Neutralization of IFN-γ reverts clinical and laboratory features in a mouse model of macrophage activation syndrome

Giusi Prencipe, PhD,[a] Ivan Caiello, BS,[a] Antonia Pascarella, PhD,[a] Alexei A. Grom, MD,[b] Claudia Bracaglia, MD,[a] Laurence Chatel, DUT,[c] Walter G. Ferlin, PhD,[c] Emiliano Marasco, MD,[a] Raffaele Strippoli, MD, PhD,[d] Cristina de Min, MD,[c] and Fabrizio De Benedetti, MD, PhD[a] *Rome, Italy, Cincinnati, Ohio, and Geneva, Switzerland*

Background: The pathogenesis of macrophage activation syndrome (MAS) is not clearly understood: a large body of evidence supports the involvement of mechanisms similar to those implicated in the setting of primary hemophagocytic lymphohistiocytosis.

Objective: We sought to investigate the pathogenic role of IFN-γ and the therapeutic efficacy of IFN-γ neutralization in an animal model of MAS.

Methods: We used an MAS model established in mice transgenic for human IL-6 (IL-6TG mice) challenged with LPS (MAS mice). Levels of IFN-γ and IFN-γ–inducible chemokines were evaluated by using real-time PCR in the liver and spleen and by means of ELISA in plasma. IFN-γ neutralization was achieved by using the anti–IFN-γ antibody XMG1.2 *in vivo*.

Results: Mice with MAS showed a significant upregulation of the IFN-γ pathway, as demonstrated by increased mRNA levels of *Ifng* and higher levels of phospho–signal transducer and activator of transcription 1 in the liver and spleen and increased expression of the IFN-γ–inducible chemokines *Cxcl9* and *Cxcl10* in the liver and spleen, as well as in plasma. A marked increase in *Il12a* and *Il12b* expression was also found in livers and spleens of mice with MAS. In addition, mice with MAS had a significant increase in numbers of liver $CD68^+$ macrophages. Mice with MAS treated with an anti–IFN-γ antibody showed a significant improvement in survival and body weight recovery associated with a significant amelioration of ferritin, fibrinogen, and alanine aminotransferase levels. In mice with MAS, treatment with the anti–IFN-γ antibody significantly decreased circulating levels of CXCL9, CXCL10, and downstream proinflammatory cytokines. The decrease in CXCL9 and CXCL10 levels paralleled the decrease in serum levels of proinflammatory cytokines and ferritin.

Conclusion: These results provide evidence for a pathogenic role of IFN-γ in the setting of MAS. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: *Macrophage activation syndrome, hemophagocytic lymphohistiocytosis, IFN-γ*

Macrophage activation syndrome (MAS) is a term used to identify hemophagocytic lymphohistiocytosis (HLH) secondary to rheumatic diseases.[1,2] It is a severe and potentially fatal condition that occurs in the context of rheumatic diseases, particularly systemic juvenile idiopathic arthritis (sJIA).[3] MAS is classified among the secondary HLHs and shares clinical and biochemical features with familial or primary hemophagocytic lymphohistiocytosis (pHLH).[4] A cytokine storm appears to be the driving feature[5]; the ensuing clinical syndrome is characterized by high fever, pancytopenia, hyperferritinemia, hypofibrinogenemia, hepatosplenomegaly, and intravascular coagulation.[6] The triggering mechanism behind pHLH is a defect in cytotoxicity caused by mutations in genes encoding proteins required for cytotoxic activity of lymphocytes and natural killer cells.[7] The pathogenesis of MAS is not clearly understood.[8] A large body of evidence supports the involvement of mechanisms similar to those implicated in patients with pHLH. Indeed, patients with JIA with MAS have been shown to have transient natural killer cell dysfunction, such as decreased cell numbers and activity, and reduced perforin expression.[9-11] Recently, in patients with sJIA and MAS, genetic studies, including whole-exome sequencing, identified rare protein-altering variants in known pHLH-associated genes and new candidate genes that are possibly involved in intracellular granule trafficking, further suggesting that MAS predisposition in patients with sJIA could be attributed, at least in part, to variants of genes involved in the cytolytic pathways.[12-14] Consistent with these data, Sepulveda et al[15] demonstrated that the accumulation of monoallelic mutations in HLH-causing genes increases susceptibility to HLH immunopathology also in mice.

