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The Role of JAK-3 in Regulating TLR-Mediated Inflammatory Cytokine Production in Innate Immune Cells

Huizhi Wang,* Jonathan Brown,† Shegan Gao,‡ Shuang Liang,* Ravi Jotwani,* Huaxin Zhou,* Jill Suttles,*† David A. Scott,*† and Richard J. Lamont*†

The role of JAK-3 in TLR-mediated innate immune responses is poorly understood, although the suppressive function of JAK3 inhibition in adaptive immune response has been well studied. In this study, we found that JAK3 inhibition enhanced TLR-mediated immune responses by differentially regulating pro- and anti-inflammatory cytokine production in innate immune cells. Specifically, JAK3 inhibition by pharmacological inhibitors or specific small interfering RNA or JAK3 gene knockout resulted in an increase in TLR-mediated production of proinflammatory cytokines while concurrently decreasing the production of IL-10. Inhibition of JAK3 suppressed phosphorylation of PI3K downstream effectors including Akt, mammalian target of rapamycin complex 1, glycogen synthase kinase 3β (GSK3β), and CREB. Constitutive activation of Akt or inhibition of GSK3β abrogated the capability of JAK3 inhibition to enhance proinflammatory cytokines and suppress IL-10 production. In contrast, inhibition of PI3K enhanced this regulatory ability of JAK3 in LPS-stimulated monocytes. At the transcriptional level, JAK3 knockout lead to the increased phosphorylation of STATs that could be attenuated by neutralization of de novo inflammatory cytokines. JAK3 inhibition exhibited a GSK3 activity-dependent ability to enhance phosphorylation levels and DNA binding of NF-xB p65. Moreover, JAK3 inhibition correlated with an increased CD4+ T cell response. Additionally, higher neutrophil infiltration, IL-17 expression, and intestinal epithelium erosion were observed in JAK3 knockout mice. These findings demonstrate the negative regulatory function of JAK3 and elucidate the signaling pathway by which JAK3 differentially regulates TLR-mediated inflammatory cytokine production in innate immune cells. The Journal of Immunology, 2013, 191: 1164–1174.

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; GSK3β, glycogen synthase kinase 3β; mTORC1, mammalian target of rapamycin complex 1; PMN, polymorphonuclear leukocyte; siRNA, small interfering RNA; TRIF, Toll/IL-1R domain–containing adapter-inducing IFN-β; WT, wild-type.

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enhanced the production of IL-12 and IL-10 in LPS-stimulated bone marrow–derived dendritic cells, which subsequently resulted in exaggerated Th1 differentiation. In contrast, Kim et al. (17) showed that JAK3 knockout in macrophages significantly reduced TLR4-mediated IL-10 production, which resulted in enhanced IL-1β–converting enzyme activity that consequently augmented the production of IL-1β. Thus, these studies showed differing effects of JAK3 on TLR4-mediated pro- and anti-inflammatory cytokine production, and the function and role of JAK3 remains to be determined.

In this study, we report that JAK3 negatively regulates TLR4-mediated inflammatory responses through differentially regulating pro- and anti-inflammatory cytokine production. Inhibition of JAK3 attenuated TLR4-mediated activation of PI3K and then suppressed the phosphorylation of Akt and its downstream constitutive active kinase, glycogen synthase kinase 3β (GSK3β), which augmented proinflammatory cytokine level and repressed IL-10 production via enhancing NF-κB activity and decreasing CREB activity, respectively. Moreover, JAK3 inhibition resulted in the increase of CD4+ T cell responses in vitro along with enhanced polymorphonuclear neutrophil (PMN) infiltration, IL-17 expression, as well as erosion of the intestinal epithelium. Collectively, these results identify the negative regulatory role of JAK3 in TLR4-mediated inflammatory responses and characterize the role of PI3K–Akt–GSK3β signaling pathway in JAK3mediated control of inflammatory responses.

