Toll-interacting protein differentially modulates HIF1α and STAT5-mediated genes in fibroblasts

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Toll-interacting protein (Tollip) deficiency has been implicated in complex inflammatory and infectious diseases whose mechanisms are poorly understood. Comparing the gene expression profiles of WT and Tollip-deficient murine embryonic fibroblasts, we observed here that Tollip deficiency selectively reduces the expression of the inflammatory cytokines interleukin 6 (IL-6), IL-12, and tumor necrosis factor α (TNFα) but potentiates the expression of fatty acid–binding protein 4 (FABP4) in these cells. We also observed that expression of hypoxia-inducible factor 1-α (HIF1α) is reduced, whereas that of signal transducer and activator of transcription 5 (STAT5) is elevated, in Tollip-deficient cells, correlating with the decreased expression of inflammatory cytokines and increased expression of FABP4 in these cells. We further found that the coupling of ubiquitin to ER degradation (CUE) domain of Tollip is required for stimulating HIF1α activity, because Tollip CUE–domain mutant cells exhibited reduced levels of HIF1α and selected cytokines. Tollip is known to mediate autophagy and lysosome fusion, and herein we observed that Tollip's autophagy function is required for modulating STAT5 and FABP4 expression. Bafilomycin A, an inhibitor of lysosome fusion, enhanced STAT5 and FABP4 expression in WT fibroblasts, whereas torin 2, an activator of autophagy, reduced STAT5 and FABP4 expression in Tollip-deficient fibroblasts. Taken together, our study reveals that Tollip differentially modulates HIF1α and STAT5 expression in fibroblasts, potentially explaining the complex and context-dependent contribution of Tollip to disease development.

Host inflammatory processes are finely modulated to elicit context-dependent responses to cope with infection and/or inflammation. Dysregulated inflammation may serve as a critical risk factor for inflammatory and infectious diseases (1–5). Toll-like–receptor (TLR)[2] signaling pathways are important networks regulating cellular inflammatory responses, capable of modulating the expression of diverse inflammatory cytokines and metabolic genes (6). TLR signaling networks may modulate differential gene expression through distinct activation of unique transcription factors such as NF-κB, STATs, and HIF1α. Although significant progress has been made in the last decade regarding the TLR signaling networks, context-dependent activation of these transcription factors and downstream gene expressions are not clearly defined.

Toll-interacting protein (Tollip) is an intracellular adaptor for the TLR signaling network with less-well-defined function. Tollip contains a C2 domain capable of binding with phosphatidylinositol phosphate (PIP) and a CUE domain capable of interacting with ubiquitin or ubiquitinated proteins (7, 8). Through its C2 domain with PIP, Tollip is involved in the fusion of lysosome with endosome, phagosome, and/or autophagosome (9, 10). Proper lysosome fusion may serve as a key mechanism for maintaining cellular homeostasis. Disrupted lysosome fusion may generate cellular stress and create signaling platforms for the activation of stress kinases. For example, the Janus kinase/STAT pathway has been shown to be assembled near lysosome (11, 12). Tollip CUE domain, on the other hand, may reduce the PIP binding capability of C2 domain and enable Tollip clearance from lysosome and shuttle to mitochondria (9, 13, 14). Mitochondrial Tollip is shown to be involved in the generation of reactive oxygen species and subsequent expression of inflammatory mediators (14). Previously, we reported that low doses of LPS cause Tollip to relocate and associate with the mitochondria rather than with late endosome/lysosomes (9). As a result, low doses of LPS induce mitochondrial reactive oxygen species and perpetuate low-grade inflammation (14).

With particular interest to this study, HIF1α can be stabilized and therefore activated by reactive oxygen species and potentiate the expression of inflammatory cytokines (15, 16). On the other hand, STAT5 is a key mediator for the expression of metabolic genes such as FABP4 and may be stabilized during disrupted autolysosome fusion (17, 18). Based on these studies, we tested the hypothesis that Tollip may differentially modulate the activation of HIF1α and STAT5. Utilizing WT and Tollip-deficient murine embryonic fibroblasts (MEFs), as well as MEFs carrying CUE–domain mutant Tollip, we examined the role of Tollip in the activation of HIF1α and STAT5, as well as the expression of selected inflammatory mediators and FABP4.

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[2] The abbreviations used are: TLR, Toll-like receptor; IL, interleukin; TNF, tumor necrosis factor; FABP, fatty acid–binding protein; HIF1α, hypoxia-inducible factor 1-α; STAT, signal transducer and activator of transcription; PIP, phosphatidylinositol phosphate; LPS, lipopolysaccharide; MEF, murine embryonic fibroblast; CMA, chaperone-mediated autophagy.

