ORIGINAL ARTICLE

Synergistic Signaling of TLR and IFNα/β Facilitates Escape of IL-18 Expression from Endotoxin Tolerance

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Abstract

Rationale: IL-18 is a member of the IL-1 cytokine family, and elevated blood IL-18 concentrations associate with disease activity in macrophage activation syndrome (MAS) and poor clinical outcomes in severe inflammatory and septic conditions.

Objectives: Although recent investigations provide mechanistic evidence for a contribution of IL-18 to inflammation and hyperinflammation in sepsis and MAS, we sought to study regulatory mechanisms underlying human IL-18 expression.

Methods: Samples from *in vivo* and *in vitro* endotoxin rechallenge experiments, patients with inflammatory disease, and isolated human monocytes treated with various stimulants and drugs were tested for cytokine gene and protein expression. Serum IL-18 expression with or without JAK/STAT inhibition was analyzed in two MAS mouse models and in a patient with recurrent MAS.

Measurements and Main Results: Peripheral blood and monocytic IL-18 expression escaped LPS-induced immunoparalysis. LPS-stimulated primary human monocytes revealed specific IL-18 expression kinetics controlled by IFNα/β signaling. JAK/STAT inhibition or IFNβ neutralization during LPS stimulation blunted cytokine expression. Similarly, microtubule-destabilizing drugs abrogated LPS-induced *IL18* expression, but this effect could be fully reversed by addition of IFNα/β. *Ex vivo* analysis of inflammatory disease patients' whole blood revealed strong correlation of type I IFN score and *IL18* expression, whereas JAK/STAT inhibition strongly reduced IL-18 serum levels in two MAS mouse models and in a patient with recurrent MAS.

Conclusions: Our data indicate that IL-18 (but not IL-1β) production from human monocytes requires cooperative Toll-like receptor and IFNα/β signaling. Interference with IFNα/β expression or signaling following JAK/STAT inhibition may control catastrophic hyperinflammation in MAS.

Keywords: experimental human endotoxemia; endotoxin tolerance; IL-18; type I IFN; macrophage activation syndrome

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At a Glance Commentary

Scientific Knowledge on the Subject: The IL-1 cytokine family member IL-18 is thought to play a key role in the pathogenesis of (hyper) inflammation in macrophage activation syndrome (MAS) and sepsis. However, regulatory mechanisms underlying human IL-18 expression remain poorly understood.

What This Study Adds to the Field: We demonstrate that IL-18 expression from human monocytes is orchestrated by synergistic Toll-like receptor and type I IFN signaling. Interference with JAK/STAT signaling can blunt IL-18 production and thus may help to control hyperinflammation in MAS.

Hyperinflammatory conditions are life-threatening complications resulting from a systemic proinflammatory response triggered by infection, injury, or chronic inflammation (1, 2). In critically ill children, the current state of knowledge suggests a significant immunophenotype overlap of hyperferritinemic sepsis–related multiple organ dysfunction syndrome, macrophage activation syndrome (MAS), and secondary hemophagocytic lymphohistiocytosis (sHLH) (3). Excessive expression of cytokines such as TNFα (tumor necrosis factor α), IL-1β, and IL-6 plays a pivotal role in the very early response of innate immune cells to infection or trauma in such conditions, but later release of these proinflammatory cytokines can be impaired by a state termed "sepsis-induced immunoparalysis" or, as the experimental analog to this clinical entity, "endotoxin tolerance" (4).

Although TNFα, IL-1β, and IL-6 are potent inflammatory mediators, the IL-1 family cytokine IL-18 has recently been suggested as the critical driver of MAS (5, 6), a severe hyperinflammatory condition characterized by a catastrophic cytokine storm resulting in multiple organ failure and high mortality. MAS is a known complication of chronic rheumatic diseases in childhood and is particularly associated with systemic juvenile idiopathic arthritis (sJIA) but also can result from bacterial or viral infections (sHLH) (7) or complicate sepsis (macrophage activation–like syndrome) (3, 4, 8). Elevated IL-18 concentrations in serum or plasma have been shown to be associated with severity of disease in MAS (5, 9) and poor clinical outcomes in severe inflammatory and septic conditions (10). Inhibition of IL-18 receptor signaling reduced inflammation in a mouse model of MAS (6), and treatment with IL-18BP (recombinant human IL-18 binding protein) can improve clinical outcome (11, 12). Similarly, targeting IL-18 in addition to neutralizing IL-1β rescued mice from inflammatory and septic shock (13).

Unlike conventional inflammatory cytokines such as TNFα or IL-6, IL-1β and IL-18 are expressed as inactive propeptides (proIL-1β, proIL-18) by monocytes, macrophages, and neutrophils following the stimulation of pattern recognition receptors such as TLRs (Toll-like receptors) by pathogen- or damage-associated molecular patterns. Both IL-1β and IL-18 propeptides then require inflammasome-mediated caspase cleavage to be secreted as mature bioactive proteins (14). In contrast to IL-1β, IL-18 secretion does not necessarily require *de novo* protein synthesis and rather is thought to be facilitated from a premade pool of cytoplasmic pro– and mature IL-18 (15, 16).

In contrast to recent advances in understanding the pathophysiologic contribution of IL-18 to (hyper) inflammation, cellular control over IL-18 expression remains poorly understood. Although the regulation of IL-1β expression highlights a critical role for NFκβ (nuclear factor κβ) (17–23), current studies on murine (24, 25) or human (25–28) cells do not provide conclusive evidence on whether and how IL-18 expression is controlled (14). In this study we demonstrated that, in contrast to TNFα, IL-6, and IL-1β, expression of human IL-18 can escape LPS-induced *in vivo* immunoparalysis and endotoxin tolerance. This is facilitated by the specific monocytic transcription kinetics of IL-18, which are controlled by cooperative type I IFN and TLR signaling. JAK/STAT inhibition in sHLH/MAS mouse models and in a treatment-refractory patient with MAS greatly reduced IL-18 serum levels and controlled MAS. Consequently, our study addresses a missing link in understanding MAS pathogenesis by providing an explanation for the clinical experience suggesting an association of MAS in autoinflammatory patients with viral infections (8, 29). In turn, this result favors JAK/STAT inhibition as a therapeutic option to control disease. Some results of these studies have been reported previously in the form of abstracts (30, 31).

Methods

Human Study Subjects

The study protocol for the *in vivo* endotoxin rechallenge was approved by the ethics committee of the Radboud University Nijmegen Medical Center and complies with the Declaration of Helsinki and good clinical practice guidelines. Healthy male volunteers gave written informed consent. Experiments were part of a larger endotoxin trial (ClinicalTrials.gov identifier NCT01374711). Patients with inflammatory disease provided written informed consent, and all studies were approved by the ethics committee of Muenster University hospital or the Cincinnati Children's Hospital Medical Center.

Mice

Wild-type (C57BL/6-J) mice were purchased from the Jackson Laboratory. Experimental mice were sex and age matched and used at 8–16 weeks of age. Mice were housed in a specific pathogen-free facility at St. Jude Children's Research Hospital. Experiments were conducted under the approval of the institutional animal care and use committee.

In Vivo Endotoxin Rechallenge

Plasma samples were obtained from an *in vivo* experimental endotoxemia rechallenge trial (32, 33). Further details are provided in the online supplement. Experimental design and blood sampling time points are depicted in Figure 1A.

Human Monocyte Isolation, Culture, and Stimulation

Detailed description is provided in the online supplement.

Quantitative Real Time–PCR Type 1 IFN Score and Secreted Cytokine Quantification

Methods are outlined in detail in the online supplement.