Materials and Methods

Mice and reagents

B6129SF2 and JAK3 homozygous mutant mice (129S4-Jak3<sup>−/−</sup>) were purchased from The Jackson Laboratory. Mice were housed in a specific pathogen-free facility at the University of Louisville School of Medicine, and the University of Louisville Institutional Animal Care and Use Committee approved all animal protocols. Pam3CSK4, Flagellin, and ultra-pure LPS from Escherichia coli 0111:B4 were purchased from Invivogen. Phosphorylated and total JAK3 Abs were purchased from Assay Biotec. Isotype-matched control Ab (IgG1) and functional-grade neutralizing anti-human IL-10 (clone JES5-9D7) mAb were purchased from ebioscience. All other Abs were obtained from Cell Signaling Technology. The GSK3β inhibitor SB216763 (Tocris) has been characterized and shown to be specific for GSK3β without discernible effects on a panel of 24 other kinases (18). JAK3 inhibitor WHIP-154 and T-1377 (CP-690550) were purchased from Tocris and LC Laboratories, respectively. PI3K inhibitor LY294002 (25 mM) was purchased from Tocris and LC Laboratories, respectively. JAK3 inhibitor LY294002 was purchased from LC Laboratories. Nontargeting pools of small interfering RNA (siRNA) and a mixture of four prevalidated siRNA duplexes specific for GSK3β or JAK3 (ON TARGET-PLUS) were purchased from Dharmacon. All ectopically expressed plasmids were purchased from Addgene. TransAM CREB and NF-κB p65 transcription factor ELISA Kits were purchased from Active Motif. The NF-κB p65 (Ser<sup>276/280</sup>) inhibitory peptide set was purchased from Imgenex. Cytokine ELISA kits were purchased from ebioscience.

Cell preparation

PBMCs were obtained from the venous blood of healthy donors as per protocols approved by the University of Louisville, Institutional Review Board, Human Subjects Protection Program, study number 12.0373. Monocytes were isolated by negative selection using the human monocyte isolation kit purchased from Active Motif. Bone marrow cells from wild-type (WT) or JAK3 knockout mice were isolated by flushing femurs and tibiae with HBSS supplemented with 2% heat-inactivated FBS. The cells were then passed through a 70-μm cell strainer. Bone marrow–derived macrophages (BMDM) were generated as previously described (19). The cells were cultured in RPMI 1640 supplemented with 10% FBS (R10), 50 μM 2-ME, 1 mM sodium pyruvate, 2 mM l-glutamine, 20 mM HEPES, 50 U/ml penicillin, and 50 μg/ml streptomycin (RPMI complete).

Transfection, cytokine detection, and Western blot

Transfection of human monocytes was carried out by electroporation using a Nucleofector device (Amaza) according to the manufacturer’s protocols. Briefly, purified 4 × 10<sup>6</sup> monocytes were resuspended in 100 μl Nucleofector solution (Human Monocytes Nucleofector kit; Amaza) along with 2 μg GFP-coding plasmid (pCMV-GFP) and 2 μg siRNA duplexes or ecotropic plasmids for each target. Immediately after electroporation, 400 μl prewarmed M-199 containing 10% FCS were added to cells that were then transferred into culture plates containing prewarmed M-199 with 10% FCS. At 48 h posttransfection, cells were exposed to LPS (1 μg/ml) with or without inhibitor. Cell lysates were prepared as previously described (20, 21). Images were acquired using the Kodak Image Station 4000MM system (Eastman Kodak, New Haven, CT). For siRNA studies, the levels of total JAK3 were assessed by Western blot at 3 d posttransfection. For experiments using inhibitors for PI3K (LY294002; 25 mM) or GSK3β (SB216763; 10 mM), control cells were pretreated for 2 h with 0.01% DMSO (organic solvent control) prior to LPS (1 μg/ml) stimulation. For siRNA studies, transfected cells were stimulated with LPS (1 μg/ml) 3 d posttransfection, and cell-free supernatants were assayed for cytokine levels by ELISA (eBioscience) 20 h after the addition of LPS.

Immunohistochemistry

Intestinal tissue samples from WT and JAK3 knockout mice were fixed in 4% formaldehyde, embedded in OCT compound, and stored at −80°C. Serial sections (8-μm thick) were stained for neutrophils and/or IL-17 using single or double immunofluorescence. For staining, slides were rehydrated, blocked, and incubated for 1 h at room temperature with primary FITC-conjugated Abs to mouse Ly6G, a specific neutrophil marker (FITC-conjugate: LifeSpan BioSciences) or with unconjugated human/mouse IL-17A (Santa Cruz Biotechnology) Abs. Alexa Fluor 594–conjugated goat anti-rabbit IgG (Molecular Probes) was used as a secondary Ab for IL-17 staining. The specificity of staining was confirmed by using appropriate FITC-conjugated isotype controls or normal rabbit IgG followed by Alexa Fluor 594-goat anti-rabbit IgG. Images were captured using a laser-scanning confocal microscope (Olympus FV1000; Olympus).

NF-κB p65 and CREB nuclear binding assay

Nuclear lysates were obtained from human monocytes using a nuclear/ cytosolic isolation kit purchased from Active Motif. Nuclear lysates were analyzed for DNA binding levels of NF-κB p65 and phosphorylated CREB (S133) using TransAM NF-κB p65 and CREB Transcription Factor ELISA Kits (Active Motif) and performed according to the manufacturer’s protocol.