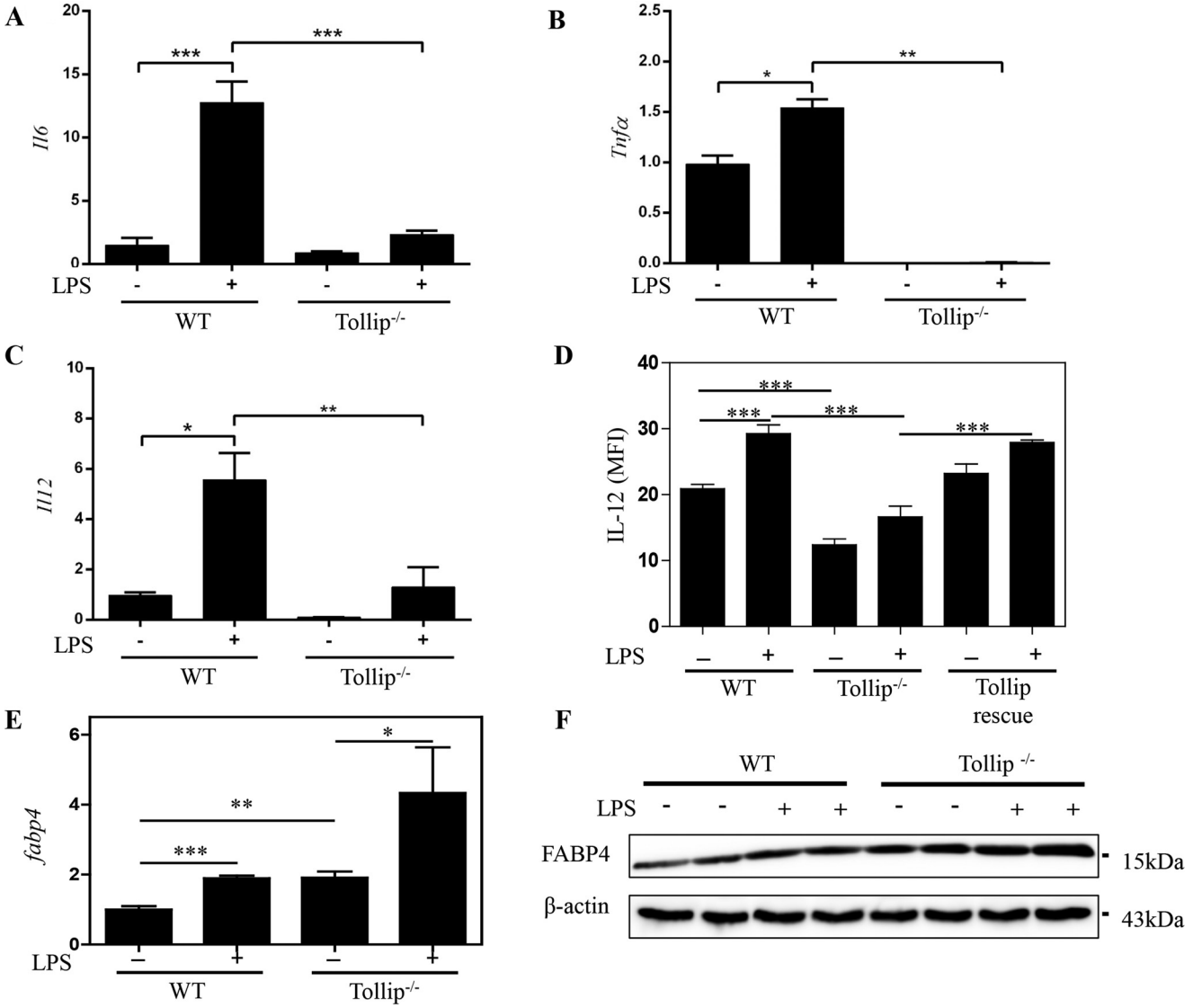

Figure 1. Tollip-deficient fibroblasts express decreased levels of pro-inflammatory cytokines and increased levels of FAPB4. WT and Tollip knockout (*Tollip*$^{-/-}$) MEF cells were untreated (−) or treated (+) with 10 ng/ml LPS for 6 h prior to RNA isolation. Real-time PCR was utilized to detect the mRNA levels of the selected inflammatory cytokines labeled accordingly. *A*, *Il6* mRNA. *B*, *Tnfα* mRNA. *C*, *Il12* mRNA levels. *D*, cellular IL-12 protein levels were detected through intracellular staining and flow cytometry. *E*, *Fabp4* mRNA expression levels in both WT and Tollip$^{-/-}$ MEF cells. *F*, FABP4 proteins were detected by Western blotting. The data represent three independent experiments processed together in each group. All values were normalized, using WT (−) as 1. Significance was determined by *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Results

Tollip differentially regulates the expression of inflammatory cytokines and FABP4

To determine the effect of Tollip deficiency on the differential gene expressions, Tollip knockout (*Tollip*$^{-/-}$) MEF cells were utilized. We first compared the expression levels of selected inflammatory cytokines such as IL-6, TNFα, IL-12, and FABP4 in WT and Tollip-deficient MEF cells treated with LPS (10 ng/ml) through RT–PCR analyses. As shown in Fig. 1, the treatment of WT MEF cells with LPS led to a significant induction of acute inflammatory cytokines, *Il6*, *Tnfα*, and *Il12* (Fig. 1, *A–C*). In contrast, Tollip deficiency significantly decreased the mRNA expression of *Il6*, *Tnfα*, and *Il12*. The basal levels of *Il6*, *Tnfα*, and *Il12* in untreated Tollip-deficient cells were also significantly lower as compared with those in WT cells. We further confirmed the involvement of Tollip in the expression of IL-12 protein through flow cytometry and documented reduced IL-12 protein levels in Tollip-deficient fibroblast cells (Fig. 1*D*). In addition, we demonstrated that reintroduction of WT Tollip back into Tollip$^{-/-}$ cells can rescue the induction of IL-12 by LPS (Fig. 1*D*). Our data demonstrate that Tollip positively regulates the expression of IL-6, TNFα, and IL-12 in cells stimulated with LPS.

We next analyzed the expression levels of FABP4, a key mediator involved in modulating both inflammation and metabolism that is selectively induced by STAT5 (18). We observed that FABP4 expression is significantly higher in Tollip-deficient fibroblast cells both at the mRNAs and protein levels (Fig. 1, *E* and *F*). Our data are consistent with the previous findings that Tollip-deficient cells have reduced endosome-lysosome fusion that causes cellular stress, and FABP4 is known to contribute to cellular stress (9, 19, 20). Taken together, our data suggest that

