Tofacitinib Suppresses IL-10/IL-10R Signaling and Modulates Host Defense Responses in Human Macrophages

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Jak inhibitors are increasingly used in dermatology. Despite broad inhibitory effects on cytokine signaling cascades, they only modestly increase the risk for infectious diseases. To address the molecular mechanisms underlying this unexpected clinical observation, we investigated how tofacintib (tofa), a first-in-class Jak inhibitor, regulates host defense responses in toll-like receptor 4—activated human macrophages. Specifically, we asked whether tofa inhibits anti-inflammatory IL-10 signaling, thereby counteracting the downregulation of inflammatory, host-protective pathways. We found that tofa blocked macrophage responses to IL-10 at the level of signal transducer and activator of transcription 3 phosphorylation. Furthermore, toll-like receptor 4—induced, autocrine/paracrine IL-10/IL-10R activation promoted the expression of hepcidin, the master regulator of iron metabolism, resulting in intracellular iron sequestration. In contrast, autocrine/paracrine IL-10/IL-10R activation repressed the expression of cathelicidin antimicrobial peptide as well as antigen-presenting molecules, thus together, inducing a pathogen-favoring environment. Although tofa further repressed cathelicidin, it prevented the induction of intracellular HAMP and restored the expression of antigen-presentation molecules in toll-like receptor 4—activated macrophages. Our study supports the concept that induction of IL-10/IL-10R signaling drives a complex immune evasion strategy of intracellular microbes. Moreover, we conclude that tofa has diverging effects on macrophage host response pathways, and we identify the toll-like receptor 4—IL-10—signal transducer and activator of transcription 3—HAMP axis as a potential therapeutic target to counteract immune evasion.

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INTRODUCTION

Inhibitors of Jaks are increasingly used to treat autoimmune and other noncommunicable inflammatory diseases in dermatology (Ciechanowicz et al., 2019; Feldman et al., 2016; Wendel et al., 2019). Jaks mediate the signal transduction of numerous type I and type II cytokine receptors lacking intrinsic catalytic activity. On ligand-mediated receptor multimerization, activated Jaks phosphorylate signal transducers and activators of transcription (STATs). Phosphorylated STATs (pSTATs) dimerize and translocate to the nucleus to regulate the activation of transcription (Rawlings et al., 2004). The Jak family consists of Jak1, Jak2, Jak3, and TYK2 (Xin et al., 2020). As a first-in-class drug, tofacitinib (tofa) was designed to inhibit Jak3 and was approved in 2012 for treating rheumatoid arthritis (Garber, 2013, 2011). However, tofa also inhibits Jak1 and Jak2 (Ghoreschi et al., 2011; Kontzias et al., 2012). In dermatology, tofa is emerging as a therapy for multiple indications, including psoriasis, atopic dermatitis, alopecia areata, granuloma annulare, and sarcoidosis (Bissonnette et al., 2016; Damsky et al., 2020; Feldman et al., 2016; Hodge et al., 2016; Hogan et al., 2019; Wang et al., 2021).

Contrary to the mAbs against specific cytokines or their receptors, Jak inhibitors affect signaling by numerous cytokines. Thus, infections during Jak-targeted therapies constitute a major concern. Nevertheless, Jak inhibitors seem unexpectedly safe in regard to infectious adverse events. For instance, infection rates in patients treated with tofa were comparable or even lower than those in patients treated with anti—TNF-α antibodies (Cohen et al., 2014; Furst et al., 2015; Pawar et al., 2020; Wollenhaupt et al., 2019). To gain better insight into these clinical observations, an increased knowledge regarding the molecular effects of Jak/STAT inhibition on human host defense is needed.

IL-10/IL-10R signaling plays a fundamental role in preventing overshooting immune activation (Grütz, 2005). However, IL-10/IL-10R signaling also suppresses

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Abbreviations: 1,25D, 1,25-dihydroxyvitamin-D; 25D, 1-α-25-hydroxyvitamin D; GC, gonococcus lysate; LPS, lipopolysaccharide; MDM, monocyte-derived macrophage; pSTAT, phosphorylated signal transducer and activator of transcription; STAT, signal transducer and activator of transcription; TLR, toll-like receptor; tofa, tofacitinib

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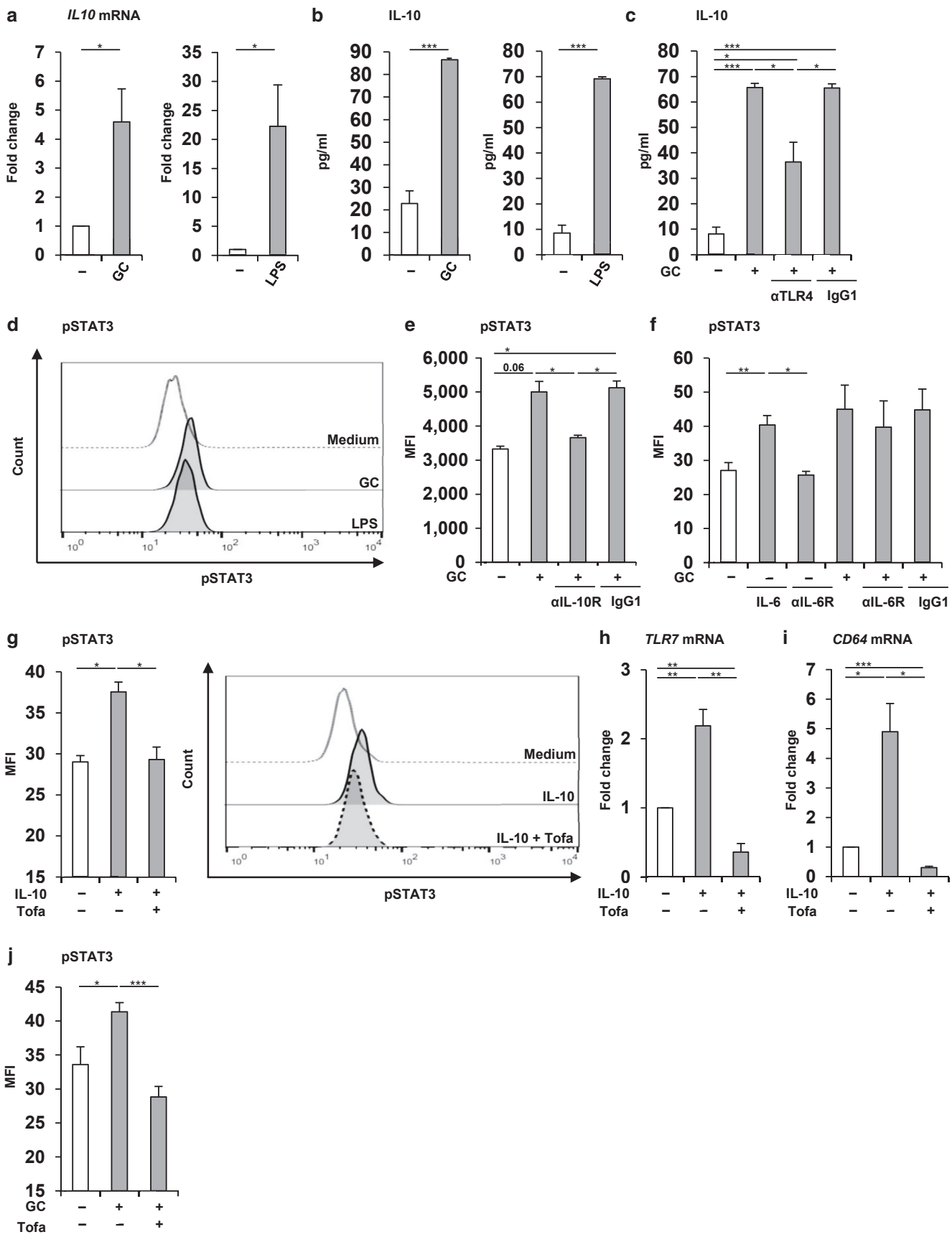

Figure 1. Tofa inhibits IL-10/IL-10R signaling in human macrophages. MDMs were treated with GC or LPS for 20–24 hours. (**a**) *IL10* mRNA expression (n = 6) and (**b**) IL-10 cytokine secretion (n = 4). (**c**) MDMs preincubated with an αTLR4 mAb or isotype control and stimulated with GC for 67 hours. IL-10 cytokine secretion (n = 4). (**d**) MDMs treated with GC or LPS, preincubated with (**e**) an αIL-10Rα mAb, or (**f**) αIL-6Rα mAb or isotype control Ab and stimulated with GC

antimicrobial mechanisms, which can be hijacked by pathogens to evade host defense (Balcewicz-Sablinska et al., 1999; Beamer et al., 2008; Carey et al., 2012; Couper et al., 2008; Gaddis et al., 2013; Higgins et al., 2003; Kane and Mosser, 2001; Mege et al., 2006). For instance, *Neisseria gonorrhoeae* triggers IL-10 secretion in murine lymph nodes to suppress adaptive immunity and induces IL-10 in human macrophages to avoid protective immunity (Liu et al., 2014; Ortiz et al., 2015; Plant and Jonsson, 2006). Moreover, IL-10 dampens the activation of CAMP in mycobacterium-infected monocytes (Teles et al., 2013). In regard to IL-10–driven immune evasion, pathogen-recognizing toll-like receptors (TLRs), including TLR2/1 and TLR4, play a central role because they not only activate proinflammatory cascades but also simultaneously trigger IL-10 (Ortiz et al., 2015; Pengal et al., 2006; Teixeira-Coelho et al., 2014). Given that IL-10/IL-10R signals are transduced by TYK2 and Jak1, we investigated how tofa regulates IL-10/IL-10R signaling and the consequences on antimicrobial pathways in TLR-activated human macrophages, key players of innate host defense.