Studies in patients and in animal models of pHLH have suggested a key role of IFN-γ in the pathogenesis of the disease. Indeed, in a large cohort of children with HLH, highly increased levels of IFN-γ correlated with disease activity have been reported.[16] Moreover, in $perforin^{-/-}$ mice infected with lymphocytic choriomeningitis virus, the high amount of IFN-γ produced by $CD8^+$ T cells has been demonstrated to be uniquely

From [a]the Division of Rheumatology, Bambino Gesù Children's Hospital IRCCS, Rome; [b]the Division of Rheumatology, ML 4010, Cincinnati Children's Hospital Medical Center; [c]NovImmune SA, Geneva; and [d]the Department of Cellular Biotechnologies and Haematology, Sapienza University of Rome.

Supported by the Italian Ministry of Health (Rome, Italy) Young Investigator Grants (GR-2011-02347874; to G.P.), by the FP7 Grant FIGHT-HLH (agreement no. 306124; to F.D.B. and C.d.M.), and by National Institutes of Health grant R01-AR059049 (to A.A.G.).

Disclosure of potential conflict of interest: G. Prencipe receives grant support from Young Investigator Grants (GR-2011-02347874). A. A. Grom receives grant support from the National Institutes of Health (R01-AR0590490); serves as a consultant for Novartis, Novimmne, and Juno; receives payment for lectures from Novartis; and receives royalties from UpToDate. C. de Min receives grant support from FP7 grant FIGHT-HLH. F. De Benedetti receives grant support from Grant FIGHT-HLH. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication October 7, 2016; revised July 17, 2017; accepted for publication July 19, 2017.

Corresponding author: Giusi Prencipe, PhD, Division of Rheumatology, Bambino Gesù Children's Hospital, Viale di S. Paolo, 15, 00146 Rome, Italy. E-mail: giusi.prencipe@opbg.net.

0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2017.07.021

Abbreviations used
ALT: Alanine aminotransferase
HLH: Hemophagocytic lymphohistiocytosis
MAS: Macrophage activation syndrome
pHLH: Primary hemophagocytic lymphohistiocytosis
sJIA: Systemic juvenile idiopathic arthritis
STAT1: Signal transducer and activator of transcription 1
WT: Wild-type

essential for disease development.[17] Similarly, in lymphocytic choriomeningitis virus/Rab27a–deficient mice, neutralization of IFN-γ has been shown to revert central nervous system involvement and reduce hemophagocytosis.[18] Neutralization of IFN-γ is able to revert hematologic abnormalities also in the mouse model of HLH secondary to infections, which was mimicked by repeated stimulation of Toll-like receptor (TLR) 9 on a normal genetic background.[19]

The aim of this study was to evaluate the pathogenic role of IFN-γ in a mouse model of MAS based on mice transgenic for human IL-6 (IL-6TG mice).[20] We have reported that IL-6TG mice, after a single administration of TLR ligands, display an increased fatality rate associated with higher levels of circulating proinflammatory cytokines and hematologic and biochemical features typically present in mice with MAS.[20] In these mice MAS is induced by mimicking an acute infection with administration of a TLR agonist (ie, LPS). This approach recapitulates what occurs in patients with sJIA: an infection is the typical trigger of MAS in the presence of active disease, which is characterized by high levels of IL-6, which appears to play a pivotal pathogenic role in the autoinflammation of sJIA.[21,22]

In this study we investigated whether levels of IFN-γ and IFN-γ–inducible chemokines are increased in the setting of MAS induced by LPS in IL-6TG mice and whether administration of an anti–IFN-γ antibody improves survival and disease parameters.

