/kg. The Mar-1 IFNAR1 blocking Ab (1 mg) or isotype control Ab (1 mg) were i.p. coinjected into mice, whereas the 2B10 IL-6R blocking Ab (1.5 mg) or isotype control (1.5 mg) Ab were i.p. injected for 1 h before LPS administration. For studies involving administration of sgp130Fc, mice were pretreated with 150 μg by i.p. injection for 16 h before LPS administration. In survival studies, mice were monitored over 72 h. In short-term studies (up to 6 h), mice were initially cheek bled (0 h time point) and then injected with LPS. At the indicated times, animals were culled and blood was collected via either cheek bleeds into EDTA-coated tubes for analysis on a Sysmex KX-21N hematology analyzer (Kobe, Japan) or cardiac puncture in preparation for serum. The peritoneal cavity of mice was lavaged with ice-cold PBS, and spleen and liver tissues were also collected and snap frozen.

Macrophage cultures

Bone marrow macrophages were generated from the flushed femurs of mice as previously described (31). Resident peritoneal macrophages were isolated by peritoneal lavage and plating cells at ~1.5 × 10⁶ cells/well in a 24-well plate in RPMI 1640 media containing 10% FCS. Medium was changed 2–4 h later to remove nonadherent cells, and stimulations were performed the following day.

Cytokine production

Murine IL-6 (Pharmingen, San Diego, CA), CCL5 (R&D Systems, Minneapolis, MN), TNF-α (Becton Dickinson Biosciences, San Jose, CA), Cxcl1 (R&D Systems), and IL-11, LIF, oncostatin-M) were quantified using commercial ELISA kits according to the manufacturers’ instructions. Cytokine production was normalized to viable cell numbers using a colorimetric MTT assay.

Generation of anti–IL-6Rα mAb, 2B10

Male Wistar rats (Charles River Laboratories, Wilmington, MA) were immunized by i.p. injection three times at three weekly intervals with 10⁷ Chinese hamster ovary (CHO) cells expressing high levels of mouse membrane–IL-6Rα in MonoPhosphoryl Lipid A plus synthetic Trehalose Dicyrornycolyl Mycolate adjuvant (Sigma-Aldrich), followed by an s.c. hyperimmunization with 10 μg sIL-6Rα. After 3 d, a fusion was performed between spleenocytes and the Sp2/0 myeloma fusion partner as previously described (32). Subsequent screening of hybridomas was performed on mock-transfected CHO cells, or CHO cells expressing mIL-6Rα, using the 8200 cellular detection system (Applied Biosystems, Zug, Switzerland). Positive clones were tested for their capacity to neutralize the IL-6-dependent proliferation of the murine plasmacytoma cell line, T1165.

Western blot analyses

Protein extracts from frozen spleen or liver tissue were prepared using ice-cold lysis buffer, after which they were precleared of cellular debris before separation by SDS-PAGE. Immunoblotting was performed with specific
Abs against ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA), STAT3, and phosphoSTAT3-Tyr705 (Cell Signaling Technology, Beverly, MA). Immunolabeled proteins were detected using Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) with the appropriate secondary Abs as per the manufacturer’s instructions.

RNA isolation and expression analyses

RNA was isolated from peritoneal macrophages using the RNeasy Mini Kit (Qiagen, Valencia, CA), as per the manufacturer’s protocol, and cDNA was prepared from 140 ng RNA using the SuperScript III System (Invitrogen Life Technologies, Carlsbad, CA). RNA was isolated from snap-frozen spleen tissue by homogenizing in TRizol (Invitrogen Life Technologies), followed by DNase treatment using the Qiagen RNAeasy column kit. cDNA was then prepared from 1 μg total RNA using the Transcription High Fidelity cDNA synthesis kit (Roche, Basel, Switzerland). Quantitative real-time PCR (qPCR) gene expression analyses were performed on triplicate samples with SYBR Green (Invitrogen) using the 7900HT Fast RT-PCR System (Applied Biosystems, Foster City, CA) over 40 cycles (95°C/15 s, 60°C/1 min) after an initial degradation step of 95°C for 10 min. Data acquisition and analyses were performed with the Sequence Detection System Version 2.3 software (Applied Biosystems). Sequences for the mouse primer sets used are available on request.

Cytospins and staining

Total cell counts of peritoneal lavage were determined using the Sysmex KK-21N automated hematology analyzer, after which cells (5 × 10⁶ per sample) were centrifuged on a Cytospin III (Shandon Scientific, Cheshire, UK). The composition of the peritoneal leukocyte infiltrate was assessed by differential cell counting of Wright-Giemsa stained slides using light microscopy. A minimum of 200 cells/slide were counted.