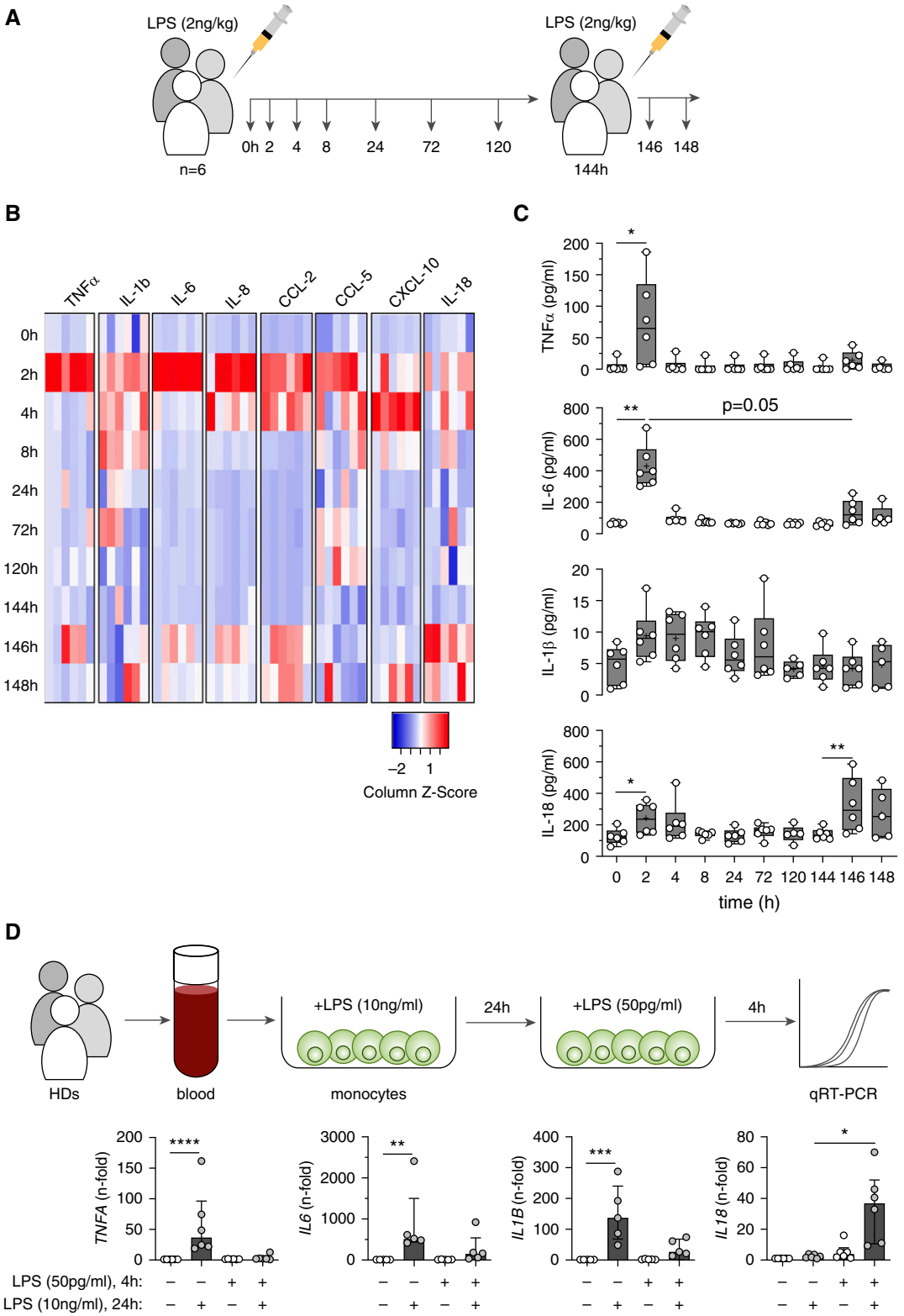

Figure 1. IL-18 expression is not affected by LPS tolerance. (*A*) Healthy human volunteers were challenged with LPS (2 ng/kg). Blood was drawn at indicated time points (arrows), and individuals ($n = 6$) were rechallenged with LPS 144 hours after the initial injection. Blood sampling was continued until 148 hours. (*B* and *C*) Plasma cyto- and chemokine levels were quantified by bead array assay. Data are presented as heat map (*B*) and box and whisker plots (*C*; 10th to 90th percentiles) of most relevant markers. The horizontal lines represent the medians, and the plus signs represent the means. Comparisons between 0 and 2, 144 and 146, and 2 and 146 hours were analyzed by Friedman followed by the Dunn multiple comparison test. (*D*) In

sHLH/MAS Mouse Models and Quantification of Murine Serum IL-8

Serum IL-18 levels were quantified from mice in sHLH/MAS models with and without ruxolitinib treatment, as described in studies by Albeituni and colleagues (34) and Weaver and colleagues (35). Mouse models and the IL-18 ELISA procedure are detailed in the online supplement.

Statistical Analysis

Data were analyzed using GraphPad Prism software (v8.0, Mac OSX; GraphPad Software), as indicated in figure legends and described in the online supplement.

Results

Human IL-18 Expression Escapes Immunoparalysis and Endotoxin Tolerance

Our studies on IL-18 were primed by observations on cytokine expression from *in vivo* and *in vitro* endotoxin desensitization experiments. In plasma of healthy individuals who underwent repeated endotoxemia experiments (32) (Figure 1A), we initially observed that IL-18 levels remained unaffected by endotoxin tolerance (Figures 1B and 1C), in contrast to other cyto- or chemokines, particularly TNFα, IL-6, and IL-1β. Similarly, in TLR4 desensitization experiments on primary human monocytes (Figure 1D), we observed that gene expression of inflammatory cytokines was blunted by repeated TLR4 stimulation. *IL18* expression, however, seemed to be induced by repeated LPS challenge (Figure 1D). With further investigation, we wondered whether human IL-18 transcription might follow different time kinetics than other inflammatory cytokines. When stimulating human monocytes with LPS, we observed that *TNFA*, *IL1B*, and *IL6* revealed maximum expression at 4 hours following endotoxin challenge; however, *IL18* expression was delayed and reached its maximum at approximately 17 hours after LPS challenge (Figure 2A). In contrast, cytokine release by LPS- and ATP-stimulated monocytes occurred readily and peaked at 4 hours after cell stimulation (Figure 2B). LPS rechallenge of monocytes at the peak of *IL18* expression at 17 hours (Figure 2A) resulted in repeated release of 75% of IL-18 (median [interquartile range] at 19 h: 215[188] pg/ml) compared with the first wave of cytokine (4 h: 288[98] pg/ml) (Figure 2D). Conversely, IL-1β expression on LPS rechallenge reached only 14% (1273[914] pg/ml) of its initially released amount (4 h: 8968[4821] pg/ml) (Figure 2C). Therefore, the delayed *IL18* gene expression pattern may allow cells to repeatedly mount IL-18 release despite endotoxin tolerance.

Interfering with JAK/STAT and Particularly Type I IFN Signaling Reduces *IL18* Expression

We next aimed at understanding the reason for the delayed *IL18* expression kinetics. Because prominent inflammatory cytokines such as TNFα, IL-1β, or IL-6 were expressed and released early on LPS stimulation of human monocytes, we wondered whether they, rather than LPS signaling, might be responsible for inducing *IL18* expression and thus explain its delayed kinetics. Monocytes were pretreated with antiinflammatory drugs neutralizing either IL-1β or TNFα or blocking the IL-1 or IL-6 receptor, followed by LPS stimulation. We observed that TNFα blockade negatively affected *IL1B* expression; however, none of the applied treatments revealed any effect on *IL18* expression (Figures 3A and E1A in the online supplement).