METHODS

Mice and *in vivo* treatments

The IL-6TG mouse has been described previously.[23] Mice between 10 and 14 weeks of age were administered intraperitoneally a single dose (7.5 or 5 μg/g body weight) of LPS (*Escherichia coli* serotype 055:B5; Sigma-Aldrich, St Louis, Mo). For treatment experiments, 100 μg/g body weight of the anti-mouse IFN-γ neutralizing antibody XMG1.2 (BioXCell, West Lebanon, NH) and of a rat IgG_1 isotype-matched control mAb35 (American Type Culture Collection, Manassas, Va) were administered to mice intraperitoneally. Mice were maintained under specific pathogen-free conditions and handled in accordance with the institutional experimental ethics committee guidelines.

Cytokine, ferritin, fibrinogen, and alanine aminotransferase measurements

Mouse plasma cytokine and chemokine levels were determined by using MILLIPLEX MAP Multiplex Immunodetection Kits (Merck, Whitehouse Station, NJ), according to the manufacturer's instructions. For all analytes, the lower detection limit was 3.2 pg/mL, and the upper detection limit was 10,000 pg/mL. CXCL9 and CXCL10 levels were further determined by using mouse Quantikine ELISA KITs (R&D Systems, Minneapolis, Minn). Serum ferritin and plasma fibrinogen concentrations were determined by using ELISA kits (ALPCO Diagnostics, Salem, NH, and Abcam, Cambridge, United Kingdom, respectively). Alanine aminotransferase (ALT) levels were determined by using an enzymatic assay kit (Bioo Scientific, Austin, Tex).

RNA isolation and quantitative real-time PCR

Total RNA was extracted from mouse liver and spleen tissues with TRIzol (Life Technologies, Grand Island, NY), and cDNA was obtained with the Superscript Vilo Kit (Invitrogen, Carlsbad, Calif). Real-time PCR assays were performed with the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, Calif) and the following gene expression assays: mouse *Ifng*, *Cxcl9*, *Cxcl10*, *Il12a*, and *Il12b* (Applied Biosystems). Gene expression data were normalized with mouse *Hprt* (Applied Biosystems) as an endogenous control. Data are expressed as arbitrary units determined by using the $2^{-\Delta ct}$ method.

Protein extraction and Western blot analysis

Total tissue proteins were extracted with RIPA Buffer (Cell Signaling, Danvers, Mass). For Western blotting, protein lysates were resolved by using 10% SDS-PAGE. Proteins were then transferred to nitrocellulose membranes (Amersham Life Sciences, Piscataway, NJ) and probed with antibodies to phospho-Tyr701 signal transducer and activator of transcription (STAT) 1, total STAT1, and glyceraldehyde-3-phosphate dehydrogenase (all from Cell Signaling) by using standard procedures.

Histology and immunohistochemical analysis

Livers from mice were drop fixed in neutral buffered formalin and then processed for paraffin embedding. Sections of 2.5 μm were stained with hematoxylin and eosin. For immunohistochemical analysis, after moist heat–induced antigen retrieval with EnVision Flex Target Retrieval Solutions High pH (DakoCytomation, Glostrup, Denmark), 2.5-μm sections were incubated with antibody to CD68 (Abcam, Cambridge, United Kingdom) overnight at 4°C. After washing, sections were incubated with appropriate horseradish peroxidase–conjugated secondary antibodies. Chromogen detection was carried out with the DAB chromogen kit (DakoCytomation). Nuclei were counterstained with hematoxylin, followed by dehydration and mounting. The number of $CD68^+$ cells was enumerated in at least 10 fields for each tissue at ×40 magnification.