RESULTS

Tofa inhibits IL-10/IL-10R signaling in human macrophages

TLRs induce IL-10 in innate immune cells (Liu et al., 2014; Ortiz et al., 2015; Pengal et al., 2006). Consistently, lysates from gram-negative *N. gonorrhoeae* (gonococcus lysate [GC]; Abcam, Cambridge, UK) and lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO) induced *IL10* gene expression and protein secretion in human monocyte-derived macrophages (MDMs) (Figure 1a and b). Moreover, GC-induced IL-10 secretion was significantly inhibited (∼50%) by a TLR4-blocking mAb (InvivoGen, San Diego, CA) (Figure 1c), showing that TLR4 is critically involved in GC induction of IL-10. In addition, LPS and GC promoted pSTAT3 measured in our assay 20 hours after stimulation (Figure 1d). IL-10 induces pSTAT3 within minutes (Braun et al., 2013) (Supplementary Figure S1a). In LPS-stimulated MDMs, IL-10 and pSTAT3 were detected after 6 hours but not 30 minutes or 1.5 hours after stimulation (Supplementary Figure S1b and c), raising the possibility that autocrine/paracrine IL-10/IL-10R signaling mediates STAT3 phosphorylation in TLR4-activated MDMs. To investigate this point, we activated MDMs with GC in the presence of a mAb against IL-10R (Sigma-Aldrich). FACS analyses revealed a block of pSTAT3 by the IL-10R blocking (Figure 1e). In addition to IL-10, IL-6 constitutes a central macrophage cytokine, which signals through pSTAT3 (Pullamsetti et al., 2018). However, in M-CSF–generated, TLR4-activated MDMs, a robust IL-10 induction dominates over an IL-6 response (Kwan et al., 2007). Consistently, we found no inhibitory effect on pSTAT3 by an IL-6R-blocking mAb (R&D System, Mineapolis, MN) in GC-activated MDMs (Figure 1f). Nevertheless, recombinant human IL-6 (Miltenyi Biotec, Bergisch Gladbach, Germany) induced pSTAT3, which was completely abrogated by blocking IL-6R (Figure 1f). Together, the data suggest that IL-10 is a central mediator of STAT3 phosphorylation in GC-activated macrophages.

Tofa, albeit designed as a Jak3 inhibitor, represses IL-10 signaling by blocking STAT3 phosphorylation, which is mediated by Jak1 and TYK2, in mouse macrophages and human leukocytes (McInnes et al., 2019; Pattison et al., 2012). To test whether tofa suppresses IL-10 signaling in human macrophages, we stimulated MDMs with recombinant IL-10 (Miltenyi) with or without tofa (Sigma-Adrich) or left cells untreated and measured pSTAT3. IL-10–induced pSTAT3 was abrogated by tofa (Figure 1g). In addition, IL-10 induction of *TLR7* and *CD64* mRNAs (Moore et al., 2001; Teles et al., 2013; te Velde et al., 1992) was prevented by tofa (Figure 1h and i). Furthermore, we showed that tofa prevented pSTAT3 induction by GC (Figure 1j). Together, our data suggest that tofa inhibits IL-10R signaling induced by either exogenous or TLR4-induced autocrine/paracrine IL-10 in human macrophages.

Autocrine/paracrine IL-10 signaling modulates human macrophage responses

CAMP is a broadly active antimicrobial peptide, which kills for instance *Mycobacteria*, *Neisseria*, *Escherichia coli*, *Listeria monocytogenes,* and methicillin-resistant *Staphylococcus aureus* (Bosch et al., 2020; Jones et al., 2009; Liu et al., 2007). TLR ligands can trigger CAMP by activating intracellular vitamin D metabolism, downstream of IL-15 (Fabri et al., 2011; Krutzik et al., 2008; Montoya et al., 2014). Meanwhile, TLR ligands induce IL-10. In turn, IL-10 inhibits CAMP in monocytes (Teles et al., 2013). This was shown by either adding exogenous IL-10 or in the context of IFN-1–mediated induction of IL-10 (Teles et al., 2013). Furthermore, *Mycobacterium tuberculosis* and *N. gonorrhoeae* can downregulate CAMP expression (Bergman et al., 2005; Fabri et al., 2011). Taken together, these findings prompted us to investigate whether TLR-induced autocrine/paracrine IL-10/IL-10R signaling dampens CAMP induction in macrophages. Thus, we stimulated MDMs with 1,25-dihydroxyvitamin D (1,25D; Biomol, Hamburg, Germany), the high-affinity ligand for the vitamin D receptor, alone and in combination with GC or left cells unstimulated. As expected, 1,25D triggered *CAMP* mRNA and protein expression compared with medium control, measured by qPCR and intracellular FACS staining, respectively (Figure 2a and b). However, GC inhibited 1,25D-induced *CAMP* mRNA and intracellular protein expression by ∼70%, respectively (Figure 2a and b). We also found that LPS completely blocked 1,25D-mediated CAMP induction (Figure 2c). To determine whether GC- and LPS-mediated CAMP repression involves IL-10/IL-10R activation, we added the IL-10R–blocking mAb to cultures in which MDMs were treated with a combination of 1,25D and GC or LPS. We found

or rIL-6 for 20 hours (n = 3). (**d–f**) pSTAT3 by FACS. (**g–i**) MDMs preincubated with tofa for 30 minutes and stimulated with rIL-10 for 20 hours. (**g**) pSTAT3 measured by FACS (n = 4). mRNA expressions of (**h**) *TLR7* and (**i**) *CD64* (n = 4). (**j**) MDMs preincubated with tofa for 30 minutes and treated with GC for 20 hours. pSTAT3 measured by FACS (n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Ab, antibody; GC, gonococcus lysate; LPS, lipopolysaccharide; MDM, monocyte-derived macrophage; MFI, mean fluorescence intensity; pSTAT, phosphorylated signal transducer and activator of transcription; rIL-6, recombinant IL-6; rIL-10, recombinant IL-10; TLR, toll-like receptor; tofa, tofacitinib.

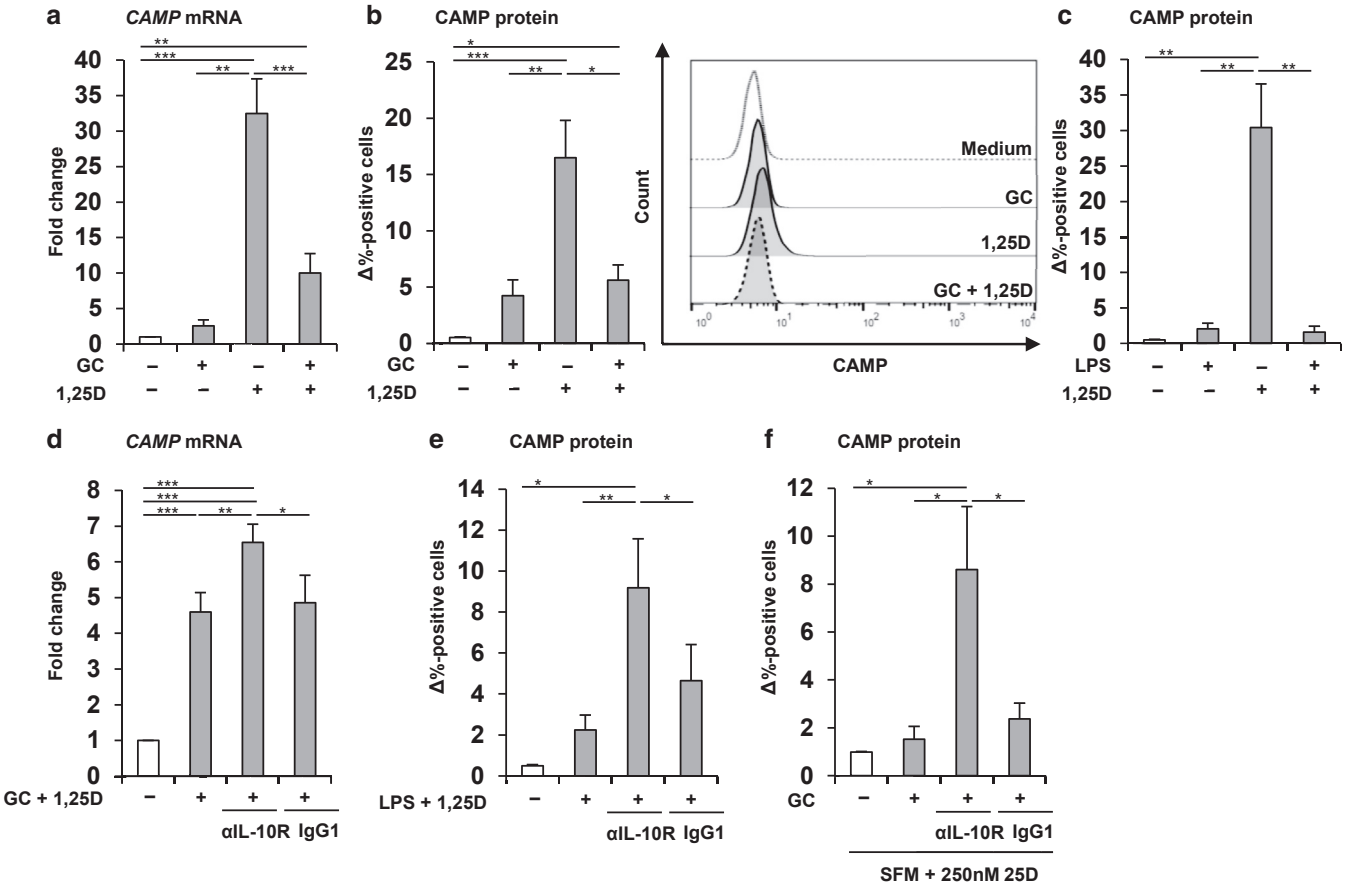

Figure 2. TLR4-induced IL-10 inhibits CAMP expression in human macrophages. (**a**) MDMs treated with GC and/or 1,25D. *CAMP* mRNA expression after 20 hours (n = 6) and (**b**) intracellular CAMP protein expression after 48 hours (n = 6) and of (**c**) LPS- and/or 1,25D-treated MDMs after 67 hours (n = 5). (**d**) MDMs preincubated with a mAb against IL-10Rα or isotype control and stimulated with or without GC and 1,25D for additional 20 hours. *CAMP* mRNA expression (n = 6) or (**e**) intracellular CAMP protein expression after 67 hours stimulation with LPS and 1,25D (n = 7). (**f**) MDMs preincubated with a mAb against IL-10Rα or isotype control and stimulated with GC for additional 67 hours in 25D-containing serum-free medium. Protein expression of CAMP (n = 8). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. 1,25D, 1,25-dihydroxyvitamin D; 25D, 1-α-25-hydroxyvitamin D; GC, gonococcus lysate; LPS, lipopolysaccharide; MDM, monocyte-derived macrophage; SFM, serum-free medium; TLR, toll-like receptor.