We also tested cytokine expression on monocyte treatment with tofacitinib, a JAK/STAT inhibitor approved for treatment of various inflammatory diseases (36). Tofacitinib pretreatment followed by LPS stimulation strongly enhanced monocytic *TNFA*, *IL6*, and particularly *IL1B* expression (Figures 3A and E1A). In contrast, JAK/STAT inhibition significantly reduced IL-18 gene expression (Figure 3A). As an inhibitor of JAK1/3 but also JAK2, tofacitinib can impair downstream phosphorylation of several STAT proteins (36, 37). Flow cytometry demonstrated that IFNα induced strong STAT1 phosphorylation, which was impaired by tofacitinib (Figures E1B and E1C). In these assays, short-term stimulation with LPS also induced some STAT1 phosphorylation, which was equally sensitive to tofacitinib treatment (Figures E1B and E1C). Cell stimulation with LPS can result in both the NFκβ-dependent transcription of classical inflammatory cytokines and the IRF3-dependent expression of type I IFN (38, 39). JAK/STAT-pathway activation can be induced by both cytokine and IFN signaling through their respective receptors. Because we did not observe any effect on *IL18* expression when interfering with receptor signaling by prominent inflammatory cytokines, we decided to further study neutralization of type I IFN, which can be expressed following LPS stimulation (38, 39). Treating endotoxin-stimulated monocytes with an IFNβ-neutralizing antibody did not affect *TNFA* and *IL6* transcription (Figure E2A) but increased *IL1B* expression by trend while greatly reducing *IL18* expression in a concentration-dependent manner (Figures 3B and 3C and E2B). At that point, we concluded that interfering with type I IFN signaling in our LPS-stimulated monocyte cultures had strong but opposing effects on *IL1β* and *IL18* gene expression.

Type I IFN Signaling Controls Monocytic IL-18 Expression

We next analyzed whether the release of IFNα/β on LPS stimulation controls monocytic *IL18* expression and thus might be responsible for the initially observed delayed expression kinetics and repeated cytokine expression. To test this hypothesis, we costimulated monocytes with LPS and IFNα/β or subjected cells to IFN priming for either 4 or 16 hours before endotoxin treatment. Compared with just LPS-stimulated cells, IFNα/β priming of monocytes enhanced *IL1B*, *TNFA*, and *IL6* expression at its maximum around 4 hours following endotoxin treatment. Beyond 4 hours, IFNα/β priming significantly reduced *IL1B* transcription (Figures 4A and E3A and E3B). In contrast, *IL18* expression

Figure 1. (Continued). *in vitro* endotoxin desensitization experiments, primary human monocytes isolated from healthy donors were subjected to 24-hour prestimulation with LPS (10 ng/ml) or left untreated. After this pretreatment, cells were restimulated with LPS (50 pg/ml) or left untreated. After 4 hours of restimulation, cellular mRNA was harvested and *TNFA*, *IL6*, *IL1B*, and *IL18* expression was quantified by qRT-PCR and is shown as *n*-fold of housekeeping gene expression. Bars show median and interquartile range of five or six independent experiments. Comparisons between 24-hour LPS pretreatment and rechallenge or with respective control conditions were analyzed by Kruskal–Wallis followed by the Dunn multiple comparison test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. HDs = healthy donors.

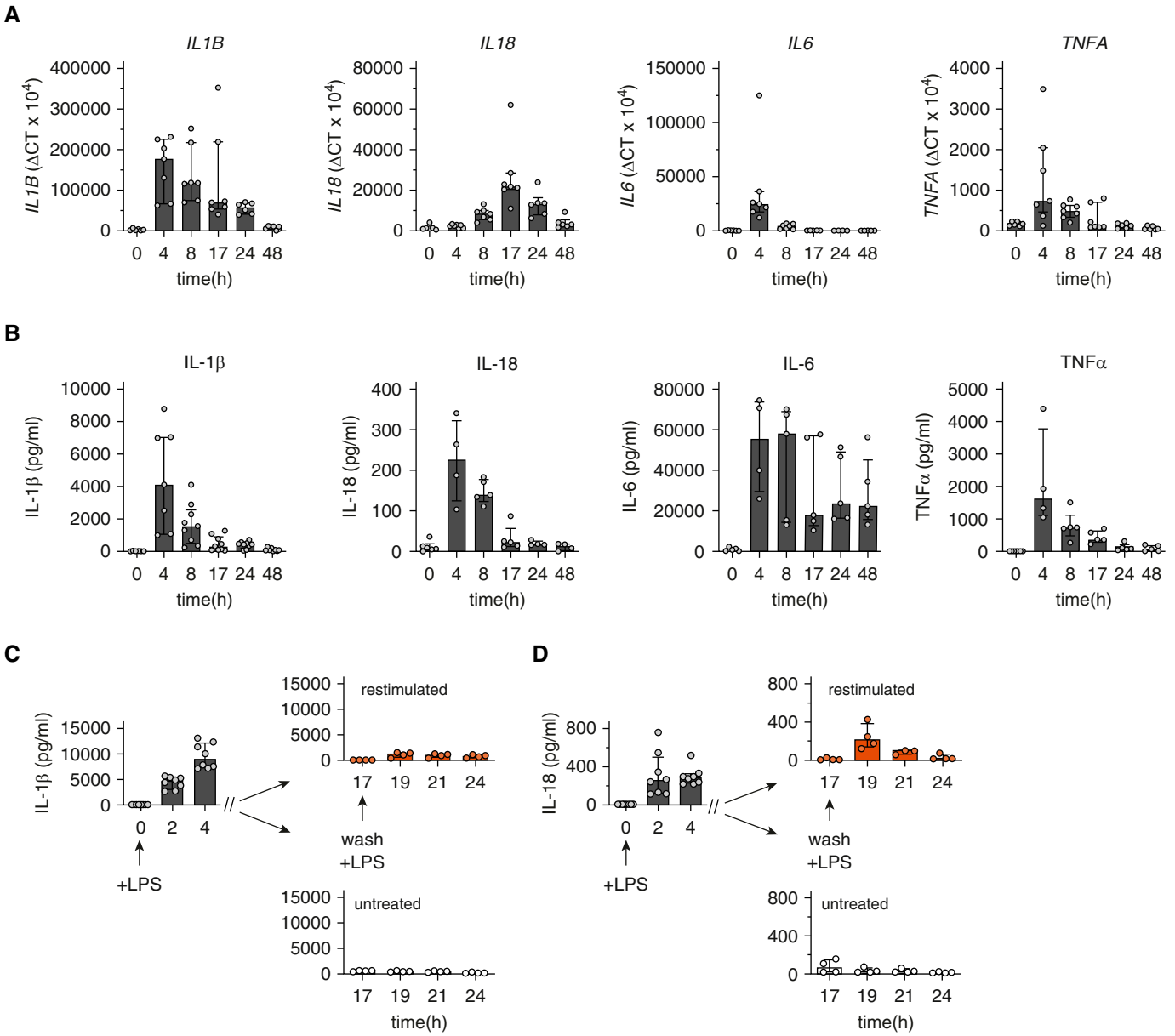

Figure 2. *IL18* gene expression follows delayed kinetics. (*A*) Primary human monocytes ($n = 4$–7 independent experiments) were stimulated with LPS (100 ng/ml), and cells were harvested for mRNA preparation at indicated time points after stimulation. *IL1B*, *IL18*, *IL6*, and *TNFA* expression was quantified by qRT-PCR. Correspondingly, supernatants of cells stimulated as in *A* were collected ($n = 4$–8 independent experiments), and (*B*) release of IL-1β, IL-18, IL-6, and TNFα was quantified. Data are presented as median and interquartile range. (*C* and *D*) Primary human monocytes ($n = 4$–8 independent experiments) were stimulated with LPS as described for *A* and *B*. At 17 hours after initial stimulation, cells from half of the experiments were washed and restimulated with LPS (100 ng/ml), and cells from remaining experiments were left untreated. Supernatants were collected at indicated time points, and (*C*) IL-1β and (*D*) IL-18 release was quantified. Data are presented as median and interquartile range. ΔCT = Δ cycle threshold.

was increased but, more important, IFNα/β priming of monocytes shifted the *IL18* expression kinetics from its 17-hour maximum to peak expression at 4 hours after LPS stimulation. The longer monocytes were primed with either IFNα or IFNβ, the more pronounced the shift in *IL18* expression kinetics (Figures 4A and E3A and E3B). Compared with IFNα/β, we also costimulated or primed cells with IFNγ. This increased *TNFA* and *IL6* but revealed no effect on *IL18* expression (Figures 4B and E3C). Apart from shifting *IL18* expression kinetics, the priming of human monocytes with IFNα/β but not IFNγ strongly increased IL-18 release on subsequent LPS stimulation. This effect was not observed with IL-1β (Figure 4C).