Statistical analysis

Statistical significance between groups was evaluated by the ANOVA and the Tukey multiple comparison test using the Instat program (GraphPad, San Diego, CA). Differences between groups were considered significant at the level of p < 0.05.

Results

Pharmacological inhibition of JAK3 enhances TLR4-mediated proinflammatory cytokine production while suppressing the production of IL-10

Although JAK3 has been reported to be involved in TLR-mediated innate immune responses, the impact of JAK3 activity on TLR-mediated inflammatory cytokine production is controversial, and at times, opposing results have been reported (14, 16, 17, 22). To determine the effects of JAK3 inhibition on TLR-mediated inflammatory cytokine production by innate immune cells, purified human monocytes were used to determine the production of TLR-mediated inflammatory cytokine production in the presence and absence of the JAK3 inhibitor T-1377 (CP-690550). Because the IC<sub>50</sub> of this inhibitor on JAK2 and JAK3 are 20 and 1 nM, respectively (23), a serial titration of T-1377 was used to minimize the influence of JAK2 on the regulatory ability of JAK3 in LPS-stimulated human monocytes. As shown in Fig. 1A–D, JAK3 inhibition with T-1377 at concentrations of 1 and 10 nM significantly enhanced the production of IL-12, TNF-α, and IL-6 in LPS-stimulated human monocytes, while suppressing the production of the prototypical anti-inflammatory cytokine IL-10. Moreover,
JAK3 inhibition with T-1377 at concentrations of 1 nM also differentially regulated TLR2- and TLR5-mediated production of IL-12 and IL-10 in human monocytes (Fig. 1E, 1F). We also tested the cellular responses with different LPS doses in the context of JAK3 inhibition and found JAK3 inhibition exhibits a significantly regulatory ability over different doses of LPS-mediated inflammatory cytokine production (Fig. 1G). To determine the influence of de novo–synthesized IL-10 on JAK3-mediated inhibition, neutralizing Ab was used to block IL-10 and then the regulatory effect of JAK3 inhibition on LPS stimulation was assessed. We found JAK3 inhibition remained capable of enhancing the production of proinflammatory cytokine in the context of IL-10 blockade, indicating JAK3 directly affects TLR4-mediated inflammatory responses (Fig. 1H). In addition, a second JAK3 inhibitor, WHIP-154, was used to confirm the regulatory function of JAK3 in TLR4-mediated cytokine production. Similar to T-1377, WHIP-154 pretreatment enhanced TLR4-mediated proinflammatory cytokine production and concurrently reduced the level of IL-10 (Fig. 1I).

**siRNA knockdown or genetic deletion of JAK3 results in elevated proinflammatory cytokine production and suppressed IL-10 production upon TLR4 stimulation**

As both T-1377 (CP-690550) and WHIP-154 can have activity against JAK2 (23), to isolate the specific influences of JAK3 inhibition on TLR4-mediated inflammatory cytokine production, we used prevalidated siRNA to knockdown JAK3 in human monocytes. As
shown in Fig. 2A, JAK3 silencing by specific siRNA resulted in a substantial decrease of total JAK3 in human monocytes, as compared with control. To exclude the possible off-target effect of JAK3 siRNA, we tested the expression of total JAK2 and found no substantial change of JAK2 in JAK3 siRNA-treated monocytes. Inhibition of JAK3 significantly enhanced TLR4-mediated IL-12, TNF-α, and IL-6 production and suppressed IL-10 level in LPS-stimulated human monocytes (Fig. 2B). These results corroborated the cytokine data obtained with the pharmacological inhibitors. Because neither pharmacological inhibition nor siRNA can completely ablate JAK3 without effects on other isoforms of JAKs, we next used JAK3-knockout macrophages to determine the effect of JAK3 in TLR4-mediated inflammatory cytokine production. As compared with WT control, JAK3 knockout resulted in significantly increased IL-12, TNF-α, and IL-6 while concurrently suppressing the production of IL-10 in LPS-stimulated macrophages (Fig. 2C–F). Taken together, these data demonstrate that JAK3 inhibition leads to elevated proinflammatory cytokine production and suppressed IL-10 production upon TLR4 stimulation.