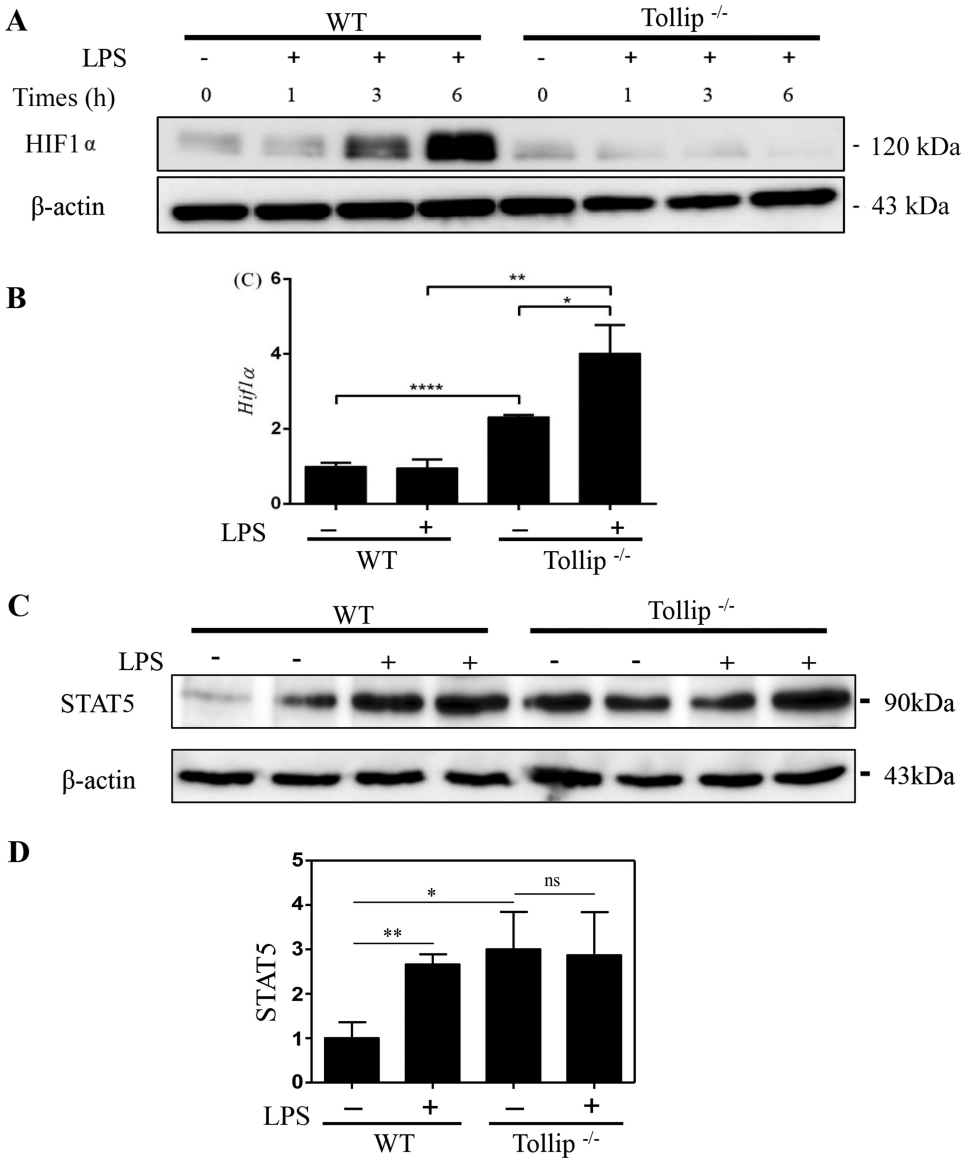

Figure 2. Key transcription factors are differentially regulated in WT and Tollip$^{-/-}$ MEF cells. WT and Tollip$^{-/-}$ MEF cells were treated with LPS (10 ng/ml) for the times indicated, and the levels of HIF1α, STAT5, and β-actin controls were determined by Western blotting analyses. Quantification of mRNA was performed for WT and Tollip$^{-/-}$ MEF cells after 6 h of LPS (10 ng/ml) treatment utilizing RT–PCR. *A*, WT and Tollip$^{-/-}$ MEF cells were treated for the indicated times with LPS, and protein levels of HIF1α were detected through Western blotting. *B*, *Hif1*α relative mRNA expression in WT and Tollip$^{-/-}$ cells treated with LPS for 6 h and normalized to β-actin. *C* and *D*, WT and Tollip$^{-/-}$ MEF cells were treated for the indicated times with LPS. STAT5 levels were detected through Western blotting (*C*) and quantitated (*D*). The data represent three separate experiments. All values were normalized, using WT (−) as 1. Significance was determined by *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Tollip contributes to the expression of IL-12 while suppressing the expression of FABP4 in MEF cells.

Tollip induces HIF1α and attenuates STAT5

To determine molecular mechanisms for the differential expression of IL-12 and FABP4 mediated by Tollip, we examined the selected transcription factors including HIF1α and STAT5. WT and Tollip-deficient MEF cells were treated with LPS (10 ng/ml) for a time course. LPS treatment significantly induced HIF1α in WT, but not in Tollip-deficient cells (Fig. 2*A*). To test whether the induction of HIF1α is at the RNA or protein levels, we tested the mRNA levels of *Hif1*α and observed higher levels of *Hif1*α mRNA in Tollip-deficient cells (Fig. 2*B*). Together, our data suggest that the reduction of HIF1α in Tollip-deficient cells is most likely due to protein post-translational degradation instead of mRNA modulation.

We also examined the levels of STAT5 because of its involvement during the expression of FABP4. We observed that STAT5 levels were significantly increased in Tollip-deficient cells as compared with WT cells (Fig. 2, *C* and *D*). The constitutive elevation of STAT5 in resting Tollip-deficient cells may allow higher induction of STAT5 downstream genes such as *fabp4*, which we further tested later.