In addition to our *in vitro* data suggesting an involvement of STAT-mediated IFNα/β signaling in controlling IL-18 gene expression, we had the opportunity to investigate a patient with a not-yet-described gain-of-function mutation in *STAT1* (*STAT1*^{GOF}, exon 10 c.838T > G p.Leu280Val) (Figure 4D). Although the patient's *STAT1* expression

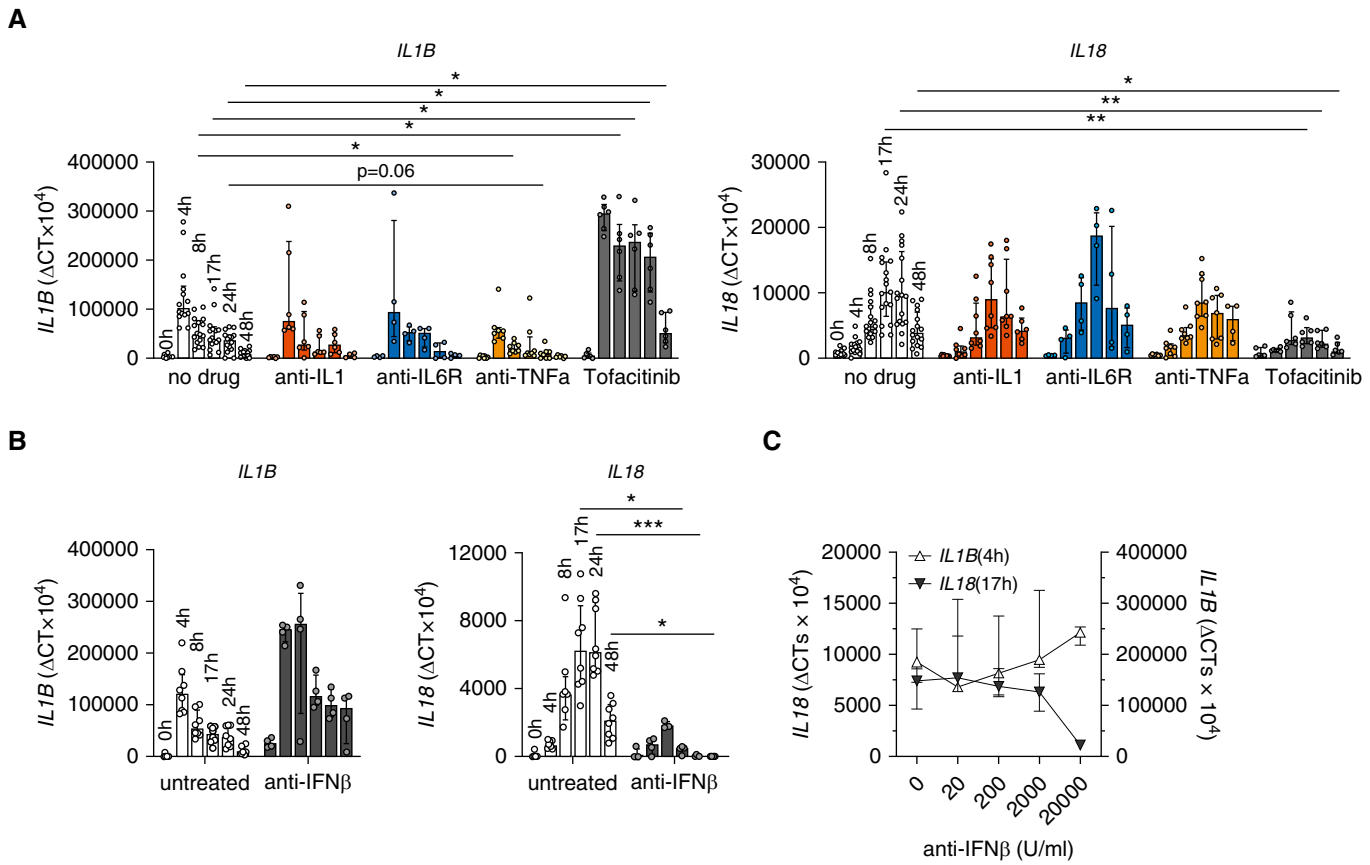

Figure 3. Interference with JAK/STAT and type I IFN signaling reduces *IL18* expression. (*A*) Primary human monocytes were stimulated with LPS (100 ng/ml) and treated with just medium ($n = 6$–12 independent experiments), anti-IL-1 (canakinumab or anakinra, both 100 ng/ml, $n = 6$–8 independent experiments), tocilizumab (anti-IL-6R, 4 μg/ml, $n = 4$ independent experiments), etanercept (anti-TNFα, 1.2 μg/ml, $n = 6$–10 independent experiments), tofacitinib (1000 nM, $n = 6$ independent experiments), or (*B* and *C*) an anti-IFNβ antibody at either 20,000 U/ml ($n = 4$–8 independent experiments) (*B*) or indicated concentrations (*C*). Cells were harvested for mRNA preparation at indicated time points after LPS stimulation, and *IL1B* or *IL18* expression was quantified by qRT-PCR. All data are presented as median and interquartile range. Effect of tested drugs on gene expression compared with untreated cells was analyzed for each time point using Kruskal–Wallis followed by the Dunn multiple comparison test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. ΔCT = Δ cycle threshold.

was already increased at baseline, we observed no differences in *IL1B* expression on LPS stimulation compared with healthy control monocytes over time (Figure 4D). In contrast, the *IL18* expression kinetics observed with *STAT1*^{GOF} monocytes differed from that of healthy controls (Figure 4D) and was shifted forward in a manner similar to IFNα/β-primed healthy monocytes (Figure 4A).

Monocytic *IL18* Expression Requires Cooperative TLR and Type I IFN Signaling

Our data indicate that manipulation of IFNα/β signaling in LPS-stimulated monocyte cultures strongly affects *IL18* expression. Therefore, we sought to test whether cell stimulation with just type I IFN would similarly induce *IL18* expression. We also aimed to study cytokine expression following stimuli such as flagellin or IL-1β, which canonically induce NFκβ activation via the MyD88-dependent pathway (40). In these assays, we observed that IFNα/β induced neither *IL1B* nor *IL18* expression, whereas NFκβ inhibition during LPS stimulation blunted *IL1B* and *IL18* expression (Figures 5A and E4A). Flagellin stimulation induced IL-1β gene expression similar to LPS, whereas rIL-1β triggered its own expression at comparably lower levels. In contrast, treatment of monocytes with flagellin or rIL-1β induced significantly less *IL18* transcription than LPS (Figure 5A). Importantly, together with remnant *IL18* expression on stimulation with flagellin, we also observed low-level induction of IFNβ gene expression. In contrast, rIL-1β failed to induce *IFNB* transcription (Figure 5B). Maximum *IFNB* expression induced by either flagellin or LPS was observed after 2 hours of stimulation (Figure 5B) and strongly correlated with respective subsequent *IL18* transcription (Figure 5C).