The effects of JAK3 inhibition on the activation of the PI3K–Akt pathway and its downstream constitutive active kinase GSK3β in LPS-stimulated innate immune cells

The PI3K pathway has been demonstrated to modulate TLR4-mediated inflammatory immune responses by differentially regulating the levels of pro- and anti-inflammatory cytokines (24–27). Our recent studies found that a downstream constitutive active kinase of the PI3K–Akt pathway, GSK3β, is responsible for the ability of the PI3K–Akt pathway to suppress the levels of proinflammatory cytokines while augmenting anti-inflammatory cytokine production upon TLR stimulation (28). Because we have observed the differential regulatory ability of JAK3 inhibition on TLR4-mediated inflammatory cytokines, and previous reports have shown the possible links between the JAK3 and PI3K pathways (22, 29–31), we next sought to determine whether JAK3 inhibition regulates the TLR4-mediated inflammatory response through PI3K and its downstream signaling components such as Akt, mammalian target of rapamycin complex 1 (mTORC1), GSK3β, and CREB. As shown in Fig. 3A and 3B, compared with the control group, T-1377–treated monocytes exhibited reduced phosphorylation of mTORC1, Akt, GSK3β, and CREB over time upon LPS stimulation. In contrast, inhibition of Akt or GSK3β has no discernible effect on the phosphorylated levels of JAK3, compared with cells stimulated with LPS alone (Fig. 3C, 3D). We next used JAK3 knockout BMDM to confirm the functional role of JAK3. As shown in Fig. 3E and 3F, compared with WT control, JAK3-knockout BMDM exhibited substantially decreased phosphorylation of mTORC1, Akt, GSK3β, and CREB over time upon LPS stimulation. Because numerous studies have shown JAK-STATs play a critical role in the cytokine-mediated signaling pathway (32–35), we next examined whether JAK3 knockout–modified autocrine inflammatory cytokine production would subsequently affect STAT phosphorylation. JAK3-knockout cells and WT controls were stimulated with LPS at different time points, and then tyrosine phosphorylation of STAT3, STAT4, STAT5, and STAT6 was examined. As shown in Fig. 3G and 3H, JAK3 knockout potently

**FIGURE 2.** JAK3 deficiency enhances the production of IL-12, TNF-α, and IL-6 while decreasing IL-10 levels in TLR4-stimulated cells. Purified human monocytes were pretreated with nontarget or JAK3-specific siRNA for 72 h and then stimulated with LPS for 24 h. Whole-cell lysates and cell-free supernatants were collected to determine the transfection efficiency and cytokine levels, respectively. (A) siRNA-mediated knockdown of JAK3 protein levels was assessed by Western blot. Total JAK2 levels were also tested to exclude an off-target effect of JAK2. The ratio of total-JAK3 to total β-actin was determined by densitometry. (B) siRNA silencing-mediated JAK3 inhibition enhances the production of IL-12, TNF-α, and IL-6 while decreasing IL-10 levels in LPS-stimulated cells. For (C)–(F), WT and JAK3-knockout (KO) BMDM were generated and stimulated with LPS for 24 h. Cell-free supernatants were collected and assayed for cytokine levels by ELISA. JAK3 knockout enhanced TLR4-induced production of IL-12 (C), IL-6 (D), TNF-α (E) and reduced IL-10 levels (F) in LPS-stimulated BMDM. Data represent the arithmetic mean ± SD of three biological replicates. *p < 0.05, **p < 0.01, ***p < 0.001. m, murine.
increased the levels of tyrosine phosphorylation of STAT3, STAT4, STAT5, and STAT6 at late time points upon LPS stimulation, as compared with WT control. This result is consistent with and expands upon an earlier study showing JAK3 inhibition resulted in enhanced STAT3 phosphorylation (35). Our previous study has shown that TLR-induced phosphorylation of STATs was mainly caused by de novo–synthesized cytokines (36). Hence, we next investigated the effect of neutralizing Abs against inflammatory cytokines (including IL-6 and IL-12) on STAT phosphorylation in the context of JAK3 inhibition. Compared with control cells, neutralization of IL-6 and IL-12 mixture for 2 h and then stimulated with LPS. Whole-cell lysates were probed for the levels of phosphorylated STATs and the ratio of phospho- to total STATs determined by densitometry (I, J). Data are representative of three to five biological replicates.

**FIGURE 3.** JAK3 inhibition suppresses TLR4-mediated activity of the PI3K pathway while enhancing the phosphorylation levels of STATs. Purified human monocytes were pretreated with JAK3 inhibitor (1 nM T-1377) for 2 h and then stimulated with LPS over a 4-h time course. (A) Total cell lysates were probed for the levels of phosphorylated mTORC1, Akt, GSK3β, and CREB by Western blot, and the ratio of phospho- to total proteins was determined by densitometry (B). (C) Purified human monocytes were stimulated with LPS in the presence or absence of PI3K or GSK3 inhibitor. The levels of phosphorylated JAK3 were analyzed by Western blot and the ratio to total JAK3 was determined by densitometry (D). From (E)–(H), WT and JAK3 knockout (KO) BMDM were treated with LPS for up to 4 h, total cell lysates were collected at the given time points, and probed for the levels of phosphorylated mTORC1, Akt, GSK3β, CREB (E), STAT3, STAT4, STAT5, and STAT6 (G) by Western blot and the ratio of phospho- to total protein determined by densitometry (F, H). Anti–IL-12 and anti–IL-6 Ab cocktails were used to determine the effect of JAK3 inhibition on the tyrosine phosphorylation of STAT3, STAT4, STAT5, and STAT6 upon the neutralization of IL-12 and IL-6 signaling. WT and JAK3 knockout BMDM were pretreated with neutralizing anti–IL6 and anti–IL-12 mixture for 2 h and then stimulated with LPS. Whole-cell lysates were probed for the levels of phosphorylated STATs and the ratio of phospho- to total STATs determined by densitometry (I, J). Data are representative of three to five biological replicates.