that IL-10R blocking significantly promoted *CAMP* mRNA and intracellular protein expression (Figure 2d and e). Nevertheless, physiologic TLR-mediated induction of CAMP is not mediated by extracellular 1,25D but rather by activation of the 1-α-25-hydroxyvitamin D (25D) hydroxylase CYP27b1, downstream of IL-15 (Chung et al., 2020; Fabri et al., 2011; Liu et al., 2006; Montoya et al., 2014), which in turn converts 25-hydroxyvitamin D intracellularly into 1,25D. Thus, we next investigated the effect of IL-10 blocking in GC-activated MDM cultures in the presence of 25D (Biomol). CAMP protein expression was observed only when IL-10R was blocked (Figure 2f). Thus, IL-10 can dampen CAMP induction driven by either exogenous 1,25D or by intracellularly converted 1,25D. Nevertheless, it seems likely that other anti-inflammatory cytokines can also contribute to the downregulation of CAMP expression. In this regard, IL-6 is one candidate because it signals through multiple pathways, including STAT3, phosphatidylinositol 3-kinase/protein kinase B, and MAPK/extracellular signal–regulated kinase, and possesses a complex dual proinflammatory and anti-inflammatory function (Pullamsetti et al., 2018). In fact, we found that IL-6R blocking enhanced intracellular CAMP protein expression (Supplementary Figure S2). In summary, our data show that TLR4-induced autocrine/paracrine signaling cascades—among which IL-10/IL-10R and IL-6/IL-6R signaling play central roles—dampen CAMP expression in human macrophages.

Tofa exerts diverging effects on human macrophage host responses

Our data showed that on one hand, tofa inhibits IL-10 signaling by dampening STAT3 phosphorylation and that on the other hand, CAMP expression is regulated by autocrine/paracrine IL-10/IL-10R signaling. Taken together, this prompted us to investigate two nonmutually exclusive hypotheses: (i) tofa repression of IL-10 may directly promote CAMP expression and (ii) tofa, being a relatively broad Jak inhibitor, may dampen CAMP induction by suppressing proinflammatory signaling cascades. To address this, we explored the effect of tofa on CAMP expression. Thus, we stimulated MDMs with 1,25D alone and in combination with GC or LPS and added tofa. Intracellular CAMP measurements by flow cytometry showed that either treatment combined with tofa suppressed 1,25D-induced CAMP expression stronger than GC or LPS alone (Figure 3a and b).

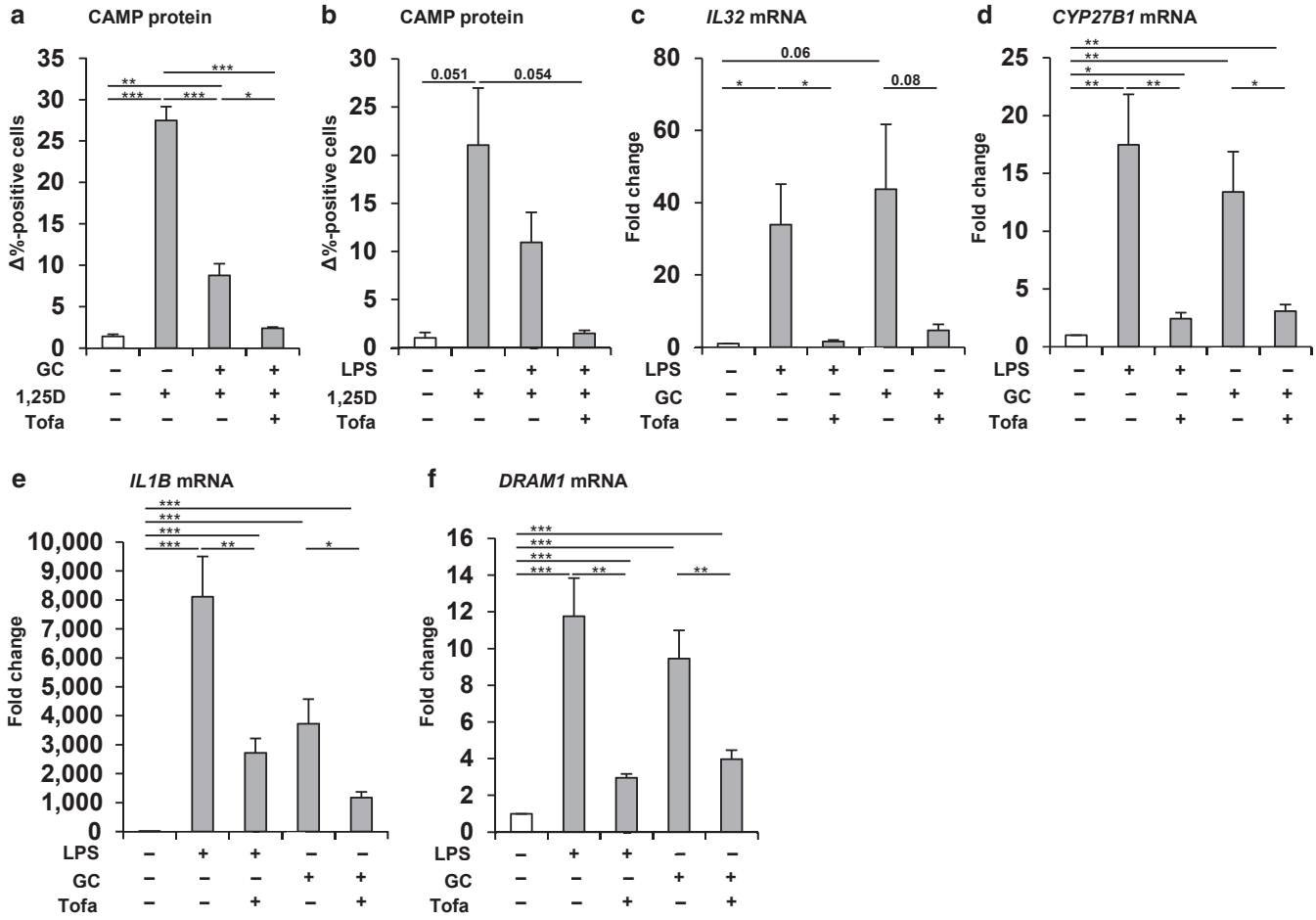

Figure 3. Tofa inhibits the expression of CAMP and the related host defense genes in human macrophages. (**a**) MDMs preincubated with tofa for 30 minutes and stimulated with 1,25D alone and with GC or (**b**) with LPS for additional 67 hours. Intracellular CAMP protein expression (n = 4 and n = 3, respectively). (**c**) MDMs preincubated with tofa for 30 minutes and stimulated with GC or LPS for 20 hours. *IL32*, (**d**) *CYP27B1*, (**e**) *IL1B*, and (**f**) *DRAM1* mRNA expression (n = 7–8). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. 1,25D, 1,25-dihydroxyvitamin-D; GC, gonococcus lysate; LPS, lipopolysaccharide; MDM, monocyte-derived macrophage; tofa, tofacitinib.

Furthermore, we investigated whether tofa may prompt the expression of other host defense genes related to the vitamin D–cathelicidin axis, specifically *IL-32*, *CYP27B1*, *IL-1B* (Liu et al., 2009), and *DRAM1* (Steiger et al., 2016). Although GC and LPS induced the mRNA expression of these genes, their expression was inhibited by tofa (Figure 3c, d, and f). In summary, these data show that despite blocking IL-10 signaling, tofa abolished the induction of CAMP and the related host defense pathway genes. That being said, IL-10 is known to regulate numerous immune pathways, including the suppression of both CD86 (Soltys et al., 2002) (Supplementary Figure S3a) as well as HLA-DR (Supplementary Figure S3b) expressions. Therefore, we next asked whether tofa also affects CD86 and HLA-DR surface expression in GC- and LPS-activated macrophages. Flow cytometry analyses showed that tofa promoted CD86 and HLA-DR expression in GC- or LPS-activated MDMs (Supplementary Figure S4a and b). Meanwhile, GC- and LPS-induced TNF-α secretion was not affected by tofa (Supplementary Figure S4c). Taken together, we found complex effects of tofa on macrophage functions. On one hand, in GC- or LPS-activated macrophages, tofa suppresses CAMP expression and defense pathway–related genes. In contrast, tofa treatment upregulates the expression of surface proteins associated with macrophage maturation and activation and antigen presentation, whereas TNF-α expression was not affected.