Statistical analyses

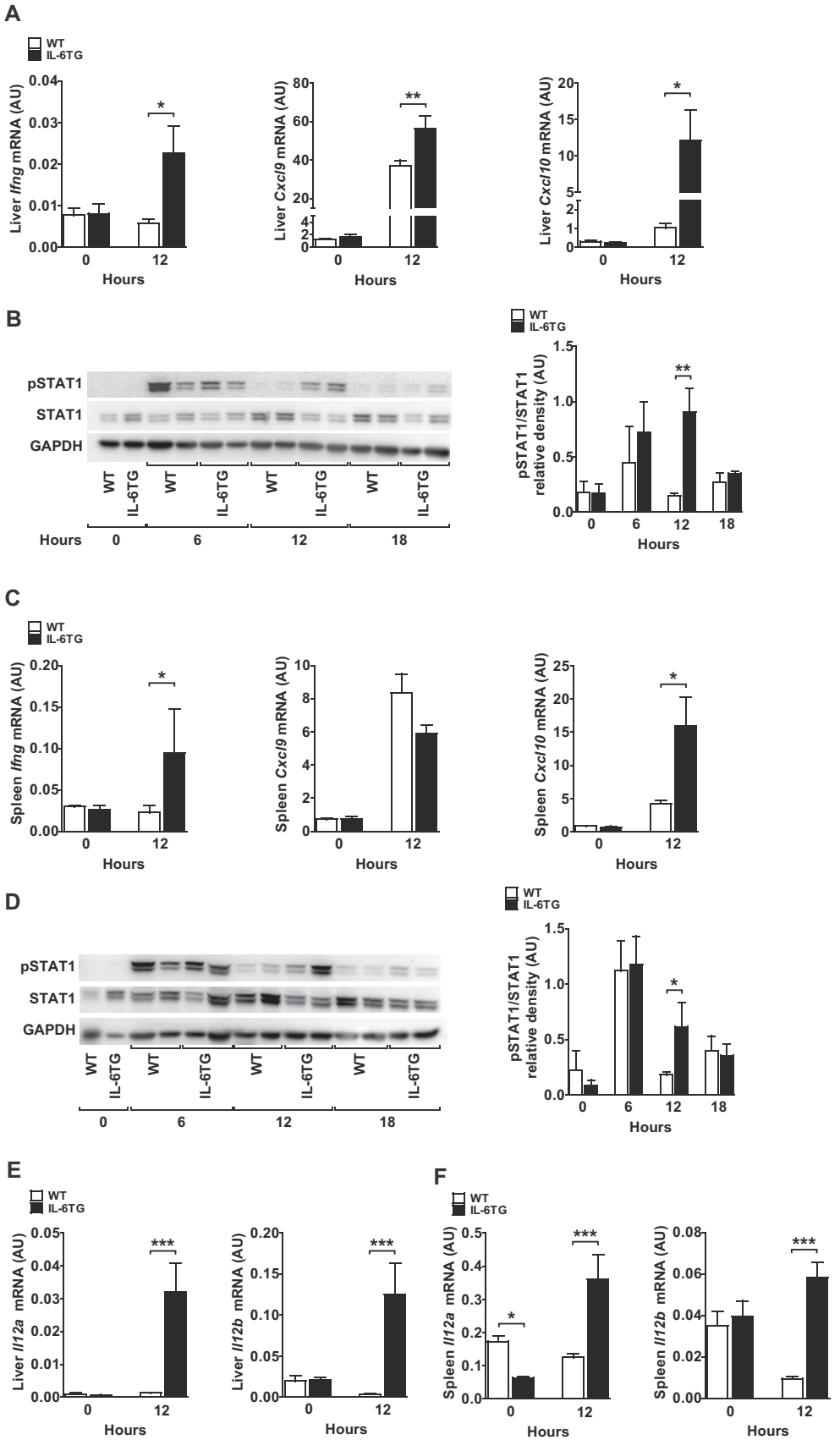
Data are presented as means ± SEMs, unless otherwise indicated. Group comparisons were performed with the nonparametric Mann-Whitney *U* test. The Spearman rank correlation coefficient *(r)* was calculated to assess correlations between variables. All statistical analyses were performed with GraphPad Prism IV software (GraphPad Software, La Jolla, Calif). A *P* value of less than .05 was considered statistically significant.