**JAK3 differentially regulates LPS-induced cytokine production in a GSK3β-dependent manner**

The PI3K pathway has been shown to play a critical regulatory role in TLR4-mediated inflammatory cytokine production via phosphorylating and then inhibiting the activity of a downstream kinase, GSK3β (28). Phosphorylation and inactivation of GSK3 is effected by Akt after activation of PI3K. Because our data have shown that inhibition of JAK3 differentially regulated LPS-induced cytokine production and decreased the LPS-induced phosphorylation of Akt and GSK3β, we next investigated the functional role of this pathway in JAK3-mediated regulation of inflammatory cytokine production. As shown in Fig. 4A, inhibition of PI3K with the pharmacological inhibitor LY294002 significantly enhanced the ability of JAK3 inhibition to augment proinflammatory cytokine production and suppress IL-10 levels in LPS-stimulated monocytes. We next asked whether overexpression of ectopic constitutively active Akt would attenuate the hyperinflammatory response induced by JAK3 inhibition. As compared with cells expressing empty vector,
expression of constitutively active Akt resulted in significantly lower production of IL-12, TNF-α, and IL-6 in LPS-stimulated monocytes (Fig. 4B, 4C). Moreover, overexpression of constitutively active Akt also abrogated the effect of JAK3 inhibition upon LPS stimulation (Fig. 4C). It suggested that the regulatory ability of JAK3 on LPS-mediated inflammatory cytokine production in human monocytes. (G) Hemagglutinin (HA) expression levels were detected by Western blot 48 h posttransfection in nontransfected monocytes and monocytes transfected to confirm the transfection efficiency of kinase-dead (K85A) plasmid encoding GSK3β. (H) Monocytes transfected with kinase-dead (K85A) plasmid exhibited a loss in the phosphorylation levels of the GSK3-specific substrate glycogen synthase (S640/641). (I) As compared with monocytes transfected with empty vector control, the kinase-dead (K85A) GSK3β mutant abrogated the ability of JAK3 inhibition to increase the production of TNF-α, IL-12, and IL-6 and decreased the IL-10 level in LPS-stimulated cells. Data represent the arithmetic mean ± SD of three biological replicates. *p < 0.05, **p < 0.01, ***p < 0.001.
JAK3 inhibition modifies TLR4-mediated DNA binding activity of NF-κB and CREB and consequently regulates inflammatory cytokine induction

We next examined whether JAK3 inhibition of Akt and GSK3β could affect downstream signaling molecules/transcription factors involved in the control of LPS-mediated inflammatory cytokine production. Our previous study (28) demonstrated that GSK3 inactivation differentially regulates TLR-mediated inflammatory cytokine production. GSK3 increases the phosphorylation of CREB that displaces NF-κB from CBP, leading to diminished proinflammatory cytokine production (28). We therefore tested whether JAK3 inhibition could affect LPS-mediated DNA binding activity of NF-κB and CREB and whether this effect was dependent on the activity of GSK3. As expected, compared with LPS simulation alone, JAK3 inhibition enhanced phosphorylation of NF-κB p65 (S536) (Fig. 5A, 5B) and its DNA binding activity (Fig. 5C) but reduced that of CREB in LPS-stimulated monocytes (Fig. 5D). We also observed that inhibition of GSK3 was able to reverse the effect of JAK3 inhibition of NF-κB and CREB DNA binding (Fig. 5C, 5D). Because our previous studies have shown that GSK3 inhibition suppresses proinflammatory cytokine production by LPS-stimulated cells via its capacity to reduce the association of CBP with NF-κB (28), we predicted TLR4-mediated activity of NF-κB would affect the capability of JAK3 inhibition to enhance the production of inflammatory cytokine production in LPS-stimulated monocytes. To test this hypothesis, we used NF-κB p65 inhibitory peptide and observed that inhibition of NF-κB p65 prevented the ability of T-1377 to increase IL-12 and decrease IL-10 production in LPS-stimulated monocytes, as compared with control (Fig. 5E, 5F).