Our data reveal that Tollip-deficient cells have drastically decreased HIF1α protein levels, which is a known transcription factor for acute inflammatory cytokines such as IL-6 and IL-12. Our study provides a potential mechanistic cause for decreased IL-12 in Tollip-deficient cells. Interestingly, HIF1α mRNA lev-

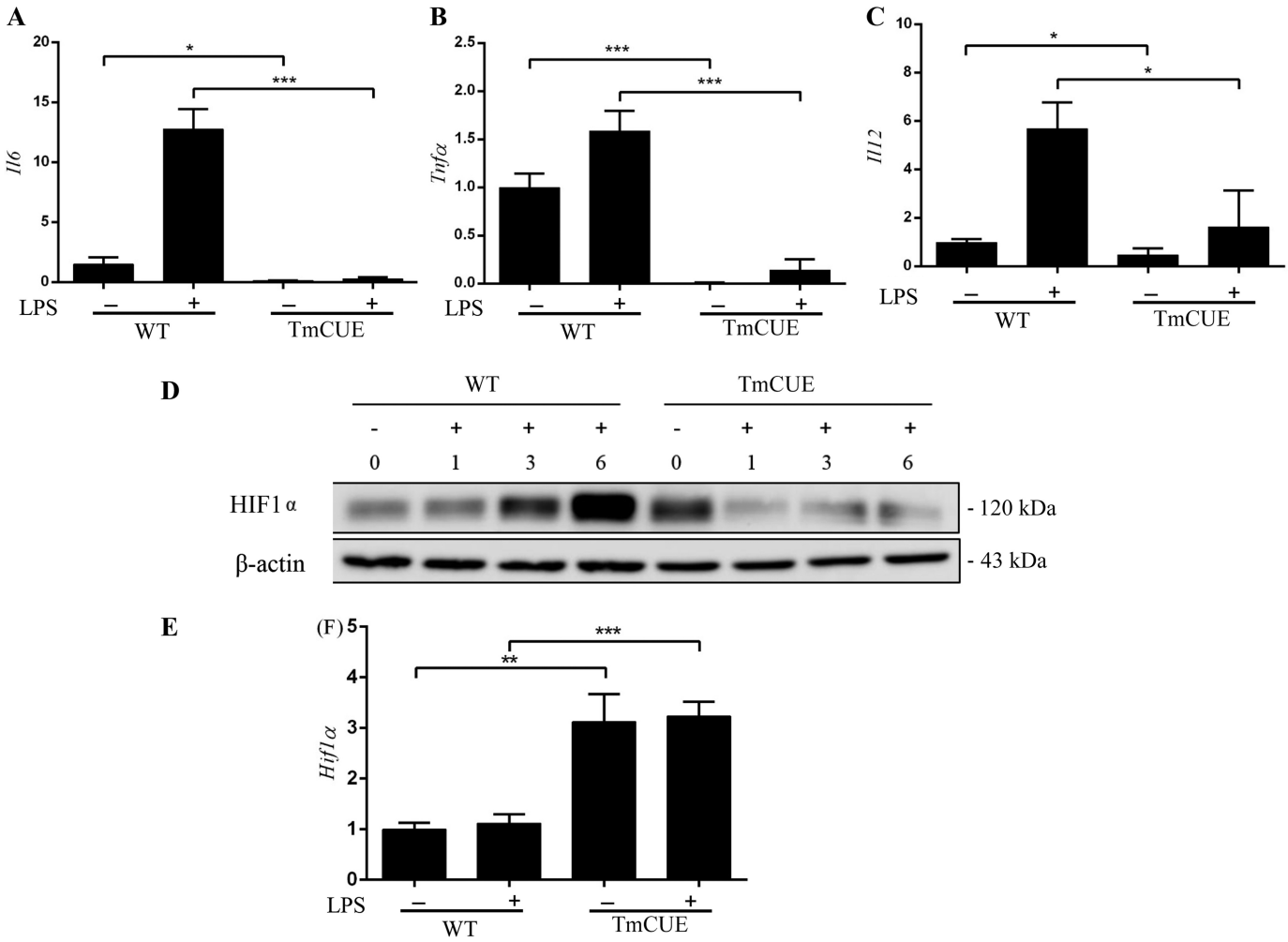

Figure 3. Dysfunctional CUE domain eliminates induction of inflammatory cytokines. WT and Tollip CUE–domain mutant (*TmCUE*) MEF cells were untreated (−) or treated (+) with 10 ng/ml LPS for 6 h prior to RNA isolation. Real-time PCR was performed to detect mRNA expression levels of select inflammatory cytokines shown above. *A*, relative *Il6* mRNA levels of WT and TmCUE with and without LPS treatment. *B*, *Tnfa* mRNA expression levels. *C*, relative *Il12* mRNA expression levels. *D*, WT and TmCUE cells were treated for times indicated and probed for HIF1α and β-actin controls. *E*, relative mRNA expression of *Hif1α*. The data represent three independent experiments. All values were normalized, using WT (−) as 1. Significance was determined by *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

els are higher in Tollip-deficient cells, which led us to hypothesize that decreased HIF1α at the protein levels, may be due to post-translational degradation. On the other hand, Tollip-deficient cells also have much higher expression levels of STAT5 even at basal levels. This gives a mechanistic explanation for increased FABP4, because of STAT5's involvement in the transcription of *fabp4* mRNA. Our data suggest that Tollip may regulate these two unique subsets of genes through differential modulation of HIF1α and STAT5.

Tollip CUE domain is involved in the differential modulation of HIF1α

Previous studies suggest that the CUE domain of Tollip may be involved in the modulation of Tollip interaction with lipid, as well as its differential subcellular localization at lysosome and mitochondria (9, 21). Because mitochondrial reactive oxygen species can induce and stabilize HIF1α, we tested whether the Tollip CUE domain may be causally associated with the activation of HIF1α. To test this, Tollip mutant MEFs harboring the CUE domain M240A/F241A mutation (TmCUE) were utilized (9). By real-time RT–PCR analyses, we observed that LPS fails to induce the expression of pro-inflammatory cytokines such as *Il6*, *Tnfα*, and *Il12* in the Tollip CUE mutant cells (Fig. 2, *A–C*). Furthermore, LPS increased the expression of HIF1α over the 6-h treatment period in WT MEF cells, whereas TmCUE MEF cells were unresponsive and showed no induction of HIF1α following LPS stimulation (Fig. 3*D*). Reduced HIF1α is consistent with reduced induction of IL-6 and TNFα in TmCUE cells. Similar to Tollip-deficient cells, TmCUE cells showed higher mRNA levels of *Hif1α*, further suggesting that HIF1 induction in WT cells may be at the protein stability level (Fig. 3*E*).