Statistical analysis

Data are expressed as means ± SEM, and statistical analyses were performed using the GraphPad PRISM software (GraphPad Software, San Diego, CA). Normally distributed data were analyzed using a paired t test, and if data did not show normal distribution, a Mann-Whitney U test was performed. One-way ANOVA was used to determine the differences between the two genotypes for all normally distributed data. If data did not show normal distribution, then a Kruskal-Wallis one-way ANOVA on ranks was performed. A P value <0.05 was considered statistically significant.

Results

Hypersensitivity of gp130F/F mice to LPS-induced endotoxic shock correlates with increased production of IL-6

To investigate whether gp130 signaling could modulate TLR4-driven inflammatory responses in vivo, we subjected gp130F/F mice displaying deregulated activation of gp130 signaling cascades to an LPS-mediated endotoxic shock model. A sublethal dose (for control gp130+/+ animals) of LPS (4 mg/kg) was i.p. injected into both gp130+/+ and gp130F/F mice, and the survival of mice was monitored over 72 h. As shown in Fig. 1A, 100% of the gp130+/+ control mice survived over 72 h after LPS administration. By contrast, gp130F/F mice displayed a remarkable hypersensitivity to LPS, with 100% of gp130F/F mice failing to survive beyond 72 h (Fig. 1A).

It is well established that hypersensitivity to LPS is characterized by augmented TLR4-driven, proinflammatory cytokine production. We therefore measured the levels of key TLR4/MyD88-dependent proinflammatory cytokines in the serum of mice up to 6 h after LPS administration. ELISA assays revealed that the serum concentration of IL-6 in LPS-treated gp130F/F mice was significantly increased by ~3.5-fold (1.5 h) and 2-fold (3 and 6 h) compared with gp130+/+ mice (Fig. 1B). By contrast, TNF-α serum levels were comparable between LPS-challenged gp130+/+ and gp130F/F mice (Fig. 1C). qPCR gene expression analyses in splenocytes from LPS-injected mice confirmed that IL-6 mRNA was induced more in gp130F/F splenocytes compared with gp130+/+ splenocytes (17-fold at 1.5 h and 12.5-fold at 3 h), whereas mRNA levels of TNFα and the LPS-responsive gene Socs3 were comparable between the two genotypes (Fig. 2A–C). We also observed augmented expression of TLR4/TRIF-dependent genes IFNβ (2.5- and 11-fold at 1.5 and 3 h, respectively) and Ccl5 (7-fold at 6 h) in LPS-treated splenocytes from gp130F/F compared with gp130+/+ mice (Fig. 2D, E).

To examine whether local TLR4-driven inflammatory responses were also heightened in gp130F/F mice, we next measured the production of IL-6 locally at the site of LPS injection (peritoneal cavity). Indeed, IL-6 protein levels in the peritoneal lavage fluid of LPS-challenged gp130F/F mice were significantly increased at 3 (3-fold) and 6 h (34-fold) compared with gp130+/+ mice (Fig. 3A). In addition, we also observed that LPS induced a 4-fold greater influx of neutrophils into the peritoneal cavity of gp130F/F mice at
3 h (Fig. 3B), which coincided with a 2-fold increase in the production of the neutrophil-attractant KC/Cxcl1 in the lavage of gp130<sup>F/F</sup> compared with gp130<sup>+/+</sup> mice in response to LPS (Fig. 3C). Collectively, these data identify that a subset of TLR4-driven local and systemic inflammatory responses in vivo are augmented in gp130<sup>F/F</sup> mice.

**Hyperresponsiveness of gp130<sup>F/F</sup> mice to LPS is not associated with impaired production and/or activity of IL-10**

IL-10 is a potent anti-inflammatory cytokine that is induced by LPS to negate the inflammatory response by suppressing the release of proinflammatory mediators (33). We therefore investigated whether the hyperresponsiveness of gp130<sup>F/F</sup> mice to LPS could be explained by the impaired production and/or biological activity of IL-10. As shown in Fig. 4A, serum levels of IL-10 in LPS-injected gp130<sup>+/+</sup> and gp130<sup>F/F</sup> mice were comparable. Furthermore, IL-10 gene expression in splenocytes from LPS-challenged gp130<sup>F/F</sup> mice was increased at all measured time points compared with gp130<sup>+/+</sup> mice (Fig. 4B), suggesting that LPS/TLR4-induced IL-10 production was not impaired in gp130<sup>F/F</sup> mice.