Microtubule-Destabilizing Drugs Impair Monocytic *IL18* Expression, Which Can Be Rescued by Type I IFN

IL-18 serum levels are greatly increased in some autoinflammatory diseases such as familial Mediterranean fever (FMF) or sJIA and particularly sJIA-associated MAS or infection-associated sHLH (5, 41, 42). Treatment with microtubule destabilizing agents (MDAs) such as colchicine can result in reduction of inflammation accompanied by decreasing IL-1β and IL-18 serum levels (41, 43, 44). In our experiments, treatment of healthy donor monocytes with colchicine before LPS stimulation had no effect on IL-1β but

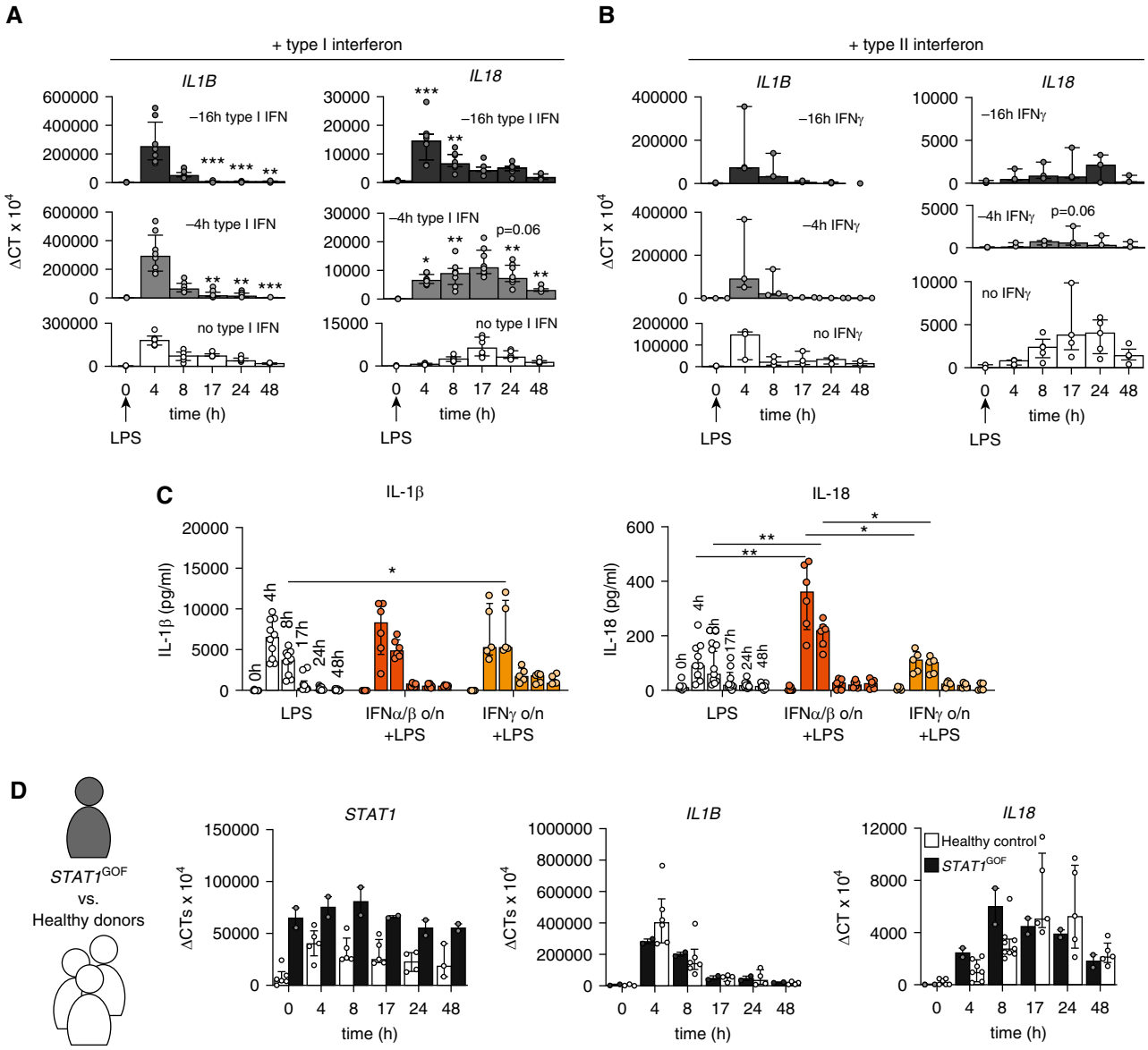

Figure 4. Type I IFN signaling controls IL-18 expression. (*A* and *B*) Primary human monocytes were left untreated or primed with type I IFN (*A*; IFNα or IFNβ, 1000 U/ml; $n = 5$–8 independent experiments) or IFNγ (*B*; 25 ng/ml, $n = 3$–5 independent experiments) for either 4 or 16 hours before LPS (100 ng/ml) stimulation. Cells were harvested for mRNA preparation at indicated time points after LPS stimulation, and *IL1B* or *IL18* expression was quantified by qRT-PCR. (*C*) Supernatants from cells treated with IFNα/β or IFNγ for 16 hours before LPS stimulation were collected at indicated time points, and IL-1β and IL-18 release was quantified ($n = 5$–9 independent experiments). Data are presented as median and interquartile range. Effect of IFNα/β/γ priming on gene expression compared with untreated cells was analyzed for each time point using Kruskal–Wallis followed by the Dunn multiple comparison test. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (*D*) Primary human monocytes were isolated from healthy controls (white bars, $n = 3$–7 independent experiments) and a 36-year-old male white patient with a STAT1 gain-of-function heterozygous mutation (exon 10 c.838T > G p.Leu280Val) at two independent clinical visits (black bars). Monocytes from the patient and healthy controls were stimulated with LPS (100 ng/ml), and cells were harvested for mRNA preparation at indicated time points after LPS stimulation. *IL1*, *IL18*, and *STAT1* expression was quantified by qRT-PCR. ΔCT = Δ cycle threshold.

increased IL-18 release (Figure E4B). At a gene-expression level, colchicine treatment of cells blunted *IL18* expression, whereas *IL1B* expression was enhanced (Figure 5D). Apart from impairment of microtubule polymerization by colchicine, we also investigated the effect of nocodazole, a synthetic microtubule polymerization inhibitor, on IL-1β and IL-18 expression. We observed that, similar to colchicine treatment, nocodazole increased IL-18 release (Figure E4B). At a gene-expression level, nocodazole amplified *IL1B* while abrogating *IL18* expression (Figure 5D).

Because our data suggested that type I IFN controlled *IL18* expression, we wondered how the observed MDA-mediated impairment might fit in this picture. In fact, reports have suggested that colchicine treatment affects IFNα/β expression or its release (45, 46). In our experiments, we found that *IFNB* gene expression peaked at 2 hours and protein secretion peaked at 3 hours following