Tofa inhibits TLR4-induced HAMP expression by blocking IL-10/IL-10R signaling in human macrophages

Intracellular pathogens commonly rely on host sources to fulfill their iron demands. Thus, the battle for iron between the host and pathogens constitutes a major determinant of defense and immune evasion (Armitage et al., 2009; Drakesmith and Prentice, 2012; Michels et al., 2015; Nairz et al., 2010, 2008; Zughaier et al., 2014b). Intracellular iron levels are controlled by the master iron regulator HAMP (Collins et al., 2008; Nemeth et al., 2004). It is known that (i) TLR4 activates HAMP-mediated iron sequestration (Andrade et al., 2016; Zughaier et al., 2014b), (ii) pathogens use this axis as an immune evasion strategy (Andrade et al., 2016; Zughaier et al., 2014b), and (iii) IL-10 regulates HAMP expression in macrophages (Huang et al., 2017, 2014). Hence, to further explore the possibility of tofa counteracting

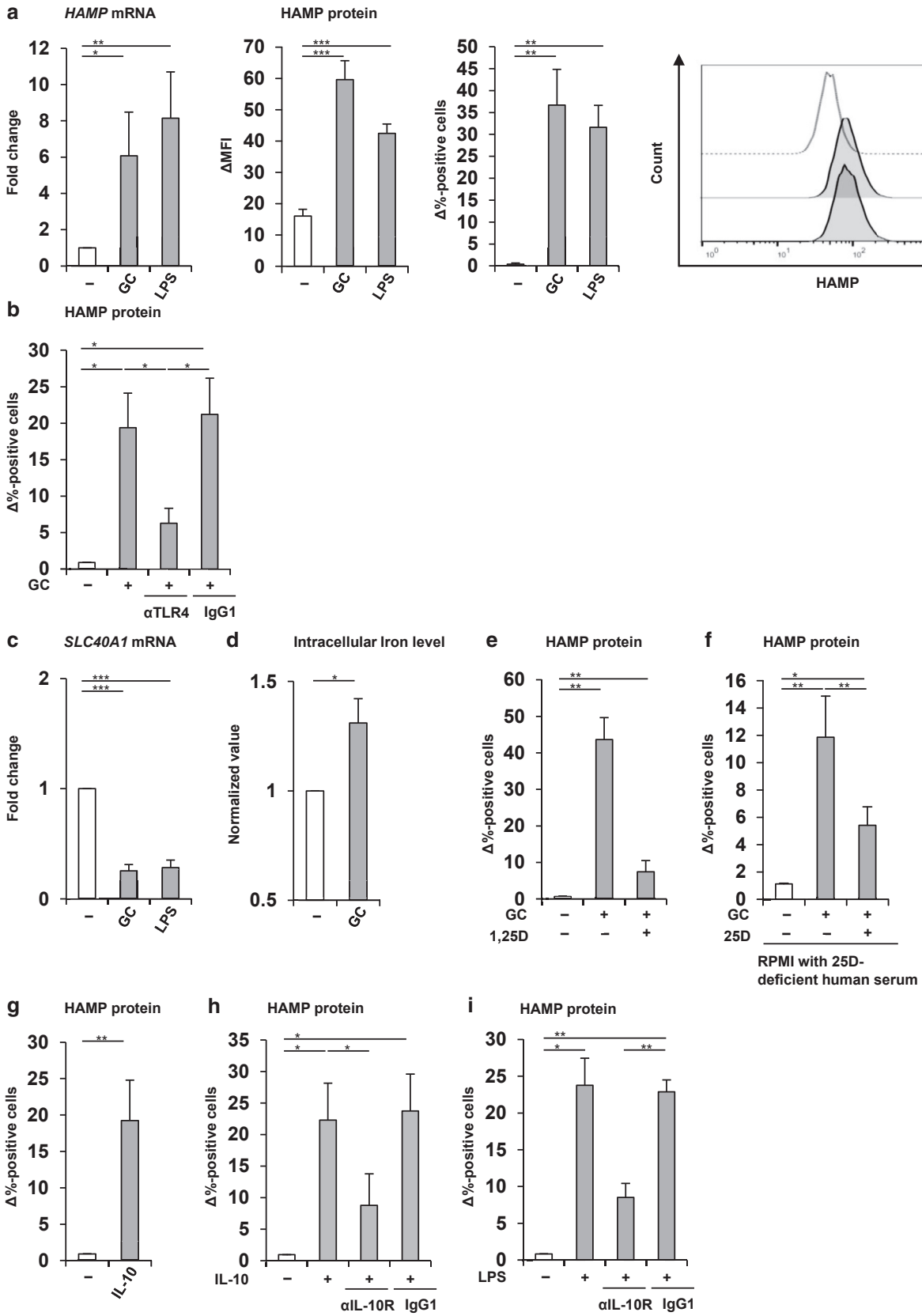

Figure 4. TLR4Ls induce HAMP expression through IL-10/IL-10R signaling in human macrophages. (**a**) MDMs stimulated with GC or LPS. *HAMP* mRNA expression for 4 hours (n = 4). Intracellular HAMP protein expression after 48 hours (n = 4). (**b**) MDMs preincubated with a mAb against TLR4 or corresponding isotype control and stimulated with GC for 48 hours. Intracellular HAMP protein expression (n = 5). (**c**) MDMs stimulated with GC or LPS for 4 hours. mRNA expression of *SLC40A1* (n = 5). (**d**) MDMs stimulated with GC for 24 hours in serum-free medium supplemented with ferric ammonium citrate. Intracellular iron levels (n = 4). (**e**) MDMs stimulated with GC or in combination with 1,25D and (**f**) with 10% 25D in 25D-deficient serum for 48 hours. Intracelluar HAMP protein

IL-10–mediated repression of macrophage defense pathways, we decided to investigate the TLR4–IL-10 axis in the context of another important host defense mechanism: the host iron milieu. We treated MDMs with GC or LPS or left the cells unstimulated and thereafter measured HAMP expression. As expected, GC and LPS induced *HAMP* mRNA and intracellular protein expression compared with medium control (Figure 4a). The same was true when cells were treated with the *M. tuberculosis*–derived 19 kD triacylated lipopeptide (EMC Microcollections, Tuebingen, Germany), a TLR2/1L (Supplementary Figure S5a). To test whether HAMP induction by GC is TLR4 dependent, we used a blocking mAb against TLR4. Blocking of TLR4 led to an inhibition of GC-induced HAMP protein expression by ∼70% (Figure 4b). Furthermore, we examined the mRNA expression of *SLC40A1*, encoding the only known iron exporter ferroportin, a HAMP target (De Domenico et al., 2011, 2007; Nemeth et al., 2004). GC, LPS, and 19 kD reduced ferroportin mRNA expression in MDMs by 4.0, 3.5, and 3.5-folds, respectively (Figure 4c and Supplementary Figure S5b). Next, we measured the iron levels in MDMs. We detected an ∼30% increase of the intracellular iron concentration in GC-treated MDMs compared with that in the medium control (Figure 4d). The fact that vitamin D regulates HAMP expression (Bacchetta et al., 2014) prompted us to analyze the effect of vitamin D on HAMP expression in MDMs by intracellular FACS staining. The 1,25D reduced GC-induced HAMP protein expression by ∼85% (Figure 4e). Moreover, GC-stimulated MDMs showed an enhanced HAMP protein expression compared with medium control when cultured in 25D-deficient human serum. Meanwhile, the addition of 25D to the serum reduced HAMP protein expression by ∼60% (Figure 4f).

We next hypothesized that IL-10 mediates HAMP upregulation on TLR4 activation. When we treated MDMs with recombinant IL-10, we found an increase of HAMP protein expression (Figure 4g), which was diminished by IL-10R blocking (Figure 4h). We showed in Supplementary Figure S1b and c that IL-10 and pSTAT3 were not detected after 30 minutes and 1.5 hours in LPS-treated MDMs; yet, we detected *HAMP* mRNA after 4 hours (Figure 4a). Thus, we asked whether the activation of IL-10/pSTAT3 occurs between 1.5 hours and 4 hours. Albeit we considered that the performed assays could not be sufficient to detect very low levels of cytokines already provoking significant biological activities. At 3.5 hours after LPS treatment, IL-10 and pSTAT3 were significantly induced in MDMs (Supplementary Figure S6a and b). This corroborated our hypothesis that IL-10 mediates HAMP upregulation. To further test this, we treated MDMs with LPS alone or in combination with an IL-10–blocking mAb. IL-10R blocking led to an inhibition of HAMP protein expression (Figure 4i). Finally, IL-10 already induced a significant increase in HAMP protein expression after 6 hours, which was yet absent in LPS-stimulated macrophages (Supplementary Figure S7). Together, our data suggest that the induction of IL-10 is an early event in response to TLR4 ligands driving HAMP upregulation in macrophages.

In this study, our data show that autocrine/paracrine IL-10 signaling regulates HAMP expression in TLR4-activated macrophages. Because tofa inhibits IL-10 signaling, we next tested the effect of tofa on HAMP expression. Therefore, we stimulated MDMs with GC alone and in combination with tofa or left the cells unstimulated. Tofa completely prevented GC-induced *HAMP* mRNA and protein expression (Figure 5a and b). Moreover, tofa almost completely blocked HAMP expression in MDMs simultaneously treated with GC and recombinant IL-10 (Figure 5c). In addition, LPS-induced HAMP protein expression was blocked by tofa (Figure 5d). Increased HAMP serum levels constitute an inflammation marker in patients with rheumatic diseases (Demirag et al., 2009; Sahebari et al., 2018; Sato et al., 2020). Thus, we next collected sera and PBMCs from patients with rheumatoid arthritis treated with tofa or rituximab, a B-cell–depleting antibody, and from a healthy group. We did not observe significant differences in intracellular HAMP protein expression within isolated blood monocytes among the three groups (Supplementary Figure S8a), albeit the range and variability were higher in the two patient groups. Furthermore, we did not observe significant differences in IL-10 serum levels within the groups (Supplementary Figure S8b). Because we did not have access to longitudinal samples from patients treated with tofa before therapy, we could not directly address the question of whether tofa reduces HAMP expression in circulating monocytes in vivo. Nevertheless, we tested the effect of serum from patients treated with tofa, from patients treated with rituximab, and from healthy individuals on *HAMP* expression in LPS-activated MDMs from different healthy donors ex vivo. For that, we cultured LPS-stimulated healthy MDMs in 20% sera from the different groups. We observed significantly lower *HAMP* mRNA expression (∼25%) in MDMs cultured in the sera of patients treated with tofa than in those cultured in the sera from the healthy and rituximab groups (Figure 5e), indicating a possible direct effect of serum tofa. Taken together, our data indicate that tofa inhibits TLR4-induced HAMP expression by blocking IL-10/IL-10R signaling in human macrophages.