We next determined whether the ability of IL-10 to inhibit TLR4-driven proinflammatory cytokine production was impaired by the gp130Y<sub>757</sub>F mutation. Treatment of gp130<sup>+/+</sup> and gp130<sup>F/F</sup> peritoneal macrophages with IL-10 led to a similar 90% inhibition of LPS-induced IL-6 mRNA expression (Fig. 4C). Furthermore, flow cytometric analyses of IL-6–stimulated gp130<sup>+/+</sup> and gp130<sup>F/F</sup> macrophages revealed a comparable induction in the intracellular levels of STAT3 tyrosine phosphorylation in response to IL-10, thus confirming that IL-10 signaling was intact in gp130<sup>F/F</sup> cells (Fig. 4D). Collectively, these data therefore suggest that augmented TLR4-mediated inflammatory responses in gp130<sup>F/F</sup> mice are not due to the impaired production or activity of IL-10.

**TLR4-driven IFNβ production is not responsible for LPS hypersensitivity in gp130<sup>F/F</sup> mice**

Activation of TLR4 by LPS also induces the expression of IFN-β via the TRIF-dependent pathway and results in the activation of STAT1, which is composed of IFNAR1 and IFNAR2 (34, 35). Our data showing that IFNβ and Ccl5 mRNA levels were increased in LPS-challenged gp130<sup>F/F</sup> mice suggest that augmented STAT signaling via IFNβ could also contribute to the LPS/TLR4-driven hypersensitivity of gp130<sup>F/F</sup> mice (Fig. 2D, E). To address this, we initially took an in vivo Ab-based approach to suppress the biological actions of type I IFNs (i.e., IFN-α/β) in gp130<sup>F/F</sup> mice challenged with LPS. Specifically, gp130<sup>F/F</sup> mice were coinjected with LPS and either an IFNAR1 blocking Ab (Mar-1) at a dose previously shown to block the actions of IFN-β (1 mg/mouse) (36), or its isotype control. In response to LPS, all gp130<sup>F/F</sup> mice treated with the isotype control Ab failed to survive past 72 h, and only 25% of gp130<sup>F/F</sup> mice treated with the Mar-1 Ab survived (Fig. 5A), thus suggesting that in vivo targeting of the type I IFN pathway did not substantially alleviate the LPS/TLR4 hypersensitivity of gp130<sup>F/F</sup> mice. To confirm the neutralizing activity of the Mar-1 Ab on type I IFN signaling in gp130<sup>F/F</sup> mice, peritoneal macrophages derived from gp130<sup>F/F</sup> mice were stimulated with either LPS or IFN-α together with the Mar-1 Ab or isotype control over 5 h, after which the expression of the IFN/STAT-dependent target gene ISG15 was examined by qPCR. Stimulation of isotype control Ab-treated gp130<sup>F/F</sup> macrophages with LPS or IFN-α led to an approximate 14- and 18-fold induction, respectively, of ISG15 mRNA (Fig. 5B). By contrast, treatment with the Mar-1 Ab suppressed the induction of ISG15 mRNA by LPS or IFN-α by ~5-fold. Notably, blocking the biological activity of type I IFNs failed to have any effect on the levels of IL-6 mRNA induced by LPS in gp130<sup>F/F</sup> macrophages (Fig. 5C).
The TLR4/MyD88-dependent pathway promotes hyperinflammatory responses in gp130F/F mice to LPS

The Mal signaling adaptor acts as a crucial bridge by recruiting MyD88 to TLR4 to facilitate LPS-induced activation of the NF-κB transcription factor and induction of proinflammatory cytokine production (MyD88-dependent pathway) (6). To determine whether gp130 signaling modulated LPS/TLR4-induced responses via Mal, we generated gp130F/F mice lacking Mal and then subjected the compound mutant gp130F/F:Mal−/− mice to LPS challenge. Notably, the genetic ablation of Mal in gp130F/F:Mal−/− mice dramatically alleviated their hypersensitivity to LPS (Fig. 6A). Furthermore, consistent with the vital role for Mal in facilitating the induction of TLR4/NF-κB–dependent proinflammatory cytokines, IL-6 serum levels were significantly reduced by 2-fold in gp130F/F:Mal−/− mice (Fig. 6B), and TNF-α production was also reduced, albeit not significantly (Fig. 6C). By contrast, serum levels of the TRIF-dependent chemokine Ccl5 were comparable between the genotypes (Fig. 6D), consistent with a lack of a role for Mal in the TRIF-dependent pathway. Collectively, these data suggest a key role for the TLR4/Mal signaling axis, rather than the TLR4/TRIF-dependent pathway, in augmenting LPS-driven IL-6–specific inflammatory responses in gp130F/F mice.