RESULTS

IFN-γ and IFN-γ–inducible chemokine levels are higher in LPS-challenged IL-6TG mice

Although *Ifng* mRNA expression levels were not different between wild-type (WT) and IL-6TG mice in both the liver (Fig 1, *A*) and spleen (Fig 1, *C*) before LPS administration, we found that they were significantly increased in IL-6TG mice compared with WT mice at 12 hours after LPS administration (Fig 1, *A* and *C*). Moreover, we found that *Cxcl9* mRNA levels were significantly higher in the liver and *Cxcl10* mRNA levels were significantly higher both in the liver and spleen compared with those in WT mice (Fig 1, *A* and *C*).

Liver and spleen protein lysates were tested by using Western blotting with a specific antibody for Tyr701-phosphorylated STAT1, which is known to mediate IFN-γ effects, to further


A
WT
IL-6TG
Liver Ifng mRNA (AU)
Liver Cxcl9 mRNA (AU)
Liver Cxcl10 mRNA (AU)
Hours
B
pSTAT1
STAT1
GAPDH
pSTAT1/STAT1 relative density (AU)
C
Spleen Ifng mRNA (AU)
Spleen Cxcl9 mRNA (AU)
Spleen Cxcl10 mRNA (AU)
D
E
Liver Il12a mRNA (AU)
Liver Il12b mRNA (AU)
F
Spleen Il12a mRNA (AU)
Spleen Il12b mRNA (AU)