Chemical inducer of HIF1α degradation reduces the expression of Il6 and Tnfα, mimicking Tollip deficiency in WT MEF cells

To independently confirm that HIF1α stabilization is responsible for the induction of *Il6* and *Tnfα*, we applied a chemical inducer of HIF1α degradation geldanamycin. WT and Tollip-deficient cells were co-stimulated with LPS and/or

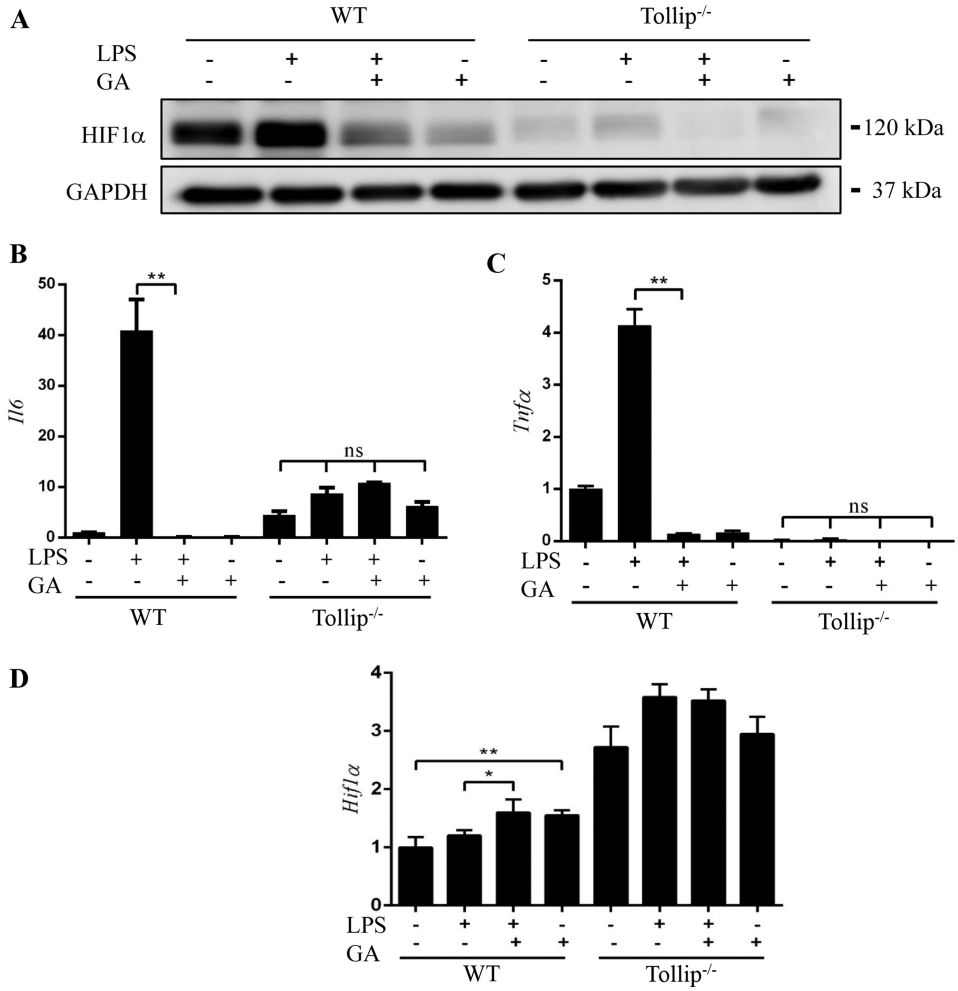

Figure 4. Geldanamycin decreases expression of HIF1α and subsequently decreases mRNA expression of *Il6* and *Tnfα* in WT cells. WT and *Tollip*$^{-/-}$ MEF cells were untreated (−) or treated (+) with LPS (10 ng/ml) and geldanamycin (*GA*; 2 μM) for 6 h prior to protein, and RNA isolation was performed. Real-time PCR was performed to detect mRNA expression levels of select cytokines and HIF1α. Protein levels of HIF1α and GAPDH controls were determined by Western blotting analyses. *A*, WT and Tollip$^{-/-}$ cells were treated with the indicated reagents and probed for HIF1α and GAPDH controls. *B*, levels of *Il6* with indicated treatments. *C*, levels of *Tnfα* with indicated treatments. *D*, levels of *Hif1α* with indicated treatments. RT–PCR data are ± S.E. of triplicates in each group. All values were normalized, using WT (−) as 1. The data represent three separate experiments. Significance was determined by *t* test. *, $p < 0.05$; **, $p < 0.01$.

geldanamycin for 6 h prior to protein and RNA analyses. WT cells showed a significant decrease in HIF1α protein expression in the presence of geldanamycin (Fig. 4*A*). Tollip-deficient cells, although already having decreased levels of HIF1α, showed a further decrease in HIF1α protein levels with the addition of geldanamycin (Fig. 4*A*). WT cells showed drastically lower transcription levels of *Il6* and *Tnfα* with geldanamycin treatment (Fig. 4, *B* and *C*). *Tollip*$^{-/-}$ cells were unaffected by the addition of geldanamycin in terms of cytokine expression, most likely because of the already low expression levels of these cytokines (Fig. 4, *B* and *C*). To show that HIF1α protein levels were decreased because of degradation and not reduced mRNA induction, RT–PCR was utilized to examine *Hif1α* mRNA expression levels. WT MEF cells showed significantly higher induction of *Hif1α* with geldanamycin treatment, potentially a compensatory effect to restore homeostasis (Fig. 4*D*). Tollip-deficient cells showed no change in *Hif1α* mRNA levels with geldanamycin treatment and showed constitutively higher mRNA levels as compared with WT cells (Fig. 4*D*).