IL-6 trans-signaling promotes LPS/TLR4-induced hyperinflammatory responses in gp130F/F mice

The augmented production of IL-6 in LPS-hypersensitive gp130F/F mice, and the reduction in IL-6 levels in gp130F/F:Mal−/− mice displaying partial protection from LPS hypersensitivity, suggests a positive correlation between the extent of LPS/TLR4-induced IL-6 production and hypersensitivity in gp130F/F mice. To provide genetic evidence for a causative pathologic role of IL-6 in the LPS/TLR4-induced hypersensitivity of gp130F/F mice, we next used gp130F/F mice in which IL-6 had been genetically ablated (29). Notably, in contrast with gp130F/F mice, all gp130F/F:IL-6−/− mice were completely resistant to LPS-induced shock (Fig. 7A),

Our observation that a small number of gp130F/F mice pretreated with the Mar-1 Ab survived suggested that the Ab only partially blocked the in vivo TLR4-mediated actions of type I IFNs or that type I IFNs do contribute (at least in part) to the impaired survival of gp130F/F mice to LPS, or both. We therefore performed genetic complementation studies to provide definitive evidence that the LPS/TLR4-driven hypersensitivity of gp130F/F mice occurred independently of type I IFN signaling. Specifically, compound mutant gp130F/F:Ifnar2−/− mice were generated in which the Ifnar2 receptor that is required for type I IFN signaling was genetically ablated. As shown in Fig. 5A, no gp130F/F:Ifnar2−/− mice survived beyond 72 h on challenge with 4 mg/kg LPS. In addition, the production of proinflammatory cytokines IL-6 and Ccl5 were not reduced in the serum of the gp130F/F:Ifnar2−/− compared with gp130F/F mice (Fig. 5D, E). Collectively, these data suggest that the modulation of LPS/TLR4-dependent inflammatory responses in gp130F/F mice occurs independently of type I IFN production and signaling via IFNAR2.

FIGURE 5. Blocking type I IFN signaling via the TLR4/IFN regulatory factor 3 pathway does not protect gp130F/F mice from LPS hypersensitivity. A, Survival over 72 h of gp130F/F (F/F) mice coinjected with LPS (4 mg/kg) and Mar-1 Ab (solid line; n = 8) or isotype control (dashed line; n = 6) (1 mg/mouse), and gp130F/F:Ifnar2−/− mice (F/F:Ifnar2−/−, dotted line; n = 7) injected with LPS alone. B, qPCR was performed on F/F peritoneal macrophages to determine ISG15 mRNA levels at 5 h after stimulation with LPS (1 ng/ml) or IFN-α (1000 IU/ml), together with the Mar-1 Ab (1 μg/ml) or isotype control (iso; 1 μg/ml). Expression data from two samples are shown after normalization to 18S expression, and are presented from replicate analysis as the mean fold induction ± SEM. C, IL-6 protein levels were assessed after 3 h by ELISA. Data are representative of three individual experiments and are expressed as the mean ± SEM relative to unstimulated macrophages. *p < 0.05. D, IL-6 and (E) Ccl5 ELISA assays on serum from LPS-injected F/F (black bars) and F/F:Ifnar2−/− (white bars) mice. Expression data from three samples per genotype are presented from replicate analysis as the mean fold induction ± SEM.

FIGURE 6. Genetic ablation of Mal alleviates LPS hypersensitivity of gp130F/F mice and reduces LPS-induced IL-6 expression. A, Survival of gp130F/F (F/F, solid line; n = 5) and gp130F/F:Mal−/− (F/F:Mal−/−, dotted line; n = 7) mice >72 h after i.p. administration of 4 mg/kg LPS. B, IL-6. (C) TNF-α, and (D) Ccl5 ELISAs were performed on serum collected from F/F (black bars) and F/F:Mal−/− (white bars) mice at the indicated time points after i.p. administration of 4 mg/kg LPS. Data are from at least three mice of each genotype and are expressed as the mean ± SEM. ***p < 0.001 versus data from F/F mice at the corresponding time point.
thus indicating that IL-6 is the primary gp130-acting proinflammatory cytokine that promotes LPS hypersensitivity in $gp130^{+/+}$ mice.