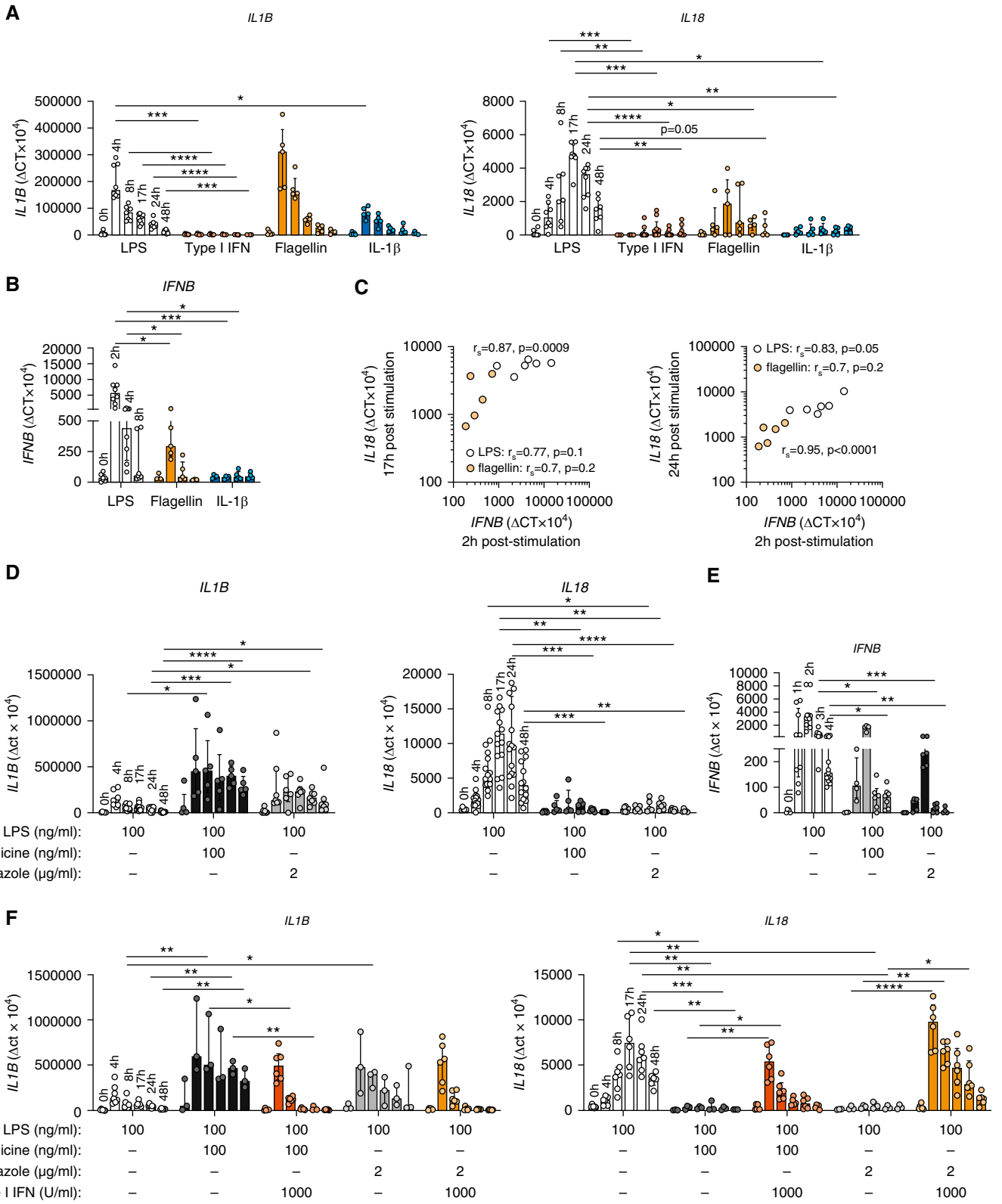

Figure 5. *IL18* expression requires cooperative TLR and type I IFN signaling. (*A* and *B*) Primary human monocytes were stimulated with LPS (100 ng/ml, $n = 7–8$ independent experiments), type I IFN (IFNα or IFNβ, 1000 U/ml; $n = 8–9$ independent experiments), flagellin (500 ng/ml, $n = 3–5$ independent experiments), or recombinant human IL-1β (10 ng/ml, $n = 4–5$ independent experiments). Cells were harvested for mRNA preparation at indicated time points after LPS stimulation, and IL-1β (*A*, left panel), IL-18 (*A*, right panel), and IFNβ (*B*) gene expression was quantified by qRT-PCR. Data are presented

LPS stimulation. Both colchicine and particularly nocodazole reduced *IFNB* expression and its release (Figures 5E and E4C). To demonstrate that lack of type I IFN signaling in our MDA-treated cells was responsible for the observed abrogation of *IL18* expression, we primed cells with IFNα/β alongside colchicine or nocodazole. Both MDA treatments and type I IFN treatment enhanced *IL1B* expression (Figures 5F and E5A and E5B). This result was similarly true for *IL6* (Figures E5G and E5H), whereas *TNFA* expression remained unaffected by MDAs and was amplified by type I IFN (Figures E5E and E5F). Strikingly, the addition of IFNα/β to MDA-treated cells was able to rescue LPS-induced *IL18* expression (Figures 5F and E5C and E5D).

Blood Cell Expression of IL-18 Correlates with Type I IFN Score and Is Sensitive to *In Vivo* JAK/STAT Inhibition

To further validate our findings, we analyzed *IL18* compared with type I IFN stimulated gene (*ISG15*, *RSAD2*, *IFIT1*, *IFI27*, *IFI44L*, and *SIGLEC1*) expression in autoinflammatory diseases (FMF and sJIA) and polygenic interferonopathies (systemic lupus erythematosus [SLE] and juvenile dermatomyositis [JDM]) in patients' whole blood. In these *ex vivo* analyses, we observed a strongly significant correlation between type I IFN score and *IL18* transcription (Figure 6A). Because we had no access to patient material before and after colchicine treatment to study the *in vivo* drug impact on type I IFN and IL-18 expression, we compared gene expression in the whole blood of patients with FMF or sJIA. Although these diseases differ in pathophysiology, they share prominent clinical phenotypes. When compared with samples from colchicine-treatment–naive patients with sJIA, samples from colchicine-treated patients with FMF revealed significantly decreased type I IFN scores and *IL18* expression (Figure 6B).

Up to this point, the majority of our analysis has focused on IL-18 gene expression. However, we anticipated that manipulations in type I IFN signaling such as JAK/STAT-inhibition would affect IL-18 serum levels and thus impact clinical outcome. To test this notion, we analyzed IL-18 serum levels in mice subjected to a model of moderate (Figure 6C) and fulminant (Figure 6D) sHLH/MAS, untreated or treated with the JAK1/2 inhibitor ruxolitinib. Mice repeatedly injected with CpG (Figure 6C) or CpG and an IL-10R–blocking antibody (Figure 6D) developed an inflammatory phenotype resembling sHLH/MAS (34, 35). Compared with untreated animals, we detected markedly increased IL-18 serum levels in diseased mice (Figures 6C and 6D). In both models, ruxolitinib treatment significantly decreased total IL-18 serum levels (Figures 6C and 6D) and improved clinical outcomes (34, 35).

Finally, we analyzed serum IL-18 levels in a 7-year-old male patient with sJIA who presented with persistently active disease, with recurrent MAS and sJIA-associated lung disease unresponsive to IL-1 and IL-6 blockade. His frequent disease flares and recurrent episodes of MAS were reduced through use of recombinant human IL-18 binding protein (12), but he continued to have persistently elevated serum IL-18 levels of 50,000–120,000 pg/ml (Figure 6E). In an effort to improve control of his overall disease and lung disease in particular, he was started on the JAK1/3 inhibitor tofacitinib. He required dose reduction and holding of medication for an episode of anemia and for an acute infection. However, once on a stable dose of tofacitinib, his markers of inflammation including white blood cell count, ferritin, and CRP stabilized. Interestingly, his serum IL-18 levels, which had remained elevated even during periods of low disease activity, showed a sustained 67% decrease during tofacitinib therapy (Figure 6E). This result highlights the effect of JAK/STAT inhibition on IL-18 regulation despite treatment interruptions. These preliminary clinical data support a model in which IFN signaling has a key role in sustaining high levels of IL-18 expression in sJIA.