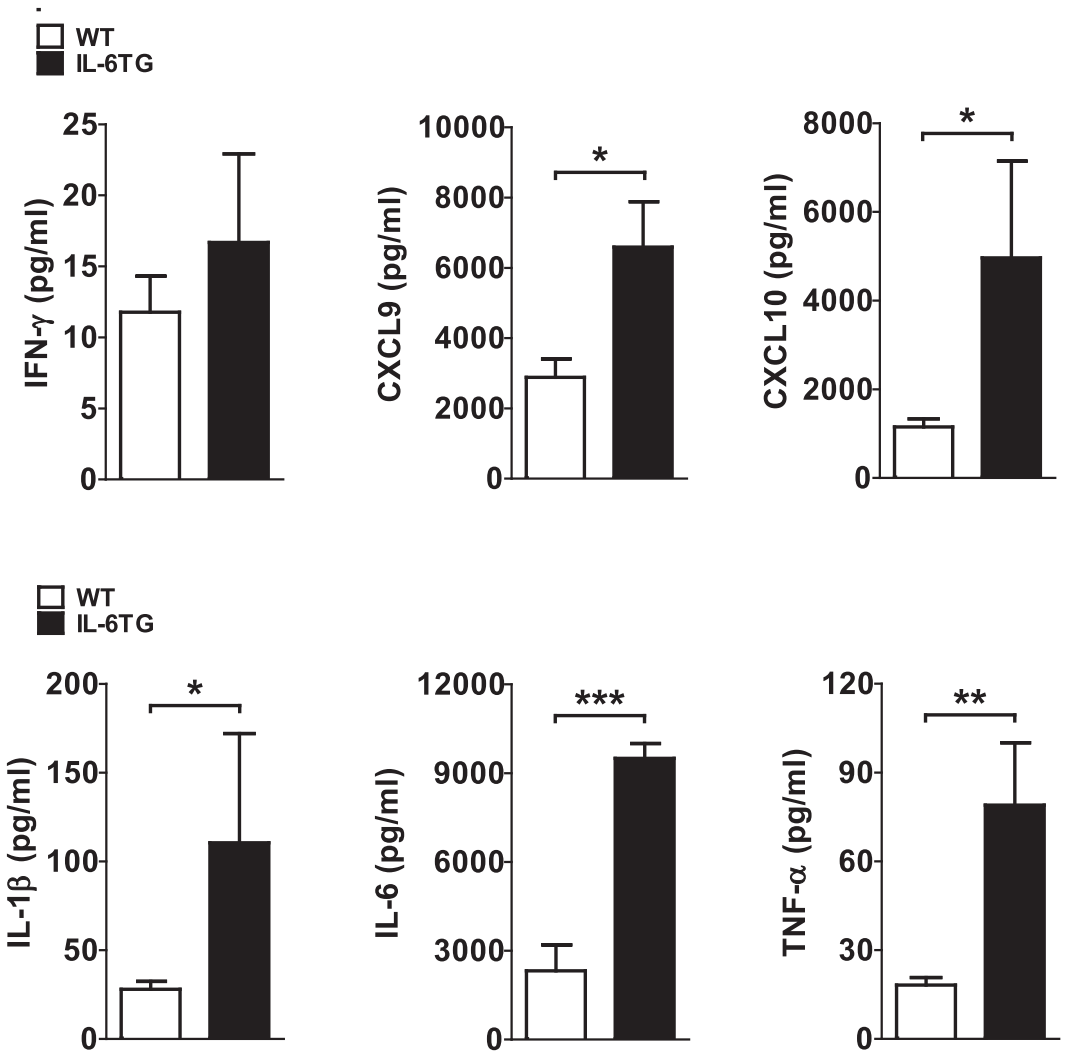


FIG 2. Circulating levels of IFN-γ–inducible chemokines and proinflammatory cytokines are increased in LPS-challenged IL-6TG mice. Plasma levels of IFN-γ, the IFN-γ–inducible chemokines CXCL9 and CXCL10, and the proinflammatory cytokines IL-1β, IL-6, and TNF-α were measured in WT and IL-6TG mice at 18 hours after challenge with LPS (5 μg/g body weight). Data (means ± SEMs) are representative of at least 2 independent experiments with at least 3 mice in each group. Statistical analyses were performed with the 1-tailed Mann-Whitney *U* test: **P* < .05, ***P* < .01, and ****P* < .001.

evaluate activation of the IFN-γ pathway in LPS-challenged IL-6TG mice compared with WT mice.[24,25] We did not detect Tyr701 phosphorylation of STAT1 before LPS challenge in both WT and IL-6TG mouse tissues. Tyr701-phosphorylated STAT1 was similarly increased both in the liver (Fig 1, *B*) and spleen (Fig 1, *D*) in WT and IL-6TG mice at 6 hours after LPS challenge. Notably, although in WT mice phospho-STAT1 levels returned to baseline at 12 hours after LPS challenge, in IL-6TG mice they remained markedly higher both in the liver and spleen (Fig 1, *B* and *D*).

Because IL-12, which is produced mainly by classically activated M1 macrophages,[26] is known to stimulate IFN-γ

FIG 1. LPS-challenged IL-6TG mice display upregulation of the IFN-γ pathway and increased mRNA expression of *Il12a* and *Il12b*. WT and IL-6TG mice were challenged with LPS (5 μg/g body weight), and at different times after the challenge, mice were killed. **A** and **C,** mRNA expression levels of *Ifng* and of the IFN-γ–regulated genes *Cxcl9* and *Cxcl10* were assayed in the liver (Fig 1, *A*) and spleen (Fig 1, *C*). Results are expressed as arbitrary units *(AU)* and obtained after normalization with the housekeeping gene *Hprt*. Tyr701-phosphorylated STAT1 protein levels were assessed by means of Western blot analysis in the liver **(B)** and spleen **(D)**. In Fig 1, *B* and *D*, at *right*, densitometric analyses confirmed the increased levels of Tyr701-phosphorylated STAT1 in the liver and spleen from IL-6TG mice compared with WT mice (n = 4 or 5 in each group). **E** and **F,** mRNA expression levels of *Il12a* and *Il12b* were also evaluated in the liver (Fig 1, *E*) and spleen (Fig 1, *F*). Data in Fig 1, *A-F* (means ± SEMs), are representative of at least 2 independent experiments with at least 3 mice in each group. Statistical analyses were performed with the 1-tailed Mann-Whitney *U* test: **P* < .05, ***P* < .01, and ****P* < .001.