Suppressor of autolysosome fusion elevates STAT5 and the expression of FABP4

To independently assess the role of autolysosome fusion in the modulation of STAT5 activation and *fabp4* expression, we applied a known chemical inhibitor of autolysosome fusion bafilomycin. Bafilomycin is a late stage autophagy blocker that prevents maturation of endosome/autophagosome by blocking fusion between the endosome/autophagosome and the lysosome, as well as preventing acidification of the lysosome. Indeed, bafilomycin and/or LPS treatment led to an elevation of STAT5 in WT cells (Fig. 5*A*). Consistent with previous observation that Tollip is required for autolysosome fusion, we observed that the STAT5 levels were constitutively high in Tollip-deficient cells (Fig. 5*A*). This is also consistent with the observation that bafilomycin treatment in Tollip-deficient cells cannot further increase STAT5 protein levels. The constitutively elevated STAT5 levels in Tollip-deficient cells may allow for enhanced induction of the STAT5 downstream gene by LPS. Indeed, we showed that WT cells had increased levels of

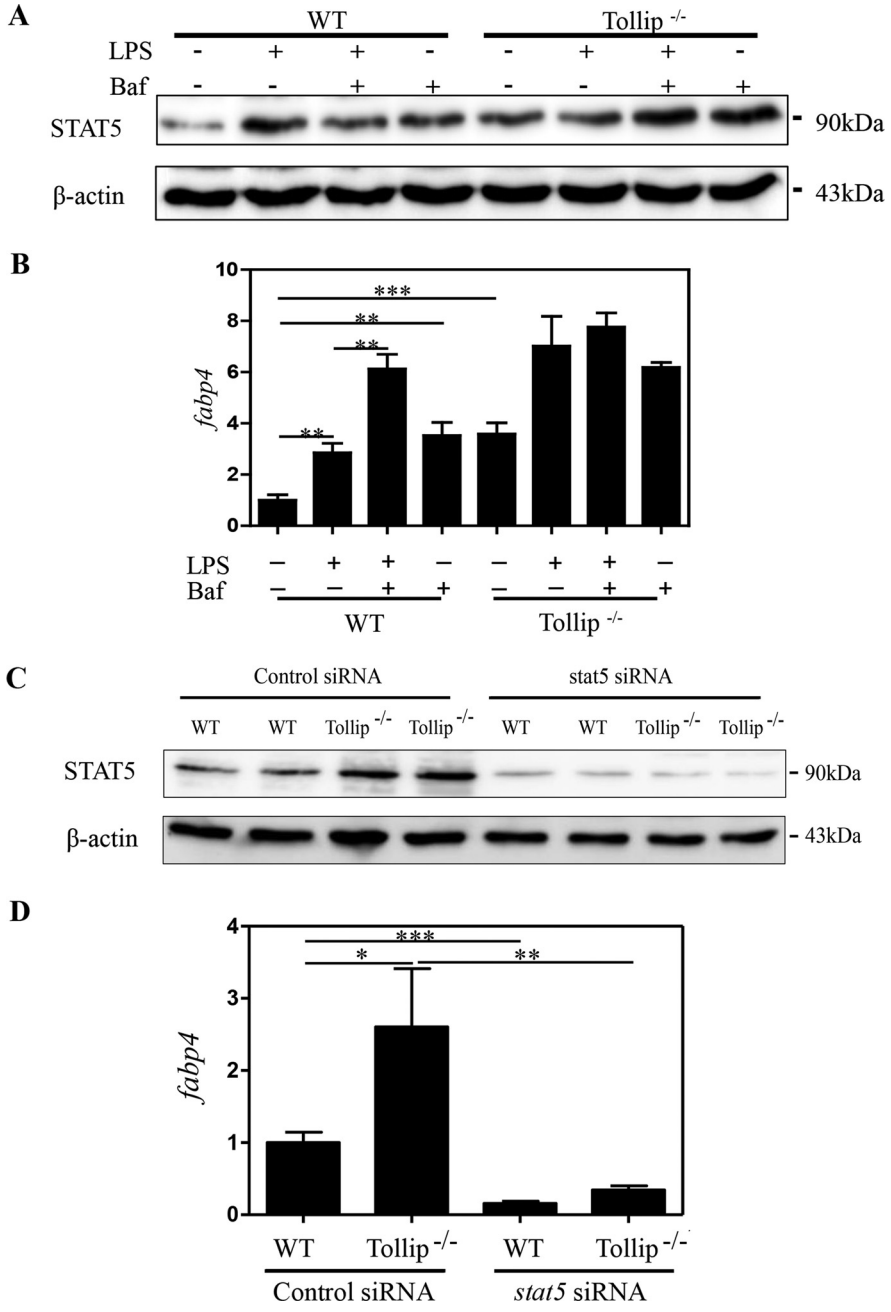

Figure 5. Constitutively elevated STAT5 levels caused by Tollip deficiency were responsible for the expression of *fabp4*. *A* and *B*, WT and Tollip$^{-/-}$ MEF cells were treated (+) or untreated (−) with LPS (10 ng/ml) and bafilomycin (*Baf*; 10 nM) for 6 h before RNA isolation was performed. *A*, protein levels of STAT5 were determined by Western blotting analyses. *B*, real-time PCR was performed to detect mRNA expression levels of *fabp4*. *C* and *D*, WT and Tollip-deficient cells were transfected with either control or *stat5*-specific siRNAs. *C*, the effective knockdowns of STAT5 in WT and Tollip$^{-/-}$ cells were verified through Western blotting. *D*, mRNA levels of *Fabp4* were determined by RT–PCR. The data represent three separate experiments. All values were normalized, using WT (−) as 1. Significance was determined by *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fabp4 with bafilomycin treatment compared with untreated cells (Fig. 5*B*).