Several previous studies have shown that low doses of JAK3 inhibitor enhanced T cell responses, whereas many others reported that JAK3 inhibition suppresses adaptive immune responses by disrupting autocrine cytokine signaling of T and B cells (16, 37–39). Because we observed pharmacological inhibition, siRNA, or genetic deletion of JAK3 enhanced proinflammatory cytokine production in innate immune cells, we next examined whether the regulatory function of JAK3 inhibition in innate immune cells also impacted subsequent T cell responses. To this end, we cultured autologous memory CD4+ T cells with supernatants isolated from human monocytes under different stimulation scenarios. We observed that supernatant from monocytes stimulated with LPS alone had the ability to enhance CD4+ memory T cell responses by augmenting IFN-γ and IL-17 production (Fig. 6A, 6B). Moreover, using supernatants from monocyte pretreatment with JAK3 inhibitor and LPS reinforced the enhancement of IFN-γ and IL-17, whereas supernatant from monocyte pretreated with JAK3 inhibitor alone reduced the production of IFN-γ and IL-17 (Fig. 6A, 6B). These results indicate that JAK3 inhibition in innate immune cells can positively regulate subsequent T cells responses by enhancing the production of IFN-γ and IL-17.

JAK3 knockout mice display high levels of intestinal inflammation and PMN infiltration

It has been reported that the intestinal lamina propria is the largest reservoir of CD4+ lymphocytes and that either IL-6 and/or IL-17 can play a crucial role in the initiation and progression of intestine bowel diseases (40–44). In the current study, we observed in vitro...
After 96 h of coculture, cell-free supernatants were collected and assayed for IFN-γ. wells of three biological replicates. * p < 0.05, ** p < 0.01, *** p < 0.001.

This suggests that JAK3 inhibition enhanced proinflammatory cytokine production including IL-6 and IL-17. Moreover, compared with WT mice, >90% knockout mice exhibited some symptoms of intestine bowel diseases including lower body weight and severe rectal prolapses after 20 wk (data not shown). These observations suggest that JAK3 inhibition may enhance intestinal microbiota-induced inflammatory responses. In an effort to investigate this possibility, we assessed the presence of IL-17 and PMN infiltration in the lamina propria of the intestine of JAK3-deficient mice. We discovered a high degree of PMN infiltration and a mild increase in IL-17 expression in the lamina propria of JAK3 knockout mice, as compared with WT mice (Fig. 7A). Moreover, obvious damage in the intestinal epithelial layer was also found in JAK3-knockout mice (Fig. 7B). Considering previous studies have demonstrated the functional role of PMN infiltration and IL-17 expression in the initiation and development of intestine bowel diseases (40, 45), these data indicated that JAK3 may participate in regulation of the initiation and development of intestine inflammation.

Discussion

In the current study, we demonstrated the negative regulatory role of JAK3 in TLR4-mediated inflammatory responses and characterized the cell-signaling pathway by which JAK3 inhibition enhanced the production of proinflammatory cytokines while concurrently suppressing the anti-inflammatory cytokine IL-10 in innate immune cells. We found inhibition of JAK3 attenuated TLR4-mediated activity of PI3K and then suppressed the phosphorylation of Akt and its downstream kinase GSK3β. Inhibition of JAK3 diminished the phosphorylation level of GSK3β, resulting in enhanced GSK3β activity and subsequent elevated TLR4-mediated inflammatory responses. This finding identified GSK3β as a downstream signaling target of JAK3 in TLR4-stimulated innate immune cells. By analyzing downstream transcription factors of GSK3β, we observed that JAK3 inhibition enhanced the DNA binding activity of NF-kB and attenuated that of CREB upon LPS stimulation, and this effect was dependent on the activity of GSK3β. Moreover, the functional role of NF-kB was verified by the finding that inhibitory peptide-mediated blocking of NF-kB attenuated the ability of JAK3 inhibition to enhance TLR4-mediated proinflammatory cytokine production. Additionally, we found JAK3 inhibition was capable of increasing CD4+ T cell responses in vitro and enhancing PMN infiltration, as well as IL-17 expression in intestine tissues. Collectively, these findings establish a negative regulatory role for JAK3 in TLR4-mediated proinflammatory responses and characterize the involvement of the PI3K–Akt–GSK3β signaling pathway in JAK3-mediated control of inflammatory responses.