To further demonstrate that IL-6 signaling is responsible for the LPS hypersensitivity of $gp130^{+/+}$ mice, we next used a preventative approach by using the 2B10 Ab raised against the ligand-binding IL-6Rα subunit that abolishes IL-6-dependent signaling. Specifically, the 2B10 Ab or isotype control Ab (1.5 mg/mouse) were i.p. injected for 1 h before LPS administration (4 mg/kg), after which $gp130^{+/+}$ mice were monitored over 72 h. As shown in Fig. 7B, $gp130^{+/+}$ mice pretreated with the isotype control Ab remained hypersensitive to LPS-induced mortality, whereas all $gp130^{+/+}$ mice pretreated with the IL-6R Ab were resistant to LPS-induced lethality.

IL-6 signal transduction occurs via two different modes, one involving the membrane-bound IL-6Rα subunit (classical signaling) and the other sIL-6Rα (trans-signaling) (20). Although a proinflammatory role for IL-6 trans-signaling has been suggested in various chronic inflammatory diseases and cancer (12, 16, 37, 38), the role of IL-6 trans-signaling in the pathogenesis of sepsis, and more specifically, LPS/TLR4-mediated endotoxic shock, is ill-defined. To examine whether trans-signaling is playing a causative role in the LPS hypersensitivity exhibited by $gp130^{+/+}$ mice, we pretreated LPS-challenged $gp130^{+/+}$ mice with sgp130Fc, a recombinant version of soluble gp130 that specifically antagonizes IL-6 trans-signaling (16). As shown in Fig. 7C, $gp130^{+/+}$ mice pretreated with sgp130Fc were completely resistant to LPS hypersensitivity over 72 h, thus revealing that IL-6 trans-signaling exacerbates TLR4-dependent inflammatory responses in $gp130^{+/+}$ mice. Moreover, the general applicability of IL-6 trans-signaling as a key proinflammatory mechanism in LPS-mediated endotoxic shock in normal mice was demonstrated by the complete protection of wild-type $gp130^{+/+}$ mice against a lethal dose of LPS on sgp130Fc pretreatment (Fig. 7D).

**FIGURE 7.** Genetic and therapeutic abrogation of IL-6 trans-signaling in mice protects against LPS hypersensitivity. Survival over 72 h of (A) $gp130^{+/+}$ (FF, solid line; $n = 11$) and $gp130^{+/+}$-IL-6$^{-/-}$ (FF-IL-6$^{-/-}$, dotted line; $n = 6$) mice after i.p. injection of 4 mg/kg LPS. (B) FF/F mice pretreated with IL-6Rα blocking Ab 2B10 (dotted line; $n = 4$) or isotype control (iso; solid line; $n = 3$) followed by i.p. injection of LPS. (C) FF/F mice untreated (solid line; $n = 4$) or pretreated with sgp130Fc (dotted line; $n = 4$) followed by i.p. injection of LPS, and (D) +/- mice untreated (solid line; $n = 4$) or pretreated with sgp130Fc (dotted line; $n = 4$) followed by i.p. injection of 6 mg/kg LPS.

**STAT3 promotes LPS/TLR4-induced hyperinflammatory responses in $gp130^{+/+}$ mice**

Among the numerous intracellular signaling cascades activated by IL-6 via the signal-transducing gp130 receptor subunit, the predominant pathway activated is JAK2/STAT3 (19). Our observations that LPS augmented IL-6 production in $gp130^{+/+}$ mice and that IL-6 trans-signaling promoted the LPS hypersensitivity of $gp130^{+/+}$ mice therefore led us to initially investigate whether STAT3 was systemically hyperactivated by LPS in $gp130^{+/+}$ mice. Indeed, Western blot analyses of spleen and liver lysates from LPS-treated mice demonstrated that STAT3 tyrosine phosphorylation (i.e., activation) was exaggerated in $gp130^{+/+}$ compared with $gp130^{+/+}$ mice in response to LPS (Fig. 8A, B).