Given the limitation of chemical inhibitors, we further performed independent experiments to confirm the causal contribution of Tollip-modulated STAT5 in *fabp4* expression. To this regard, we used the siRNA approach to knock down *stat5* expression in both WT and Tollip-deficient cells. WT and Tollip-deficient cells were treated with either control or *stat5*-specific siRNAs. As shown in Fig. 5*C*, Tollip-deficient cells had elevated resting levels of STAT5 protein in the presence of control siRNA. In contrast, in the presence of *stat5*-specific siRNA, the STAT5 levels were drastically reduced and comparable between WT and Tollip-deficient cells (Fig. 5*C*). We further tested the expression of *fabp4* in these cells. Consistent with the above findings, the expression of *fabp4* was significantly higher in Tollip-deficient cells as compared with WT cells treated with control siRNA (Fig. 5*D*). In sharp contrast, the expression of *fabp4* was significantly reduced in cells treated with *stat5*-specific siRNA. Furthermore, the expression levels of *fabp4* between WT cells and Tollip-deficient cells treated with *stat5*-

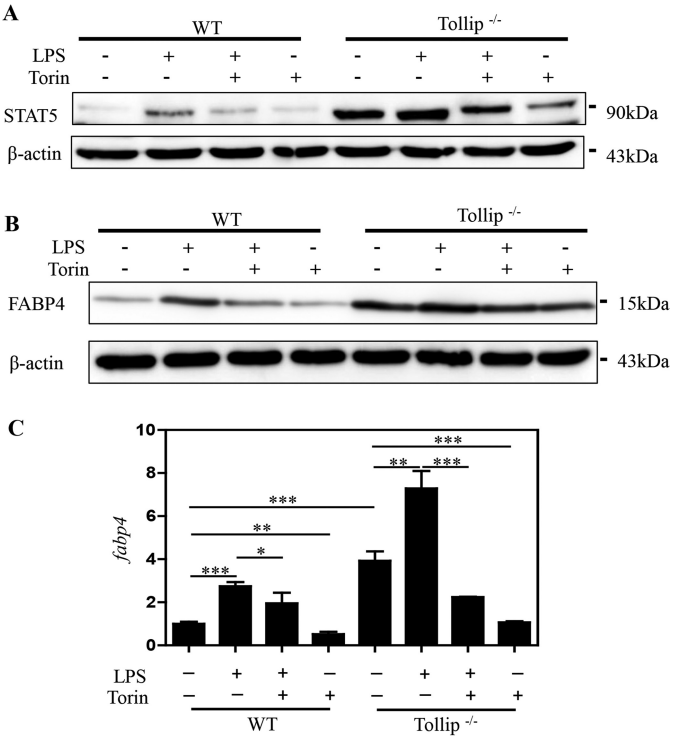

Figure 6. Application of autophagy inducer Torin 2 reduces STAT5 and FABP4 expressions. WT and Tollip$^{-/-}$ MEF cells were treated with LPS (10 ng/ml) and Torin 2 (10 mM) for 6 h prior to protein and RNA extraction. The protein levels of STAT5 and FABP4 were determined by Western blotting analyses. *A* and *B*, relative protein expression of STAT5, FABP4, and β-actin. *C*, *Fabp4* mRNA expression were determined by RT–PCR. The data represent three separate experiments. All values were normalized, using WT (−) as 1. Significance was determined by *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

specific siRNA were comparable (Fig. 5*D*). Our data further corroborate our conclusion that STAT5 elevation caused by Tollip deficiency is responsible for the enhanced induction of *fabp4*.

Induction of autolysosome fusion reduced STAT5 activation and FABP4 expression

To independently corroborate the contribution of lysosome fusion with the modulation of STAT5 and FABP4 expression in WT and Tollip-deficient cells, we applied the chemical inducer of autophagy torin 2 (22). In contrast to bafilomycin, torin is known to restore defective autophagy and lysosome fusion. We thus hypothesize that torin may reduce *fabp4* induction by LPS in both WT and Tollip-deficient cells by restoring autolysosome-mediated degradation of STAT5. Indeed, we observed that the addition of Torin 2 reduced LPS-induced elevation of STAT5 in both WT and Tollip-deficient cells (Fig. 6*A*). Functionally, the addition of Torin 2 significantly reduced the induction of FABP4 in both WT and Tollip-deficient cells (Fig. 6, *B* and *C*). Collectively, our data suggest that Tollip suppresses FABP4 expression through facilitating lysosome fusion and STAT5 degradation.

Discussion

Our data reveal novel roles of Tollip during the expression of IL-12 and FABP4 within murine fibroblasts. Tollip-deficient fibroblasts subjected to LPS challenge were unable to express IL-6, TNFα, and IL-12. On the other hand, Tollip-deficient cells had increased expression levels of FABP4. We demonstrate that Tollip may facilitate the low-grade expression of IL-6, TNFα, and IL-12 through stabilizing HIF1α while suppressing the expression of FABP4 through suppressing STAT5 in fibroblasts.