Discussion

Unopposed IL-18 signaling has been shown to trigger hyperinflammation in MAS (5, 6) and to play a key role in the pathogenesis of inflammatory and septic shock (13). However, transcriptional control over IL-18 expression by monocytes as a primary source for release of bioactive cytokine is unknown. In this study, we demonstrated that—unlike TNFα, IL-6, and IL-1β—human IL-18 expression escapes LPS-induced immunoparalysis and endotoxin tolerance. We found this could be due to delayed IL-18 gene expression kinetics, providing mRNA for repeated protein production. Furthermore, we showed that transcription of the IL-18 gene by human monocytes is orchestrated by inflammatory TLR and type I IFN signaling. MDAs such as colchicine that are used to treat diseases with a hallmark of IL-18 overexpression abrogate its transcription by interfering with type I IFN expression. *IL18* expression correlates with type I IFN score in inflammatory disease patients, and JAK/STAT inhibition greatly decreases serum IL-18 levels in sHLH/MAS murine models and in a patient with treatment-refractory MAS.

Several studies have shown elevated IL-18 concentrations to be associated with disease activity in MAS (5, 9) or poor clinical outcome in severe inflammatory and septic conditions (10). However, apart from our observations, we know of no human data on IL-18 expression in a context with LPS-induced immunoparalysis or endotoxin tolerance. Observations in

Figure 5. (Continued). as median and interquartile range. Effect of tested stimuli on gene expression compared with untreated cells was analyzed for each time point using Kruskal–Wallis followed by the Dunn multiple comparison test. (*C*) *IFNB* expression after 2 hours of LPS or flagellin stimulation was correlated (Spearman) with *IL18* transcription induced by either LPS or flagellin after 17 hours (left panel) or 24 hours (right panel). (*D* and *E*) Primary human monocytes were stimulated with LPS (100 ng/ml) following 16 hours of pretreatment with just medium ($n = 5$–16 independent experiments), colchicine (100 ng/ml, $n = 3$–7 independent experiments), or nocodazole (2 μg/ml, $n = 3$–7 independent experiments). (*D*) IL-1β and IL-18 or (*E*) IFNβ gene expression was analyzed as described above. (*F*) Primary human monocytes were stimulated with LPS (100 ng/ml; $n = 6$–7 independent experiments) following 16 hours of pretreatment with just medium or colchicine and nocodazole ($n = 3$ independent experiments each) or colchicine and nocodazole in combination with IFNα/β ($n = 5$–6 independent experiments), all at indicated concentrations. *IL1B* or *IL18* expression was quantified as described above. Data are presented as median and interquartile range. Effects of colchicine/nocodazole treatments compared with untreated or IFNα/β-treated cells were analyzed for each time point using Kruskal–Wallis followed by the Dunn multiple comparison test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. ΔCT = Δ cycle threshold.

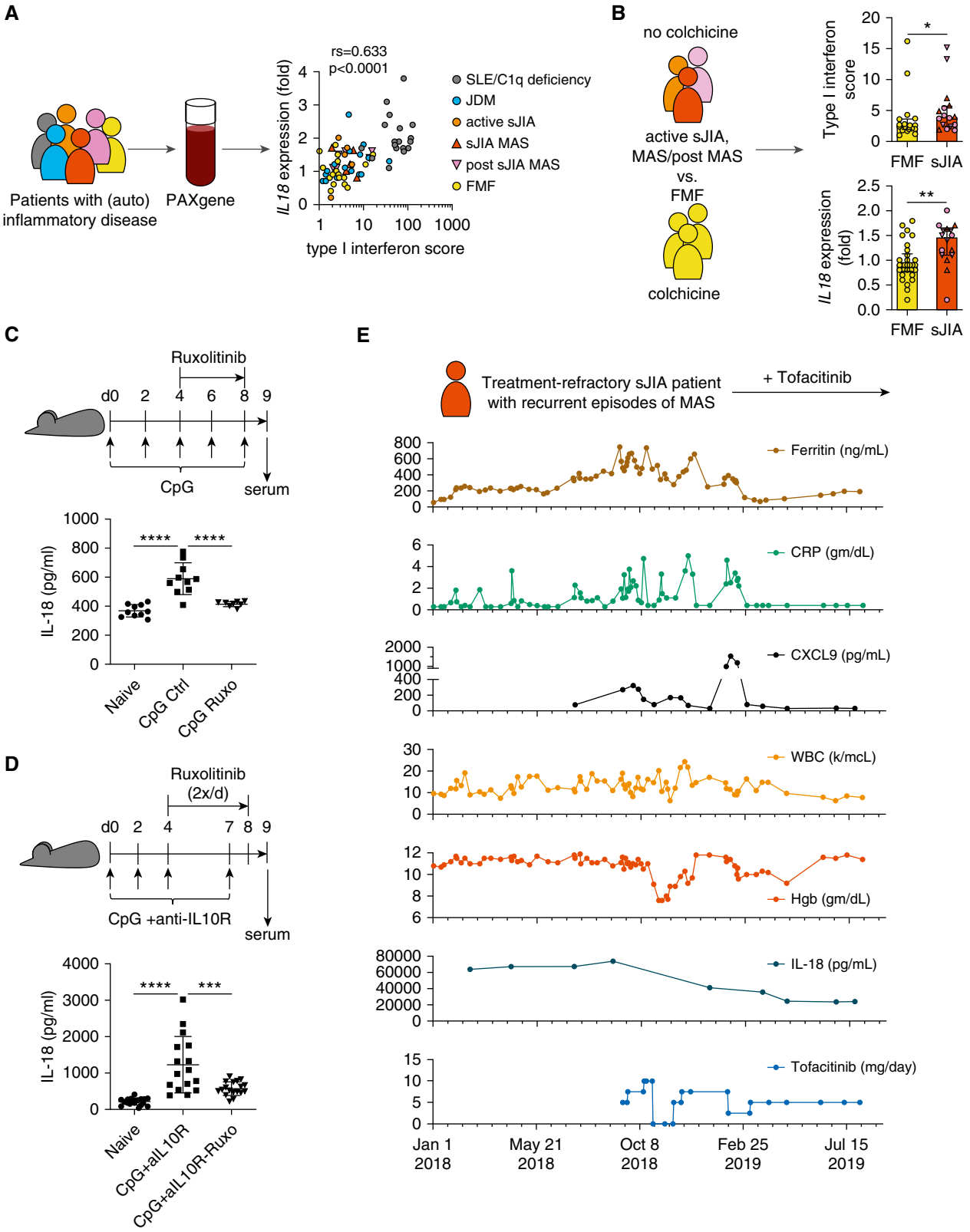

Figure 6. IL-18 *in vivo* expression correlates with type 1 IFN score and is sensitive to JAK/STAT blockade. (*A*) Spearman correlation of *IL18* expression and type I IFN score according to *ISG15*, *RSAD2*, *IFIT1*, *IFI27*, *IFI44L*, and *SIGLEC1* expression in whole-blood samples obtained from patients with systemic lupus erythematosus/C1q deficiency ($n = 18$), juvenile dermatomyositis ($n = 16$), active systemic juvenile idiopathic arthritis (sJIA; $n = 5$),

mice mimic our findings, as serum or cellular IL-18 levels in animals rechallenged with LPS are reported to remain unaffected or to increase compared with mice receiving only single-endotoxin conditioning (47, 48).

When further studying possible contributors to the observed delayed *IL18* gene expression, we identified a critical requirement of type I IFN for monocytic *IL18* expression. Apart from our data on human cells, several studies suggest a relationship between IFNα/β signaling and IL-18 secretion in mice. In *Ifnar*$^{-/-}$ macrophages responding to *Streptococcus pneumoniae* infection, IFNα/β signaling has been observed to be required for IL-18 release (49). Similarly, TRIF deficiency has been observed to abrogate both IL-1β and IL-18 release during systemic gram-negative infection (50). However, both of these studies attribute their findings to IFNα/β-dependent inflammasome activation and caspase licensing rather than cytokine gene expression. Also focusing on *IL18* gene expression rather than its secretion, a recent murine study by Zhu and Kanneganti (16) describes findings that are strikingly similar to our observations in human cells. After stimulation of bone marrow–derived macrophages with LPS, they also observed divergent kinetics in *Il1b* and *Il18* expression. IL-18 but not IL-1β gene and propeptide expression was abrogated in *Ifnar1*$^{-/-}$, *Ifnar2*$^{-/-}$, *Irf9*$^{-/-}$, and *Stat1*$^{-/-}$ cells (16).