JAK3 has been demonstrated to be involved in cytokine signaling through coupling with the γc receptor and subsequently to play a critical role in the development, proliferation, and differentiation of B cells and T cells (38, 46). Despite numerous studies noting the immunosuppressive effect of JAK3 inhibition in the treatment of organ transplantation and several other inflammatory diseases (47–49), there is no consistent evidence for a role of JAK3 inhibition in TLRs mediated inflammatory responses. Yamaoka et al. (14) reported that a genetic deficiency of JAK3 or its γc chain receptor resulted in the elevation of inflammatory cytokines including IL-12 and IL-10 in LPS-stimulated mice bone marrow–derived dendritic cells, whereas other groups have shown JAK3 inhibition or knockout reduced the production of IL-10 in TLR4-mediated innate immune cells (17). In our current study, using...
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primary human monocytes and gene knockout Jak3 macrophages, we found Jak3 inhibition or genetic deficiency resulted in enhanced production of TNF-α, IL-6, and IL-12 but reduced IL-10 production. The discrepancy in IL-10 production among the studies is likely due to several factors such as different cell types, different maturation stage of dendritic cells, and the influence of Jak3 deficiency on other signaling pathways. Our current findings highlight the functional role of Jak3 in innate immune cells and elucidate the signaling pathway by which Jak3 negatively regulates TLR-mediated inflammatory cytokine production.

The PI3K–Akt pathway has been demonstrated to negatively regulate TLR-mediated inflammmatory responses (28). However, how TLRs initiate the activation of PI3K and the possible role of tyrosine kinases like Jak3s in this process are less known. Previous studies have demonstrated that activation of Jak3 occurs via its regulatory subunit p85 binding to a phosphotyrosine residue (YXXM) present within activated cellular receptors (50). Upon TLR stimulation, PI3K was activated through binding with the YXXM motif of the adaptor molecules such as MyD88 or the intracellular component of TLR receptors such as TLR2/3 (51, 52). Several lines of evidence showed Jak family members are involved in the regulation of TLR signaling (53, 54). However, no study to date has identified how Jak3s are involved in the signaling events of TLRs, if Jak3s are involved in the tyrosine phosphorylation of TLRs, or how they may act in a synergistic, independent, or opposing manner in innate immune cells. In this study, we demonstrated Jak3 inhibition suppressed activity of the PI3K–Akt–GSK3β pathway, which enhances the production of proinflammatory cytokines while decreasing IL-10 levels in innate immune cells. Our unpublished data showed that Jak3 inhibition attenuated tyrosine phosphorylation of TLR4 and Jak3 at early time points, which indicated Jak3-mediated tyrosine phosphorylation is involved in TLR-initiated signaling events. Although the connectivity between Jak3 and TLR4 is still not known, TLR4 receptor stimulation induces a conformational change, which may bring Jak3 into close proximity, resulting in activation via trans phosphorylation. Upon TLR-mediated phosphorylation, activated Jak3 could phosphorylate tyrosine residues within the cytoplasmic domains of associated receptor or adaptor molecules that allow for the recruitment of downstream signaling molecules.

Mapping of the PI3K pathway by several groups revealed that the ability of the PI3K pathway to dictate regulation of pro- and anti-inflammatory cytokines largely depended upon the inactivation of the serine/threonine kinase GSK3β (19, 20, 28). Several groups have shown that Jak3 activation is involved in the activation of the PI3K pathway following various stimuli (22, 29–31). In particular, inhibition of Jak3 was shown to downregulate PI3K activation and Akt phosphorylation in mast cells in the present of Ag stimulation (30). However, it was unclear if Jak3 was required for the TLR-mediated action of the PI3K pathway. Our present study has provided evidence for the first time, to our knowledge, demonstrating the PI3K–Akt–GSK3β pathway is responsible for Jak3-mediated differential regulation of inflammatory cytokine production by LPS-stimulated innate immune cells. Moreover, we observed several signaling molecules, such as mTORC1 and P70S6K, involved in Jak3-mediated regulation of cytokine production (data not shown).

This finding is consistent with our previous published results on the regulatory effect of the PI3K–mTORC1 pathway and its downstream serine/threonine kinases P70S6K and GSK3β in TLR4-mediated human monocytes (36, 55). Because our previous publication has demonstrated that mTORC1 inhibition led to differential cytokine production (36), and because inhibition of Jak3 resulted in dephosphorylation of mTORC1 and its downstream signaling molecules P70S6K and GSK3β, it is reasonable to predict that Jak3 exhibits its regulatory role through this pathway. Our current data support this concept, because either pharmacological inhibition or ectopic expression of kinase dead GSK3β abrogated the differential regulatory ability of Jak3 inhibition upon LPS stimulation. Considering the similarity of Jak3 and MtorC1 inhibitors on suppressing adaptive immune responses while enhancing TLR4-mediated proinflammatory cytokine levels, our study suggested this could be a common feature for molecules that possess the capability to suppress adaptive immune responses while concurrently enhancing the immune responses of innate immune cells. At the transcriptional level, we found that Jak3 inhibition differentially altered the TLR4-mediated DNA binding activity of CREB and NF-κB. Furthermore, peptide-mediated inhibition of NF-κB suppressed proinflammatory cytokine production in Jak3 inhibited cells stimulated with LPS. These observations suggest that Jak3 differentially regulates TLR4-mediated inflammatory responses through modifying the activity of NF-κB and CREB. Taken together, our current study characterized the signaling pathway, PI3K–Akt–GSK3β, that Jak3 depends upon to affect TLR4-mediated inflammatory cytokine production.