The in vitro pharmacological inhibition or in vivo conditional genetic ablation of STAT3 has assigned both anti-inflammatory and proinflammatory roles for STAT3 in the context of LPS/TLR4-induced inflammatory responses (23, 24, 39), although the mechanistic basis for these opposing roles of STAT3 in modulating TLR4-driven inflammatory responses remains poorly understood. To formally define the contribution of endogenous STAT3 hyperactivation (via IL-6 trans-signaling) to the TLR4-driven hyperinflammatory phenotype of LPS-challenged $gp130^{+/+}$ mice, we used $gp130^{+/+}$:Stat3+/- mice in which the level of IL-30-dependent STAT3 activation has been genetically reduced (28). As predicted, compared with the exaggerated STAT3 tyrosine phosphorylation observed in LPS-treated $gp130^{+/+}$ mice, levels of tyrosine phosphorylated STAT3 were substantially lower in LPS-treated $gp130^{+/+}$:Stat3+/- mice and comparable with $gp130^{+/+}$ mice (Fig. 8D). Moreover, compared with $gp130^{+/+}$ mice, the genetic reduction of STAT3 activity in $gp130^{+/+}$:Stat3+/- mice dramatically alleviated the LPS hypersensitivity, with 78% of $gp130^{+/+}$:Stat3+/- mice surviving over 72 h (Fig. 8C). Furthermore, LPS-induced STAT3 tyrosine phosphorylation was dramatically suppressed in the $gp130^{+/+}$:IL-6$^{-/-}$ mice that were resistant to LPS-induced shock (Fig. 8D), thus revealing an essential role for IL-6 in promoting STAT3 tyrosine phosphorylation in response to LPS.

In further support of our earlier data, and consistent with the notion that augmented IL-6 production correlates strongly with the LPS-induced hypersensitivity of $gp130^{+/+}$ mice, the improved survival of LPS-challenged $gp130^{+/+}$:Stat3+/- mice correlated with a reduction in serum protein, peritoneal lavage protein, and splenocyte mRNA levels of LPS-induced IL-6 (compared with $gp130^{+/+}$ mice) that was similar to those measured in $gp130^{+/+}$ mice (Fig. 8E–G). Collectively, these data demonstrate for the first time, to our knowledge, a causal role for exaggerated IL-6–induced trans-signaling via STAT3 in augmenting TLR4-driven inflammatory responses.

**Discussion**

In this study, we have combined both genetic and therapeutic approaches to demonstrate in vivo for the first time, to our knowledge, that the IL-6/gp130/STAT3 signaling axis modulates LPS/TLR4-driven inflammatory responses. The hyperresponsiveness of $gp130^{+/+}$ mice to LPS involved the specific upregulation of IL-6 in a gp130/STAT3- and TLR4/MyD-dependent manner, suggesting both pathways synergize to promote the production of IL-6 in response to LPS (Fig. 9). Moreover, we have made the discovery that IL-6 trans-signaling via the sIL-6R is the primary mode of signaling that elicits the potent proinflammatory actions of IL-6 during LPS/TLR4-driven endotoxic shock. This latter finding contributes to the growing body of evidence implicating a key role for IL-6 trans-signaling in the pathogenesis of experimentally induced inflammation models, as has previously been shown for peritonitis, colitis, and inflammatory arthritis (12, 16, 37). Because upregulated...
production of IL-6 and increased activation of STAT3 are common traits of these human chronic inflammatory states, our study is also likely to provide important mechanistic insights of potential clinical relevance to such disorders driven by microbial or endogenous TLR ligands, or both.

Another important finding of this study was the preferential upregulation of IL-6 after LPS stimulation compared with TNF-α in an in vivo disease model (i.e., gp130F/F mice). Although the mechanistic rationale for this observation remains unclear, it is likely to reflect subtle differences in the transcriptional regulation of specific proinflammatory genes produced via TLR4 signaling cascades. For instance, activation of p38 MAPK is required for the LPS/TLR4-induced expression of TNF-α, but not IL-6 (40). Moreover, consistent with our in vivo data, in vitro studies have shown that blocking STAT3 activity preferentially inhibits LPS-mediated IL-1β and IL-6 production, but not TNF-α, in RAW264.7 cells (39), and STAT3 activation does not directly regulate LPS-induced TNF-α production in human monocytes (41). Our data demonstrating that genetically reducing the level of STAT3 activity in gp130F/F mice reduced IL-6 expression in response to LPS further supports a role for STAT3 in promoting IL-6 gene transcription. In addition, our observations that genetic targeting of Mal, which primarily facilitates NF-κB activation via the LPS/TLR4/MyD88 signaling axis, also led to a reduction in the levels of IL-6 produced in response to LPS is consistent with previous studies invoking the involvement of MyD88 and NF-κB in the production of IL-6 in response to LPS (42, 43). We do note, however, that LPS-induced IL-6 production was not completely ablated in gp130F/F:Mal−/− mice, suggesting that other TLR4 ligands, or both.