DISCUSSION

Given their broad inhibitory effects on the signaling of protective proinflammatory cytokines, it is surprising that Jak inhibitors only moderately increase the risk for infectious disease. To get a better understanding of the underlying reasons for these clinical observations on a molecular level, we studied the effect of Jak inhibition by tofa on human macrophages. Tofa abolished TLR4-induced autocrine/paracrine signaling by IL-10, an anti-inflammatory cytokine pivotal to immune evasion. However, we found disparate effects of tofa on two central, IL-10–regulated host defense mechanisms: tofa blocked the induction of CAMP, a broadly active antimicrobial peptide, and the related host defense

expressions (n = 4, n = 6). (**g**) MDMs stimulated with rIL-10 for 48 hours. Intracellular HAMP protein expression (n = 8). (**h**) MDMs preincubated with a mAb against IL-10Rα or corresponding isotype control and stimulated with rIL-10 for 48 hours and (**i**) LPS for 67 hours. Intracellular HAMP protein expression (n = 4, n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. 1,25D, 1,25-dihydroxyvitamin D; 25D, 1-α-25-hydroxyvitamin D; GC, gonococcus lysate; LPS, lipopolysaccharide; MDM, monocyte-derived macrophage; rIL-10, recombinant IL-10; TLR, toll-like receptor; TLR4L, toll-like receptor 4 ligand; tofa, tofacitinib.

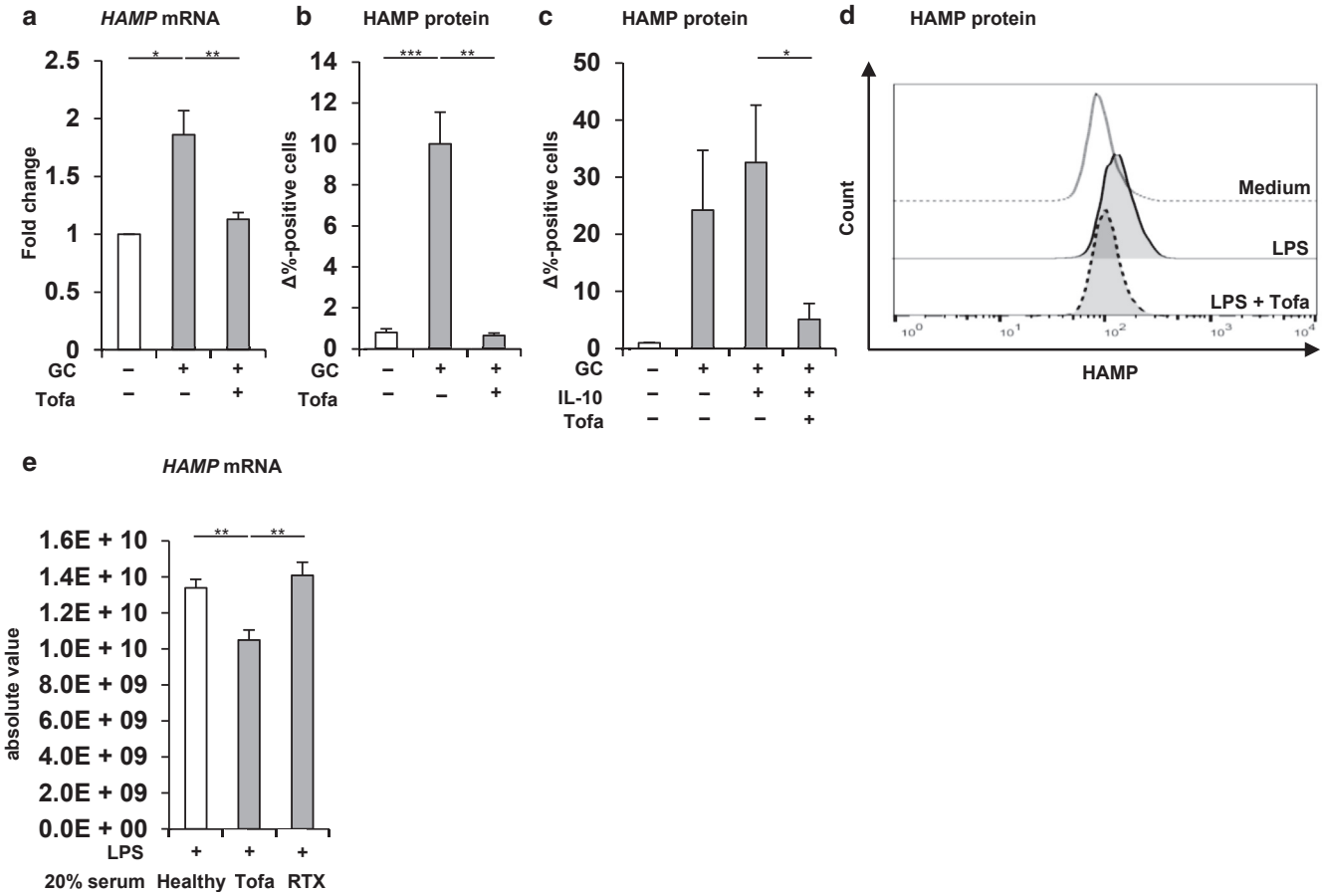

Figure 5. Tofa inhibits TLR4-induced HAMP expression in human macrophages. (**a**) MDMs preincubated with tofa for 30 minutes and stimulated with or without GC for 4 hours. *HAMP* mRNA expression (n = 4) and (**b**) intracellular HAMP protein expression after 20 hours (n = 5). (**c**) MDMs preincubated with tofa for 30 minutes and stimulated with GC alone or in combination with rIL-10 for 67 hours. Intracellular HAMP protein expression (n = 5). (**d**) MDMs preincubated with tofa for 30 minutes and stimulated with or without LPS for 20 hours. Intracellular HAMP protein expression. (**e**) MDMs stimulated with LPS and cultured in serum-free medium with 20% serum of patients with rheumatic diseases treated with tofa or RTX, together with the serum of healthy donors for 4 hours. Intracellular *HAMP* mRNA expression (n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. GC, gonococcus lysate; LPS, lipopolysaccharide; MDM, monocyte-derived macrophage; rIL-10, recombinant IL-10; RTX, rituximab; TLR, toll-like receptor; tofa, tofacitinib.

genes. On the contrary, tofa counteracted IL-10–mediated induction of HAMP, the master regulator of iron metabolism. Given that intracellular pathogens commonly exploit host cell iron metabolism through HAMP induction for their requirements (Ben-Othman et al., 2014; Chlosta et al., 2006; Sow et al., 2007; Zughaier et al., 2014b), our results suggest an antimicrobial, prohost effect of tofa on macrophage-mediated host defense. Therefore, we provide one possible explanation for the limitedly increased incidence of infections in patients receiving Jak inhibitors.

Our study defines a link between TLR4 induction of autocrine/paracrine IL-10/IL-10R signaling and upregulation of HAMP along with downregulation of CAMP expression. It is well-established that TLR4 ligands trigger IL-10, which in turn plays a fundamental role in pathogen-induced immune evasion. Importantly, bacterial pathogens express several pattern-associated molecular patterns that activate specific recognition receptors. For *N. gonorrhoeae* recognition, these receptors include TLR4, cyclic GMP-AMP Synthase, NOD2, TLR9, and CEACAM, of which several induce IL-10 expression, underscoring the relevance of IL-10 in host evasion (Ahn et al., 2017; Andrade et al., 2016; Dobson-Belaire et al., 2010; Mavrogiorgos et al., 2014; Sadarangani et al., 2011; Schmitt et al., 2020). In this study, we showed that IL-10 induces HAMP, consistent with an earlier in vitro finding (Huang et al., 2014) as well as in vivo data linking IL-10 signaling to systemic iron metabolism (Tilg et al., 2002). Moreover, IL-10 represses CAMP (Teles et al., 2013). Using a blocking mAb against IL-10R, we showed that TLR4 induction of HAMP is mediated by autocrine/paracrine IL-10 signaling. Meanwhile, autocrine/paracrine IL-10 signaling dampens the proinflammatory activation of CAMP by TLRs (Fabri et al., 2011; Teles et al., 2013). Using an IL-6R–blocking mAb, we also found that IL-6 contributed to the repression of CAMP in TLR4-activated macrophages, probably mediated by signaling cascades different from Jak/STAT (Pullamsetti et al., 2018), because STAT3 phosphorylation was not affected. Furthermore, blocking IL-10 promoted CD86 and HLA-DR expression in TLR4-activated macrophages. Together, we showed that TLR4-induced autocrine/paracrine IL-10/IL-10R signaling affects macrophage host defense at multiple levels. Moreover, other pattern recognition receptors contribute to the induction of IL-10 and other regulatory cytokines, such as IL-6 or IFN-1s (Andrade

et al., 2016; Ozato et al., 2002; Schmitt et al., 2020), indicating a complex intracellular bacterial survival plan. From a host perspective, protective signaling cascades (e.g., those driven by IL-15 and IL-1β [Fabri et al., 2011]) are induced in parallel, and the dynamic regulation of such cytokine networks finally determines the outcome of human infections.

Tofa shows cellular specificity for Jak1/3 over Jak2 (Changelian et al., 2003; T Virtanen et al., 2019). De Vries et al. (2019) suggested Jak1 as the main target of tofa. Consistently, we showed that tofa blocks IL-10R signaling, which is mediated by TYK2 and Jak1. Furthermore, we provide evidence that this prevents IL-10–driven upregulation of HAMP in TLR-activated macrophages. In addition, blocking IL-10R or adding tofa promoted CD86 and HLA-DR expression. In contrast, tofa inhibits CAMP expression and other related host defense genes stronger than TLR4 ligands alone. This is not surprising given that STATs are involved in the signaling of positive cytokine feedback loops, induced by TLRs, such as IL-15 (Krutzik et al., 2008). Thus, it is likely that the suppression of positive feedback signals from other cytokines masks the effects of IL-10 suppression on CAMP expression by tofa (Pattison et al., 2012; Zavala et al., 2018). Moreover, STATs have been directly linked to TLR signal transduction (Balic et al., 2020; Luu et al., 2014; Samavati et al., 2009).