Although numerous reports have shown Jak3 inhibition suppresses inflammation responses in several autoimmune diseases (47, 49), whether this is truly attributable to inhibition of Jak3 has not yet been established. It has been reported that the IC50 value of the most widely investigated Jak3 inhibitor, T-1377 (CP-690550), is 1 nM for Jak3 but 20 nM for Jak2 in lymphocytes (23). Additionally, as accurate concentrations of inhibitors are difficult to achieve in vivo, it is unlikely that all of the inflammatory suppressive effects of T-1377 can be attributed to Jak3 inhibition. Because several recent studies (56–58) have established a suppressive function for Jak2 inhibition in TLR-mediated immune responses, the suppressive effect of T-1377 on inflammatory responses might be mainly due to Jak2, not Jak3, inhibition. A recent study by Yoshida et al. (16) substantiated this possibility, showing that a low dose of T-1377 enhanced the inflammatory responses and accelerated the onset of experimental autoimmune encephalomyelitis. A very recent published abstract also reported that specific inhibition of Jak1 and Jak2 is more effective than inhibition of Jak3 in protecting mice from acute graft-versus-host disease by significantly decreasing alloreactive CD4+ T cells (59). These observations indicated Jak2 and Jak3 might have distinct regulatory function in inflammatory responses. Our current study demonstrated Jak3 inhibition, either by titrated concentrations of pharmacological inhibitor or genetic knockdown, had an ability to enhance the proinflammatory cytokine production and related T cell responses. As a negative regulator of inflammation, Jak3 knockout also resulted in the high expression of IL-17 and infiltration of PMN in the lamina propria of the distal intestine. These results identified the negative regulatory role of Jak3 in TLR-induced inflammatory cytokine production.

Whereas numerous studies have reported that pharmacological inhibition of Jak3 was capable of suppressing the inflammatory immune response by disrupting the development of lymphocytes, the effect of Jak3 on innate immune cells and the cellular mechanism responsible for this effect have remained obscure. As it is well known that the innate immune responses prime the adaptive immune cells to enhance the host defense ability, elucidating the indirect influence of Jak3 inhibition on the responses of autologous CD4+ T cells will be helpful to comprehensively assess the regulatory effect of Jak3 inhibition on TLR-mediated immune responses. The work presented in this study demonstrated that Jak3 inhibition enhanced TLR4-mediated proinflammatory cytokine production while concurrently reducing the IL-10 levels in innate immune cells. We also discovered that the PI3K–Akt–GSK3β signaling pathway
is used by JAK3 to differentially regulate TLRL4-mediated cyto-
kin production. JAK3 inhibition induced enhancement of proin-
fammatory cytokine production, and this correlated with elevated CD4+ T cell responses and the inflammation status of the intestine. Our further studies elucidated that JAK3 inhibition enhanced GSK3β activity and was capable of altering the DNA binding activity of NF-κB and CREB, which resulted in elevated production of TLRL4-
mediated proinflammatory cytokines. Thus, the function of JAK3 in immune cells related to the dampening of the proinflammatory responses and elevating production of anti-inflammatory cytokine IL-10. Understanding the mechanism regulating inflammatory cytokines by JAK3 inhibition provides a logical strategy for designing novel treatment strategies to combat immune-related inflamma-
tory diseases.

Disclosures
The authors have no financial conflicts of interest.

References
5. Lee, M. S., and J. Y. Kim. 2007. Signaling pathways downstream of pattern-
necrosis factor alpha production and microlgia activation in the brainstem and restricts West Nile Virus pathogenesis. J. Virol. 83: 9329–9338.
8. Belinda, L. W., X. Wei, B. T. Hanh, L. X. Lei, H. Bow, and D. J. Ling. 2008. SAPK/JNK a novel Toll-like receptor adaptor, is functionally conserved from ar-
18. Wang, K. S., D. A. Frank, and J. Ritz. 2000. Interleukin-2 enhances the response of natural killer cells to interleukin-12 through up-regulation of the interleukin-