![FIGURE 8](http://www.jimmunol.org/)

**FIGURE 8.** Genetic reduction of STAT3 in gp130F/F mice protects against LPS hypersensitivity and suppresses augmented IL-6 production. A, Gp130+/+ (+/+) and gp130F/F (F/F) mice were i.p. administered with LPS (4 mg/kg), and at defined intervals, STAT3 tyrosine phosphorylation, total STAT3, and ERK1/2 levels were measured by immunoblotting spleen lysates. Results shown are representative of three mice per genotype per time point. B, Liver lysates from LPS-treated +/-, F/F, and gp130F/F:Stat3−/− (F/F:St3−/−) mice were immunoblotted as in A. Results shown are representative of three mice per genotype per time point. C, Survival of +/- (dashed line; n = 9), F/F (solid line; n = 11), and F/F:Stat3−/− (dotted line; n = 9) mice over 72 h in response to 4 mg/kg LPS. D, Liver lysates from LPS-treated +/- (lanes 1, 4, 7, 10), F/F (lanes 2, 5, 8, 11) and gp130F/F, IL-6−/− (F/F:IL-6−/−: lanes 3, 6, 9, 12) mice were immunoblotted as in A. Results shown are representative of three mice per genotype per time point. E, Serum and (F) peritoneal lavage fluid was collected from +/-, F/F, and F/F:Stat3−/− mice in response to 4 mg/kg LPS, and IL-6 ELISA was performed. Data are from three mice of each genotype and are expressed as the mean ± SEM. G, qPCR analyses of IL-6 gene expression in whole spleen tissue from mice after i.p. administration of 4 mg/kg LPS. Expression data from three samples per genotype are shown after normalization to 18S expression and are presented from replicate analysis as the mean fold induction ± SEM relative to unstimulated samples. *p < 0.05; **p < 0.01; ***p < 0.001 versus data from F/F mice at the corresponding time points.

![FIGURE 9](http://www.jimmunol.org/)

**FIGURE 9.** Proposed model for regulation of LPS/TLR4 signaling by IL-6 and STAT3. Before LPS engagement of TLR4, basal IL-6-mediated STAT3 activation selectively modulates the TLR4/Mal signaling axis for potentiated production of IL-6. On LPS-induced activation of TLR4/Mal (and NF-κB) signaling, upregulated IL-6 complexes with sIL-6R (trans-signaling) and the gp130Y757F receptor to augment STAT3 activation. Hyperactivation of STAT3 further upregulates IL-6 production, both directly and via TLR4/Mal signaling.
signaling mediators also contribute to LPS-induced IL-6 production, such as TRIF, albeit in a cell type-specific manner (43).

Importantly, our gp130F/F mice build on the current paucity of genetically defined mouse models to directly investigate the mechanistic basis by which overactivated endogenous IL-6/STAT3 signaling promotes chronic inflammation. Curiously, a recent study has demonstrated an anti-inflammatory role for hepatic gp130 signaling in septic inflammatory responses (44). Mice with hepatocyte-specific deletion of gp130 were highly susceptible to sepsis-associated mortality induced by cecal ligation and puncture (CLP; non–TLR4-specific) and were characterized by increased systemic levels of IL-6 and TNF-α, whereas knock-in mice harboring the gp130Y757F mutation exclusively in hepatocytes were protected against CLP-induced mortality. However, the latter mice were not challenged with LPS, thus preventing a direct comparison with our gp130F/F mice, and a causal role for STAT3 signaling via IL-6 contributing to the septic phenotype of hepatocyte-specific gp130 mutant mice was not investigated. An obvious explanation for the disparity between these observations and those of our study is the differences in experimental models (poly-microbial, non–TLR4-specific versus LPS, TLR4-specific) used to trigger sepsis. We also note that another conditional mouse model in which the IL-6 signaling negative regulator Socs3 was targeted in macrophages, thus indirectly leading to increased macrophase-specific IL-6/STAT3 activation, was shown to be largely protected against LPS-mediated endotoxic shock (45). A possible explanation for this finding that contradicts our study is most likely the use by Yasukawa and colleagues (45) of the low-dose LPS model, which, in contrast with our study (high-dose LPS model), involves macrophase-mediated acute liver injury in mice on d-galactosamine sensitization. However, the same authors also reported that Socs3−/− mice, which presumably would display global increased IL-6/STAT3 signaling akin to our gp130−/− mice, were hypersensitive to LPS (45), an observation that is supported by studies demonstrating that global overexpression of Socs3 in vivo either via gene delivery (46) or intracellular protein delivery (47) protects mice against LPS challenge. Thus, these latter findings support our study that global IL-6/STAT3 hyperactivation sensitizes mice to LPS/TLR4-driven inflammatory responses, and imply that the artificial compartmentalization of IL-6/STAT3 hyperactivation to a specific subset of mutant cells (e.g., Socs3-deficient macrophages or gp130Y757F-bearing hepatocytes only) is in response to systemic inflammatory insults may elicit an alternate anti-inflammatory environment (44, 45).