Given that HAMP is beneficial, but CAMP is detrimental for many intracellular pathogens, their reciprocal regulation constitutes an interesting therapeutic target. An immune modulator known to repress HAMP and promote CAMP is vitamin D (Bacchetta et al., 2014; Fabri et al., 2011; Liu et al., 2007; Moran-Lev et al., 2018; Smith et al., 2017; Zughaier et al., 2014a), which is confirmed by this study. Thus, vitamin D and IL-10 show opposing effects on the reciprocal regulation of HAMP and CAMP. However, clinical trials yet could not show a benefit of vitamin D supplementation in infectious diseases (Ganmaa et al., 2020; Martineau et al., 2015). Meanwhile, tofa suppressed HAMP induction but likewise CAMP expression. In this regard, more specifically inhibiting signal transduction of IL-10, for instance, at the level of STAT3 by TYK2 inhibitors, constitutes one interesting, alternative target for this concept, which should be tested in future studies. Furthermore, it remains to be shown that Jak inhibitors suppress HAMP induction in circulating monocytes or even in tissue macrophages. In this study, we did not have longitudinal monocyte preparations from patients before and after the initiation of tofa treatment. However, given that HAMP expression is also regulated by the underlying disease (Demirag et al., 2009; Sahebari et al., 2018; Sato et al., 2020), direct effects of tofa on circulating monocytes would likely be indirectly altered by disease-modifying effects. Thus, analyses of cells from healthy individuals should be considered in future phase I clinical trials with Jak inhibitors. We showed a reduction of HAMP expression in healthy MDMs treated with sera from tofa patients in comparison with expression in those cultured with sera from healthy or rituximab-treated individuals. This could suggest that a direct effect of serum tofa but other factors may also play a role, including 25D (Sato et al., 2020; Smith et al., 2017) or cytokines, which should be addressed in future studies.

In summary, we provide evidence that tofa suppresses IL-10/IL-10R signaling in human macrophages and that tofa has a major impact on innate host responses. Thereby, our study deepens our knowledge of the effects of Jak inhibitors on human macrophage responses on a molecular level. Such knowledge is crucial for a better understanding of the clinical safety profile of Jak inhibitors in regard to infectious adverse events. Furthermore, we identified STAT3 as a potential target for host-directed therapies to interrupt iron acquisition by intracellular pathogens and simultaneously enhance antimicrobial peptide expression in macrophages, warranting further investigations.

MATERIALS AND METHODS

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki and approved by the local Ethics Committee (Ethikkommission) of the University of Cologne (Cologne, Germany). All donors provided written informed consent for the collection of blood and subsequent analyses.

Macrophage culture

MDMs were prepared by culturing peripheral blood monocytes in RPMI (Thermo Fisher Scientific, Waltham, MA) containing 10% fetal calf serum (PAN-Biotech, Aidenbach, Germany) for 4−6 days with M-CSF (Miltenyi). MDMs were cultured in Macrophage-SFM (Thermo Fisher Scientific), unless indicated otherwise, without supplements or were either supplemented with 10% vitamin D–deficient human AB serum (PAN-Biotech) (25D level 33,75 nmol/l), 250 nM 25D, or 1,000 μM ferric ammonium citrate (Sigma-Aldrich) and incubated with *N. gonorrhoeae* whole-cell lysate, LPS, TLR2/1L Pam_3Cys-SKKK, 25D, 1,25D, recombinant IL-10, anti–IL-10Rα, recombinant IL-6, IL-6Rα, anti-TLR4, IgG1, or tofa. Protein expression was analyzed by FACS (shown as Δ%-positive cells or mean fluorescence intensity + SEM in bar graphs) and mRNA expression (shown as fold change + SEM) by qPCR. Supernatant cytokine levels (shown as mean cytokine levels + SEM) were measured by ELISA.

Iron quantification

Intracellular iron levels were quantified using the Iron Colorimetric Assay (BioVision, Milpitas, CA). MDMs were lysated in Iron Assay Buffer for 45 minutes on ice. Iron levels were normalized to total protein by BCA Protein Assay (Thermo Fisher Scientific).

Statistics

P-values were calculated using two-tailed Student's *t*-tests. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. n refers to the number of biological replicates.

See the Supplementary Materials and Methods for further details on Material and Methods.

Data availability statement

No large datasets were generated; all related data are included in this article or in Supplementary Materials.

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CONFLICT OF INTEREST

EJP is a founder of Rheos Medicines. ELP is a Scientific Advisory Board member of Immunomet Therapeutics and a founder of Rheos Medicines. The remaining authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: KK, MF; Data Curation: KK, MF; Formal Analysis: KK, KMG, MF; Funding Acquisition: MF; Investigation: KK, RRR, KMG, RS, BL, DMK; Methodology: KK, KMG, RS, DMK, MF; Project Administration: KK, MF; Resources: KK, KMG, RS, BL, ELP, EJP, DMK, MF; Supervision: ELP, EJP, MF; Validation: KK, MF; Visualization: KK, MF; Writing - Original Draft Preparation: KK, MF; Writing - Review and Editing: KK, RRR, KMG, RS, BL, NY, ELP, EJP, DMK, MF

Disclaimer

The funders had no role in study design, data collection and analysis, manuscript preparation, or the decision to publish the study.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.07.180.

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SUPPLEMENTARY MATERIALS AND METHODS

Reagents and antibodies are provided in Supplementary Tables S1 and S2 and were purchased from Biomol (Hamburg, Germany), PAA Laboratories (Cölbe, Germany), Miltenyi Biotec (Bergisch Gladbach, Germany), Sigma-Aldrich (St. Louis, MO), GE Healthcare (Chicago, IL), Abcam (Cambridge, United Kingdom), EMC Microcollections (Tübingen, Germany), Life Technologies (Carlsbad, CA), BD Bioscience (Heidelberg, Germany), Cell Signaling Technology (Frankfurt am Main, Germany), InvivoGen (San Diego, CA), and R&D Systems (Minneapolis, MN).

Cell preparation

Whole blood or buffy coats from healthy donors or patients were obtained with informed consent. PBMCs were separated from other components (erythrocytes, granulocytes) of the blood by density gradient centrifugation. $CD14^+$ monocytes were isolated from PBMCs through MidiMACS cell separation system (Miltenyi Biotec) using CD14 MicroBeads according to the manufacturer's instructions.

Real-time quantitative PCR

mRNA was prepared from monocyte-derived macrophages (MDMs) using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized from mRNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA), and mRNA levels were calculated by quantitative PCR as previously described (Fabri et al., 2011). Sequences for human primers (Supplementary Table S3) were previously reported (Fabri et al., 2011; Krutzik et al., 2003; Liu et al., 2006; Montoya et al., 2014; Zughaier et al., 2014) and were purchased from Metabion International AG (Planegg, Germany). QuantiTect Primer Assays for *DRAM1*, *IL1B*, *IL-10*, *IL32*, and *SLC40A1* were from Qiagen.

Serum collection

Serum was collected from patients with rheumatic diseases treated with tofacitinib or rituximab as well as from healthy donors. MDM preparations (biological replicates from healthy donors) were treated with sera from n = 4 different patients. The value for each MDM serum treatment was averaged before calculating the averages of the different MDM preparations.

Flow cytometry

Surface staining was performed in PBS (Thermo Fisher Scientific, Waltham, MA) containing 2% fetal calf serum and 0.1% sodium azide (Sigma-Aldrich). For intracellular measurement of CAMP and HAMP or the appropriate isotype control, cells were stained using Cytofix/Cytoperm Fixation/Permeabilization Kit (BD, Franklin Lakes, NJ) according to the manufacturer's instructions. MDMs staining using phosphorylated signal transducer and activator of transcription 3 and FcR blocking reagent (Miltenyi Biotec) was conducted by 4% formaldehyde fixation followed by permeabilization in 90% methanol. Flow cytometry was accomplished on an Attune NxT (Thermo Fisher Scientific) (Figure 1e), on an LSR II (BD Biosciences, Heidelberg, Germany) (Supplementary Figure S8), or on a FACS Calibur (BD Biosciences) (all other figures). Data were analyzed using FlowJo software (Tree Star, Ashland OR). In some experiments, FACS values were calculated as follows:

$$\Delta MFI = MFI^{specific\ \ mAb} - MFI^{corresponding\ \ isotype\ \ control},$$

where MFI is the mean fluorescence intensity, or $\Delta\% - positive\ cells = \%-positive\ cells^{specific\ mAb} - \%-positive\ cells^{corresponding\ \ isotype\ \ control}$.