Although our study demonstrates IFNα/β dependence of human *IL18* transcription predominantly by utilizing JAK/STAT inhibition, IFNβ neutralization, and IFNα/β priming, we similarly speculate on a role of STAT1 in *IL18* gene expression. Strikingly, the human *IL18* promoter region (27, 28) is predicted to contain both NFκβ binding sites and an accumulation of GAS (IFNγ-activated sequence) elements (51) required for STAT1, STAT1α, STAT1β, and IRF-1 binding. In contrast, next to NFκβ response elements, the approximately threefold larger *IL1B* promoter region (18, 23) contains only a few GAS elements (Figure E6).

Despite high frequency of potential STAT binding sites within the *IL18* promoter, exclusive IFNα/β signaling did not induce *IL18* expression. Similarly, IL-1β as the exclusive NFκβ activator failed to initiate *IL18* transcription. In contrast, flagellin as the canonical TLR5 ligand induced decreased but still detectable *IL18* transcription. In line with this result, we also observed remnant *IFNB* expression following flagellin stimulation of human monocytes; indeed, TLR5 stimulation by flagellin has been reported to result in predominant NFκβ activation but can activate the type I IFN–inducing TRIF pathway via TLR4/TLR5 heterodimers (52). Therefore, we conclude that both STAT and NFκβ activation are required to cooperate in facilitating *IL18* transcription. Likewise, such unconventional transcription initiation complex assembly by both STAT and NFκβ has been suggested to regulate nitric oxide synthase expression (53). Apart from *in vitro* experiments on healthy donor cells, we observed LPS-stimulated monocytes with *STAT1*^{GOF} mutation to reveal distinct IL-18 expression, and *ex vivo* analysis of patients with systemic (hyper)inflammation demonstrated a strong correlation of type I IFN score and *IL18* transcription.

Importantly, and apart from direct IFN neutralization or JAK/STAT inhibition, we observed MDAs selectively abrogating *IL18* expression. This finding is of particular clinical relevance because inflammatory and autoinflammatory diseases that are marked by IL-1β and IL-18 overexpression can be treated with colchicine. In these diseases, colchicine-mediated microtubule depolymerization is thought to interfere with nonclassical release of IL-1β, IL-18, or S100 proteins and thus contribute to alleviating inflammation (54). Recent findings suggest that colchicine specifically inhibits the pyrin inflammasome (44, 55). However, cell stimulation with LPS and ATP, as used by us, activates the NLRP3 rather than the pyrin inflammasome (44). Thus, both IL-1β and IL-18 are released via a pyrin-independent mechanism and secretion is unaffected by colchicine. In line with this finding, we and others observed no or an enhancing effect of MDAs on monocytic *IL1B* transcription (56) or IL-1β (57–60) and IL-18 release on NLRP3 inflammasome activation. Despite subsequent inhibition of *IL18* transcription by colchicine, cytokine release immediately after LPS stimulation is likely constituted from the premade pool of cytoplasmic pro– and mature IL-18 (15, 16).

In addition to inhibition of *IL18* transcription, we observed that MDAs interfered with IFNβ gene expression and protein release. Data from the 1970s described an effect of microtubule integrity on IFN production (46), and recent evidence suggests that MDAs inhibit type I IFN translation and secretion (45). Moreover, we demonstrated that blunting of *IL18* expression in LPS-stimulated cells following MDA treatment can be fully rescued by IFNα/β supplementation. In support of this result, colchicine-treated patients with FMF revealed significantly decreased type I IFN scores and *IL18* expression compared with patients with active sJIA disease or during or after MAS who were not exposed to colchicine.

Our findings have significant implications for clinical conditions with a hallmark of high IL-18 serum levels. Clinical experience suggests that episodes of MAS in

Figure 6. (Continued). sJIA-associated macrophage activation syndrome (MAS, $n = 5$), sJIA status after MAS ($n = 6$), or familial Mediterranean fever (FMF, $n = 18$). (*B*) Type I IFN score and *IL18* expression in whole blood of colchicine-treated patients with FMF compared with active sJIA ($n = 5$), sJIA-associated MAS ($n = 5$), or sJIA status after MAS ($n = 6$). Data are presented as median and interquartile range and were analyzed by Mann-Whitney *U* test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (*C*) Wild-type mice were injected with phosphate-buffered saline (naive) or CpG on days 0, 2, 4, 6, and 8 or (*D*) CpG plus aIL-10R on days 0, 2, 4 and 7. (*C*) CpG-injected mice were treated daily with control chow (CpG Ctrl) or ruxolitinib (CpG Ruxo) starting Day 4 after the first injection. (*D*) Mice injected with CpG and aIL-10R were treated with vehicle control (captisol in 0.1 M citrate buffer, pH = 3.5) or ruxolitinib (90 mg/kg) twice daily starting Day 4 after the first injection. All mice were killed on Day 9 after the first CpG (plus aIL-10R) injection, and IL-18 serum levels were quantified by ELISA. Results in *C* and *D* are combined from two (*C*, total of 9–10 mice/group) or four (*D*, naive and CpG plus aIL-10R: 16–17/group; Ruxo: 18/group) independent experiments. Statistical significance was calculated using one-way ANOVA combined with the Tukey multiple comparison test. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. (*E*) Laboratory parameters and CXCL-9 and IL-18 serum levels of a treatment-refractory patient with sJIA with recurrent episodes of MAS treated with tofacitinib. JDM = juvenile dermatomyositis; SLE = systemic lupus erythematosus; WBC = white blood cell.

sJIA are associated with viral infections (29). Intriguingly, MAS can also occur in polygenic type I interferonopathies such as SLE or JDM (61–63). These diseases also link to type I IFN, and disease activity in SLE and JDM correlates with serum IL-18 levels (64–66). In sJIA-associated MAS, IFNα/β signaling resulting from viral infection can come together with NFκβ activation due to background inflammatory activity constituted from, for example, S100 proteins as endogenous TLR4 ligands (67–69). In combination, this can potentiate and sustain IL-18 expression as a suggested promoter of MAS (5). Along these lines, repeated CpG challenge to induce type I IFN expression in mice has been shown to result in MAS-like disease (70), which can be exacerbated by increasing the bioavailability of IL-18 (6) and is sensitive to JAK/STAT inhibition (71). In these models, TLR9 stimulation by CpG renders an otherwise survivable sepsis insult induced by mild cecal ligation and puncture or low-dose staphylococcal toxin or endotoxin overwhelmingly lethal (3, 70). Importantly, we demonstrated that JAK/STAT inhibition in two sHLH/MAS mouse models (34, 35) greatly reduces IL-18 serum levels. We also showed that JAK/STAT inhibition successfully reduced serum IL-18 in a previously treatment-refractory patient with sJIA who had recurrent episodes of MAS and consistently high levels of IL-18 (12).

Linking this to our initial observations on IL-18 expression in LPS-induced immunoparalysis and endotoxin tolerance, serum IL-18 levels have been reported to increase in animals rechallenged with LPS compared with mice receiving only single-endotoxin conditioning. Importantly, priming of animals with CpG resulted in extreme overexpression of IL-18 on subsequent LPS challenge (47). Consequently, it is conceivable that at the level of IL-18 expression, type I IFN and TLR4 signaling can cooperate in facilitating sustained or "second-wave" systemic inflammation that promotes autoinflammation- or infection-associated MAS. ■

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