Our study clarifies the complex functions of Tollip in the modulation of innate immune responses in fibroblasts. Tollip can serve as either a positive or negative regulator of LPS signaling processes in myeloid cells such as macrophages, dependent upon the signal strength of LPS. Higher LPS levels can induce compensatory endotoxin tolerance, and elevated Tollip levels may contribute to endotoxin tolerance by suppressing IRAK-1–mediated NF-κB activation (23). In contrast, super-low-dose LPS does not trigger robust NF-κB activation nor endotoxin tolerance (24). Instead, super-low-dose LPS triggers prolonged low-grade inflammatory gene expression through translocating Tollip from cellular and/or lysosomal membranes to mitochondria and facilitating reactive oxygen species generation (9). Thus, Tollip serves as a context-dependent adaptor for either endotoxin tolerance (when localized at cellular/lysosome membrane in cells challenged with strong LPS signals) or nonresolving low-grade inflammation (when translocated to mitochondria in cells challenged with weaker LPS signal). We also reported that the functional CUE domain is required for the signal-dependent translocation to mitochondria (9). Tollip CUE mutant constitutively resides at lysosome (9). Although the role of cellular/lysosomal membrane-localized Tollip has been relatively well-defined in the context of endotoxin tolerance in macrophages, the role of Tollip in fibroblast is not defined. To better address this issue, our current study utilizes fibroblast cells, which are known to be nontolerizable to LPS and thus not sensitive to LPS dosage variations (25–27). Fibroblast cells not only can provide a robust system for the focused study of the nonresolving inflammation without the complication of compensatory tolerance but also are highly relevant in chronic diseases such as fibrosis, asthma, and arthritis (25–27). In agreement with studies conducted in macrophages (9, 28), we observed that Tollip is a positive regulator for the expression of inflammatory IL-6, TNFα, and IL-12 in fibroblasts induced by LPS.

Our mechanistic study reveals that Tollip fulfills its role of inducing inflammatory cytokines by stabilizing HIF1α. Although HIF1α is known to be induced by LPS from previous studies and is involved in the expression of inflammatory cytokines, our current data provide evidence that demonstrates Tollip as a key mediator for LPS-induced HIF1α activation in fibroblasts under low-grade nonresolving inflammatory condition. At the mechanistic level, HIF1α is known to be regulated at multiple steps including gene transcription and post-translational stabilization/degradation (29, 30). Our data reveal that Tollip-mediated HIF1α activation in inflammatory fibroblasts is due to HIF1α protein stabilization instead of mRNA expression. HIF1α protein degradation occurs because of elevated chaperone-mediated autophagy (CMA) that is not only distinct from but also may compete with the classical microautophagy involving lysosome fusion with autophagosome (29). Under inflammatory conditions, Tollip in fibroblasts was shown to

translocate away from lysosome, and thus the traditional microautophagy/lysosome fusion is compromised (31). Our current study confirms the functional consequence of Tollip deletion for enhanced CMA and HIF1α degradation. Our data using the CMA activator geldanamycin showing a blockage of HIF1α activation by LPS further support this conclusion.

Our study also reveals the less-recognized role of Tollip in modulating the expression of FABP4. FABP4 is critically involved in cellular stress, inflammation, and metabolic reprogramming (19, 20). Dysregulated expression of FABP4 has been associated with diverse inflammatory diseases such as atherosclerosis and asthma (32, 33). Tollip deletion has been previously shown to contribute to elevated atherosclerosis (31). Human Tollip polymorphisms have been linked with elevated risks of asthma and chronic obstructive pulmonary disease (34, 35). Our current study complements these pathophysiological studies and reveal potential mechanistic cause of Tollip-mediated regulation of FABP4. We observed that Tollip dampens the activation of STAT5, and this may help to prevent excessive activation of cellular mediators associated with the chronic inflammatory diseases. Through the application of autolysosome inhibitor and activator, our data demonstrate that the disruption of autolysosome fusion caused by Tollip deficiency is key for STAT5 activation. Our work is consistent with previous studies that proper lysosome fusions with endosomes/autophagosomes are critically important to maintain cellular homeostasis (36, 37).

Taken together, our current study demonstrates that Tollip is differentially involved in the activations of HIF1α and STAT5 in fibroblasts, causing differential expression of IL-12 and FABP4. Future translational and basic studies are warranted to further clarify the distinct involvements of various human Tollip variants during the pathogenesis of complex inflammatory diseases such as atherosclerosis and respiratory syndromes.

Materials and methods

Reagents

LPS (*Escherichia coli* O111:B4), bafilomycin, LiCl, and geldanamycin were obtained from Sigma–Aldrich. Anti-β-actin antibody was obtained from Santa Cruz Biotechnology. Anti-HIF1α, and anti-STAT5 were obtained from Cell Signaling Technology.

Cell culture

The cells were harvested using 0.05% trypsin; resuspended in DMEM supplemented with 2% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin, and 1% (v/v) L-glutamine; and allowed to equilibrate overnight before additional treatments were performed. WT and Tollip-deficient MEF cells and Tollip mutant MEFs harboring the CUE domain M240A/F241A mutation were cultured in DMEM supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine, and 1% (v/v) streptomycin/penicillin.

Immunoblotting

The cells were washed with DMEM containing no FBS; harvested in SDS lysis buffer containing protease inhibitor mixture (Sigma) and phosphatase inhibitor cocktails 1 and 2 (Sigma); and subjected to SDS-PAGE. The protein bands were transferred to an Immun-Blot polyvinylidene difluoride membrane (Bio-Rad). Western blotting analyses were performed with the specified antibodies according to the manufacturer's instructions. Graphical analyses were performed using the ImageJ program.

mRNA expression analysis

Total RNA was extracted after specified treatments using an Isol-RNA lysis reagent (Invitrogen), and cDNA was generated with a high-capacity cDNA reverse transcription kit (Applied Biosystems). This was then followed by analysis using SYBR Green Supermix on an iQ5 thermocycler (Bio-Rad). The relative levels of mRNA expression were calculated using the $\Delta\Delta$Ct method, and results were normalized based on the expression of β-actin within the same experimental setting. The relative level of mRNA in untreated WT MEF cells was adjusted to 1 and served as the basal reference value for all subsequent samples tested.

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