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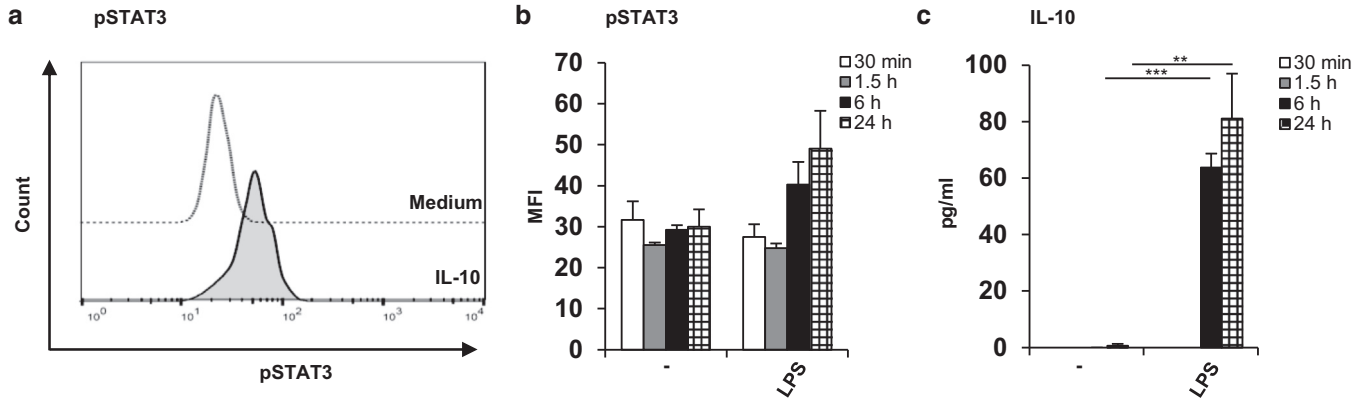
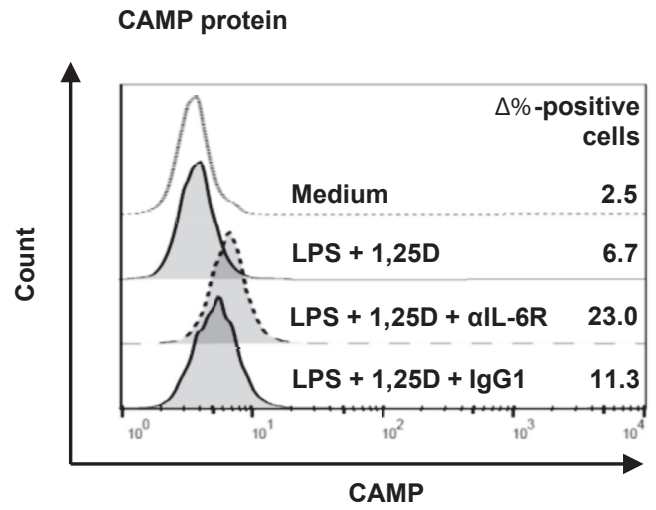
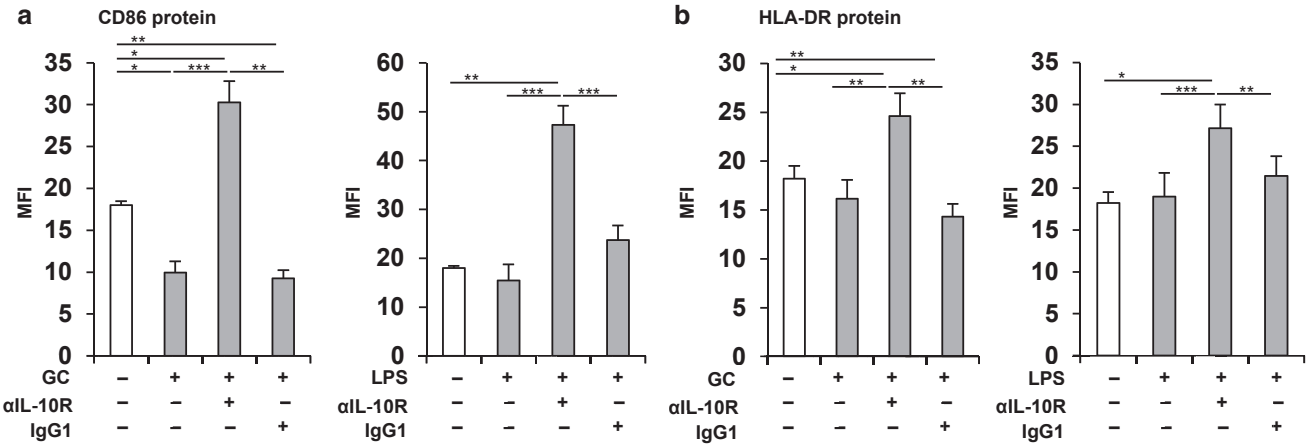


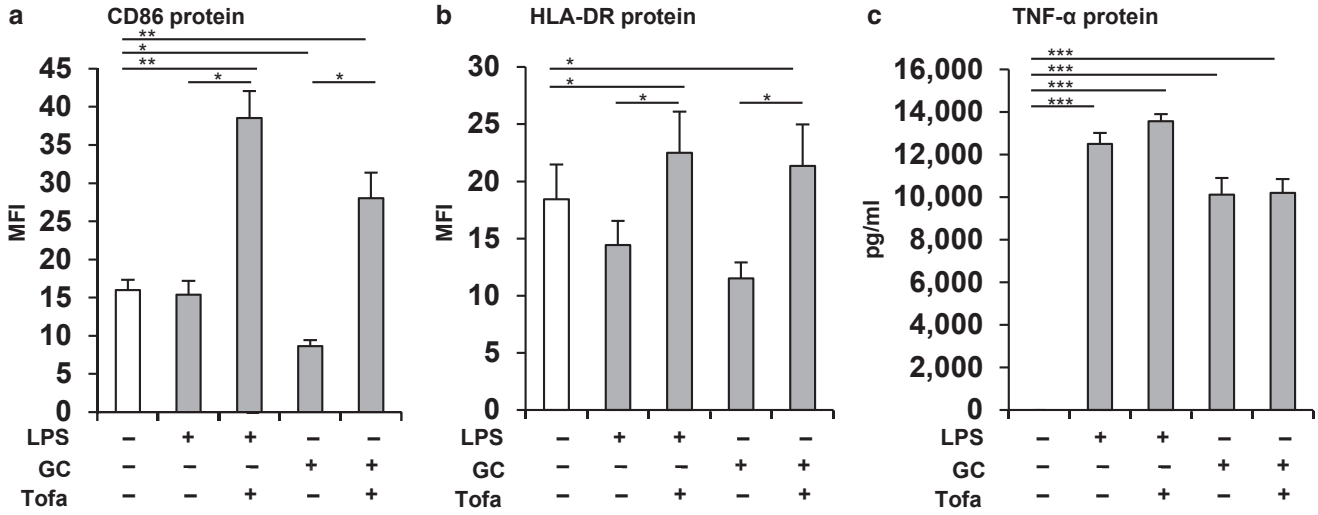
Supplementary Figure S1. pSTAT3 and IL-10 induction by LPS and GC (**a**) MDMs were incubated with rIL-10 for 30 min or (**b**) MDMs were treated with LPS for 30 min, 1.5 h, 6 h, and 24 h. Phosphorylation of STAT3 was measured by FACS (MFI + SEM, n = 3), and **(c)** IL-10 cytokine levels in culture supernatants were measured by ELISA (mean of cytokine levels in pg/ml + SEM, n = 3). $^{**}P < 0.01$, $^{***}P < 0.001$. GC, gonococcus lysate; h, hour; LPS, lipopolysaccharide; MDM, monocyte-derived macrophage; min, minute; MFI, mean fluorescence intensity; pSTAT, phosphorylated signal transducer and activator of transcription; rIL-10, recombinant IL-10; STAT, signal transducer and activator of transcription.



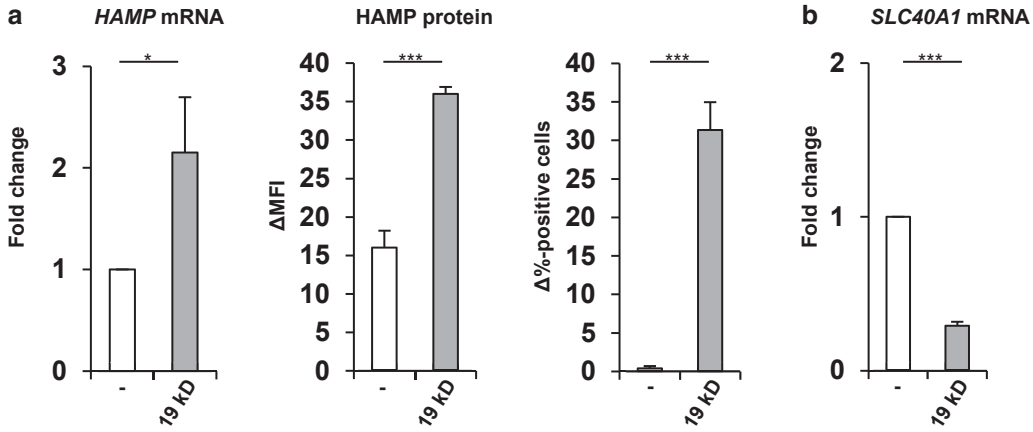
Supplementary Figure S2. IL-6R blocking promotes CAMP expression in TLR4-activated human macrophages. MDMs were preincubated with a mAb against IL-6Rα or corresponding isotype control for 1 hour and stimulated with LPS and 1,25D for additional 67 hours. CAMP protein expression was determined by intracellular FACS staining (Δ%-positive cells + SEM, 1 of 3). 1,25D, 1,25-dihydroxyvitamin D; LPS, lipopolysaccharide; MDM, monocyte-derived macrophage; TLR, toll-like receptor.



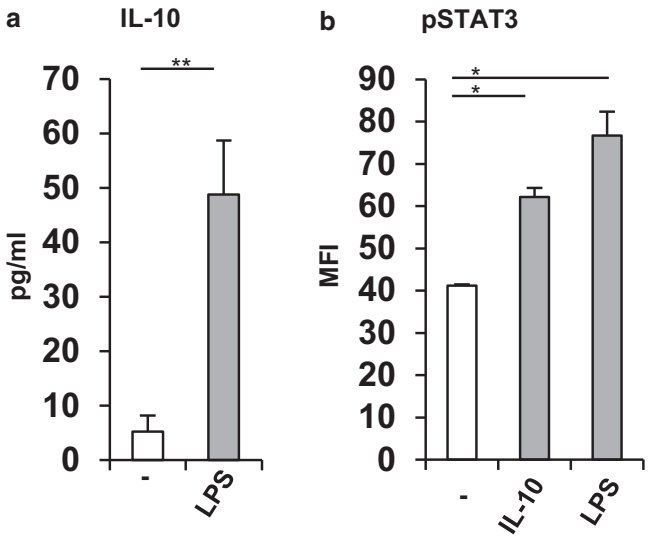
Supplementary Figure S3. IL-10R blocking promotes CD86 and HLA-DR expression in TLR4-activated human macrophages. (**a**) MDMs were preincubated with a mAb against IL-10Rα or the corresponding isotype control for 1 hour and stimulated with GC or LPS for additional 24 hours. Surface protein expression of CD86 and (**b**) HLA-DR was determined by FACS (MFI + SEM, n = 4). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. GC, gonococcus lysate; LPS, lipopolysaccharide; MDM, monocyte-derived macrophage; MFI, mean fluorescence intensity; TLR, toll-like receptor.