To date, direct exploration of the role STAT3 plays during the inflammatory response has largely relied on a host of genetic mouse models in which STAT3 has been conditionally deleted in a cell type- or organ-specific manner, because of the embryonic lethality associated with the global genetic ablation of STAT3 in mice (22). For instance, mice harboring macrophage/neutrophil- or endothelial cell-specific deletion of Stat3 have assigned an anti-inflammatory role for STAT3, irrespective of the initiating mode of “septic” inflammation (e.g., LPS, CLP) (23, 24, 48), as a consequence of the inability of IL-10, which predominantly signals via STAT3, to mount a potent anti-inflammatory response. Furthermore, the specific deletion of Stat3 in hematopoietic progenitors (49) or the myeloid lineage (23) in mice spontaneously leads to chronic intestinal inflammation similar to that observed in IL-10–deficient mice (50). In this respect, we note that both the production of IL-10 and its ability to suppress cytokine production in response to LPS were unaffected in gp130F/F mice, thus eliminating any potential involvement of impaired anti-inflammatory activity in the LPS hypersensitivity phenotype of gp130F/F mice. In addition, our data demonstrating that LPS-induced STAT3 tyrosine phosphorylation is dramatically suppressed in gp130F/F mice lacking IL-6 implies that IL-6 is the predominant cytokine required for LPS-induced STAT3 activation.

Interestingly, our data revealing that hemizygous deletion of STAT3 partially rescued the LPS hypersensitive phenotype of gp130F/F mice suggests either a gene dosage effect whereby sufficient levels of STAT3 “proinflammatory” signals still emanate from the gp130Y757F receptor in a STAT3 hemizygous state, or alternatively, that there are signaling mediators downstream of gp130Y757F other than STAT3 that can potentiate LPS-induced inflammatory responses. Regarding the latter scenario, a likely candidate may be STAT1, which has potent proinflammatory activities and plays a significant role in the pathogenesis of LPS/TLR4-induced endotoxic shock. For instance, hyperactivation of STAT1 in Socs1−/− mice leads to multiorgan inflammation, as well as hypersensitivity to LPS (51), whereas STAT1 deficiency in mice protects against LPS-driven endotoxic shock (34). Although the link between STAT1 and TLR4 signaling was proposed to be due to type I IFN-induced hyperactivation of STAT1 via the TRIF-dependent pathway (52), in our study, both Ab-mediated blockade and genetic ablation of type I IFN signaling failed to protect gp130F/F mice from LPS hypersensitivity, suggesting STAT1 signaling in response to LPS may not have any pathologic involvement in our model.

In summary, our current data led us to propose a mechanism whereby IL-6 trans-signaling via STAT3, activated downstream of TLR4 in response to LPS, feeds back into the Mal/NF-κB pathway to specifically modulate TLR4/IL-6-driven IL-6 production and therefore the inflammatory response (Fig. 9). We note that ongoing DNA microarray and proteomic approaches in our laboratory are designed to uncover STAT3-regulated genes and/or interacting protein partners whose expression, activity, or both are influenced by IL-6 trans-signaling in the gp130F/F mouse model. In this respect, it is tempting to speculate that such a candidate may include NF-κB, which can form a transcripational complex with STAT3 to induce a specific subset of genes, albeit in immortalized human mammary epithelial cells, including IL-6 (53). The identification of such mechanisms that underlie the key role for IL-6 trans-signaling and STAT3 in the molecular pathogenesis of LPS/TLR4-mediated endotoxic shock should be of clinical relevance, especially in Gram-negative cases of sepsis/septic shock characterized by increased IL-6 production. Indeed, because IL-6 trans-signaling blockade constitutes a potential therapeutic strategy against this and other systemic inflammatory disorders exhibiting increased IL-6 production and/or STAT3 hyperactivation (e.g., RA, IBD), our study validates the gp130F/F mouse as a unique preclinical model for further translational research into the potential therapeutic benefits of targeting IL-6 trans-signaling in patients exhibiting symptoms of bacterial shock. Considering the opposing proinflammatory and anti-inflammatory roles of IL-6, selective therapeutic targeting of proinflammatory IL-6 trans-signaling with sgp130Fc rather than “global” IL-6 signaling (i.e., trans-signaling and classical signaling) with Abs against IL-6 or the IL-6R provides a critical advantage in a chronic inflammatory disease setting where increased IL-6 production is associated with disease progression.

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Disclosures

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