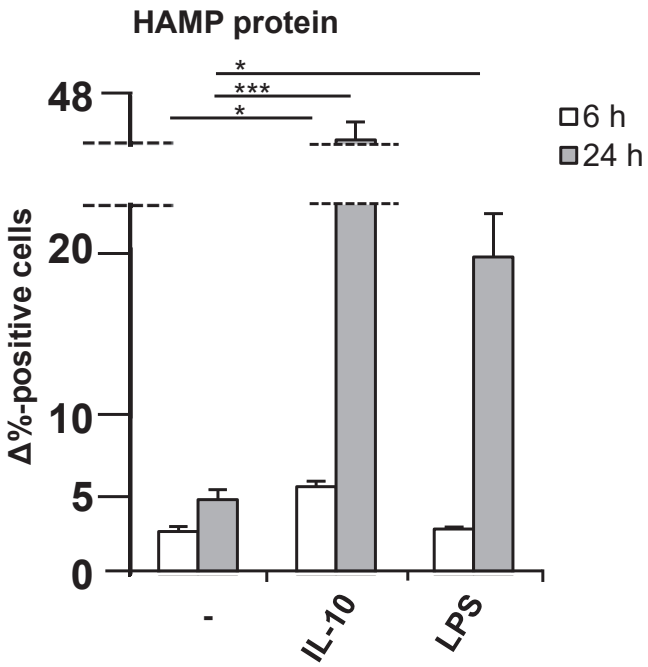
Supplementary Figure S4. Tofa promotes CD86 and HLA-DR expression in TLR4-activated macrophages. (**a**) MDMs were preincubated with tofa for 30 minutes and stimulated with GC or LPS for additional 24 hours. Protein expression of CD86 and (**b**) HLA-DR was determined by surface FACS (MFI + SEM, n = 4), and (**c**) TNF-α cytokine levels in culture supernatants were measured by ELISA (mean of cytokine levels in pg/ml + SEM, n = 4). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. GC, gonococcus lysate; LPS, lipopolysaccharide; MDM, monocyte-derived macrophage; MFI, mean fluorescence intensity; TLR, toll-like receptor; tofa, tofacitinib.



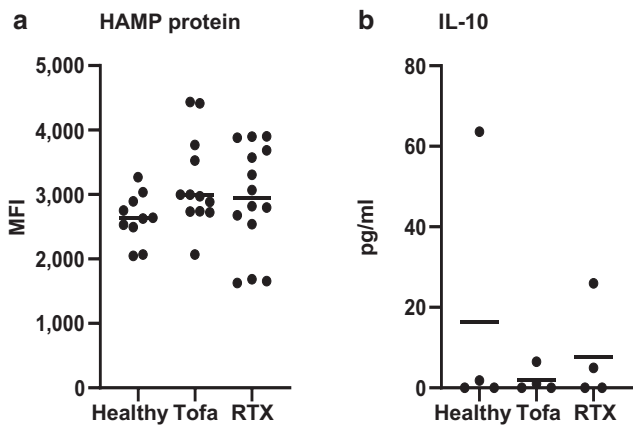
Supplementary Figure S5. TLR2/1 ligand induces HAMP expression in human macrophages. (**a**) MDMs were stimulated with 19 kD for 4 hours. mRNA expression of *HAMP* was assessed by qPCR (mean fold change + SEM, n = 4). HAMP protein expression was examined by intracellular FACS after 48 hours (ΔMFI/Δ%-positive %-positive cells + SEM, n = 4). (**b**) MDMs were stimulated with 19 kD for 4 hours. mRNA expression of *SLC40A1* was assessed by qPCR (mean fold change + SEM, n = 6). (**c**) MDMs were stimulated with 19 kD or in combination with 1,25D for 48 hours. HAMP protein expression was examined by intracellular FACS (Δ%-positive cells + SEM, n = 4). $^{*}P < 0.05$, $^{***}P < 0.001$. 1,25D, 1,25-dihydroxyvitamin D; MDM, monocyte-derived macrophage; MFI, mean fluorescence intensity; TLR, toll-like receptor.



Supplementary Figure S6. IL-10 induction and subsequent STAT3 activation is an early event in TLR4-activated human macrophages. (**a**) MDMs were treated with LPS for 3.5 hours. IL-10 cytokine levels were measured by ELISA (mean cytokine levels in pg/ml + SEM, n = 8). (**b**) STAT3 phosphorylation was determined after MDMs were treated with rIL-10 and LPS for 3.5 hours by FACS analyses (MFI + SEM, n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$. LPS, lipopolysaccharide; MDM, monocyte-derived macrophage; MFI, mean fluorescence intensity; pSTAT, phosphorylated signal transducer and activator of transcription; rIL-10, recombinant IL-10; STAT, signal transducer and activator of transcription; TLR, toll-like receptor.



Supplementary Figure S7. IL-10–induced HAMP expression precedes TLR4-mediated HAMP activation in human macrophages. MDMs were stimulated with rIL-10 or LPS for 6 h and 24 h. HAMP protein expression was examined by intracellular FACS (Δ%-positive cells + SEM, n = 3). $*P < 0.05$, $***P < 0.001$. h, hour; LPS, lipopolysaccharide; MDM, monocyte-derived macrophage; rIL-10, recombinant IL-10; TLR, toll-like receptor.



Supplementary Figure S8. IL-10 levels in sera and intracellular HAMP levels in circulating monocytes of patients with rheumatoid arthritis and healthy controls. (**a**) $CD14^+$ monocytes were isolated, and HAMP protein expression was assessed by intracellular FACS (MFI + SEM). (**b**) IL-10 levels were measured in pooled sera according to the pools used in Figure 5e from healthy donors and tofa-treated or RTX-treated patients with rheumatoid arthritis by IL-10 ELISA (mean cytokine levels in pg/ml + SEM, n = 4 for each group). MFI, mean fluorescence intensity; RTX, rituximab; tofa, tofacitinib.

Supplementary Table S1. Reagents Used in this Study

Reagent	Concentration	Company
1,25-dihydroxyvitamin D	10 nM	Biomol
25-hydroxyvitamin D	100/250 nM	Biomol
AB serum	10%	PAA Laboratories
FcR blocking reagent	1:200	Miltenyi Biotec
Ferric ammonium citrate	1,000 μM	Sigma-Aldrich
Fetal calf serum	10%	PAA Laboratories
Ficoll-Paque PLUS	—	GE Healthcare
LPS (*E. coli* 0111:B4)	1 ng/ml	Sigma-Aldrich
M-CSF	50 ng/ml	Miltenyi Biotec
Neisseria gonorrhoeae whole-cell lysate	10 ng/ml	Abcam
Pam_3Cys-SKKK (TLR2/1 ligand)	10 μg/ml	EMC Microcollections
rIL-10	10 ng/ml	Miltenyi Biotec
rIL-6	10 ng/ml	Miltenyi Biotec
RPMI and serum-free media	—	Life Technologies
Tofa	10 μM	Sigma-Aldrich

Abbreviations: LPS, lipopolysaccharide; rIL-6, recombinant IL-6; rIL-10, recombinant IL-10; Tofa, tofacitinib.

Supplementary Table S2. Antibodies Used in this Study

Antibody	Clone	Company
For surface staining		
FITC-labeled anti–HLA-DR	G46-6	BD Bioscience
PE-labeled anti-CD86	2331	BD Bioscience
For intracellular staining		
Cathelicidin (primary)	OSX12	Abcam
IgG1 pure (isotype control)	X40	BD Bioscience
PE Anti-Mouse IgG1 (secondary)	A85-1	BD Bioscience
Hepcidin (primary)	EPR18074	Abcam
IgG pure (isotype control)	EPR25A	Abcam
PE Anti-Mouse Fluor 488 IgG (secondary)	Polyclonal	Abcam
pSTAT3 (Tyr705)	D3A7	Cell Signaling Technology
For blocking (concentration used)		
anti–IL-6Rα (10 μg/ml)	17506	R&D Systems
anti–IL-10Rα (10 μg/ml)	37607	Sigma-Aldrich
anti-TLR4 (500 ng/ml)	W7C11	InvivoGen
IgG1 pure (isotype control)	11711	R&D Systems
For ELISA		
anti–TNF-α (coating)	AHC3712	Invitrogen
anti–TNF-α (secondary)	AHC3419	Invitrogen

Abbreviations: p, phosphorylated; STAT3, signal transducer and activator of transcription 3; TLR, toll-like receptor.

Supplementary Table S3. Primer Sequences Used for RT-qPCR

Target	Forward	Reverse
36B4	CCACGCTGCTGAACATGCT	TCGAACACCTGCTGGATGAC
CAMP	GGACCCAGACACGCCAAA	GCACACTGTCTCCTTCACTGTGA
CD64	TGGTTCTTGACAACTCTGCTC	AGATGGAGCACCTCACAATG
CYP27B1	ACCCGACACGGAGACCTTC	ATGGTCAACAGCGTGGACAC
HAMP-25	GACCAGTGGCTCTGTTTTCC	CACATCCCACACTTTGATCG
TLR7	TCACCAGACTGTTGCTATGATGC	CAGCCAAAACCCACTCGGT

Abbreviation: TLR, toll-like receptor.