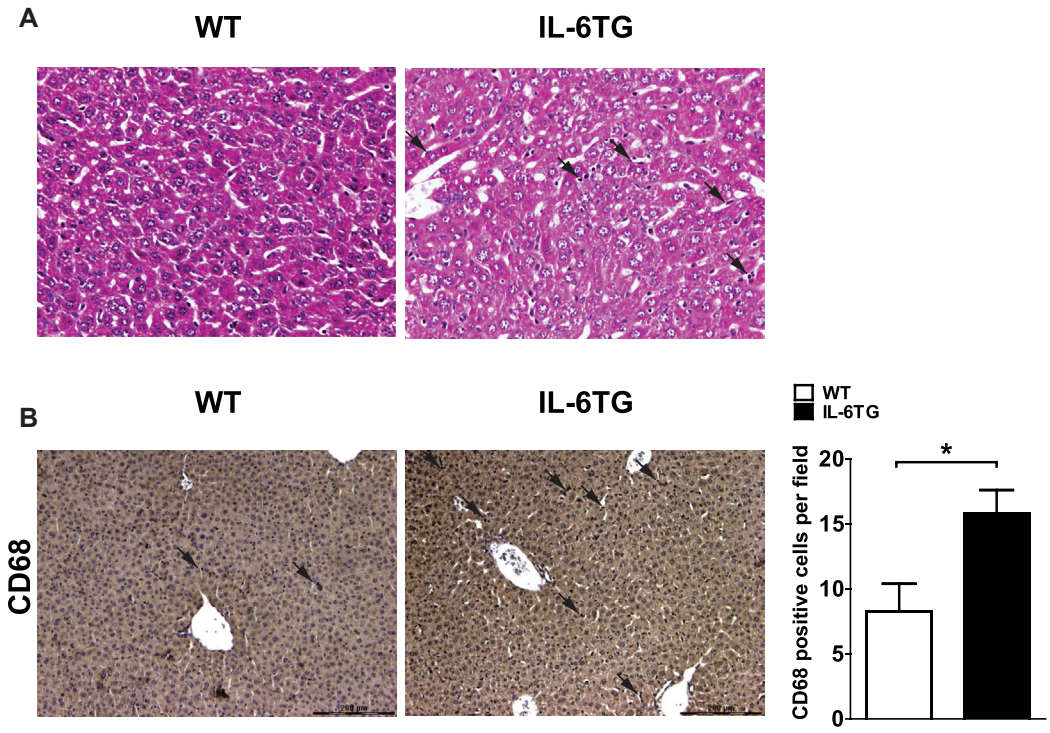


FIG 3. Presence of dilated sinusoids and infiltration by $CD68^+$ macrophages in livers of LPS-challenged IL-6TG mice. **A,** Liver sections from WT and IL-6TG mice at 12 hours after LPS challenge (5 μg/g body weight) were stained with hematoxylin and eosin *(H&E)*. Dilated sinusoids populated by mononucleated cells are indicated by *arrows*. Representative sections are shown at a magnification of ×40. **B,** Representative images of liver sections from WT and IL-6TG mice stained for total macrophages by using the CD68 antibody at 12 hours after the challenge with LPS (5 μg/g body weight). $CD68^+$ cells are indicated by *arrows*. *Scale bars* = 200 μm. At *right,* the number of $CD68^+$ cells per field was reported. Data (means ± SEMs) are representative of at least 2 independent experiments with at least 2 mice in each group. Statistical analyses were performed with the 1-tailed Mann-Whitney *U* test: $*P < .05$.

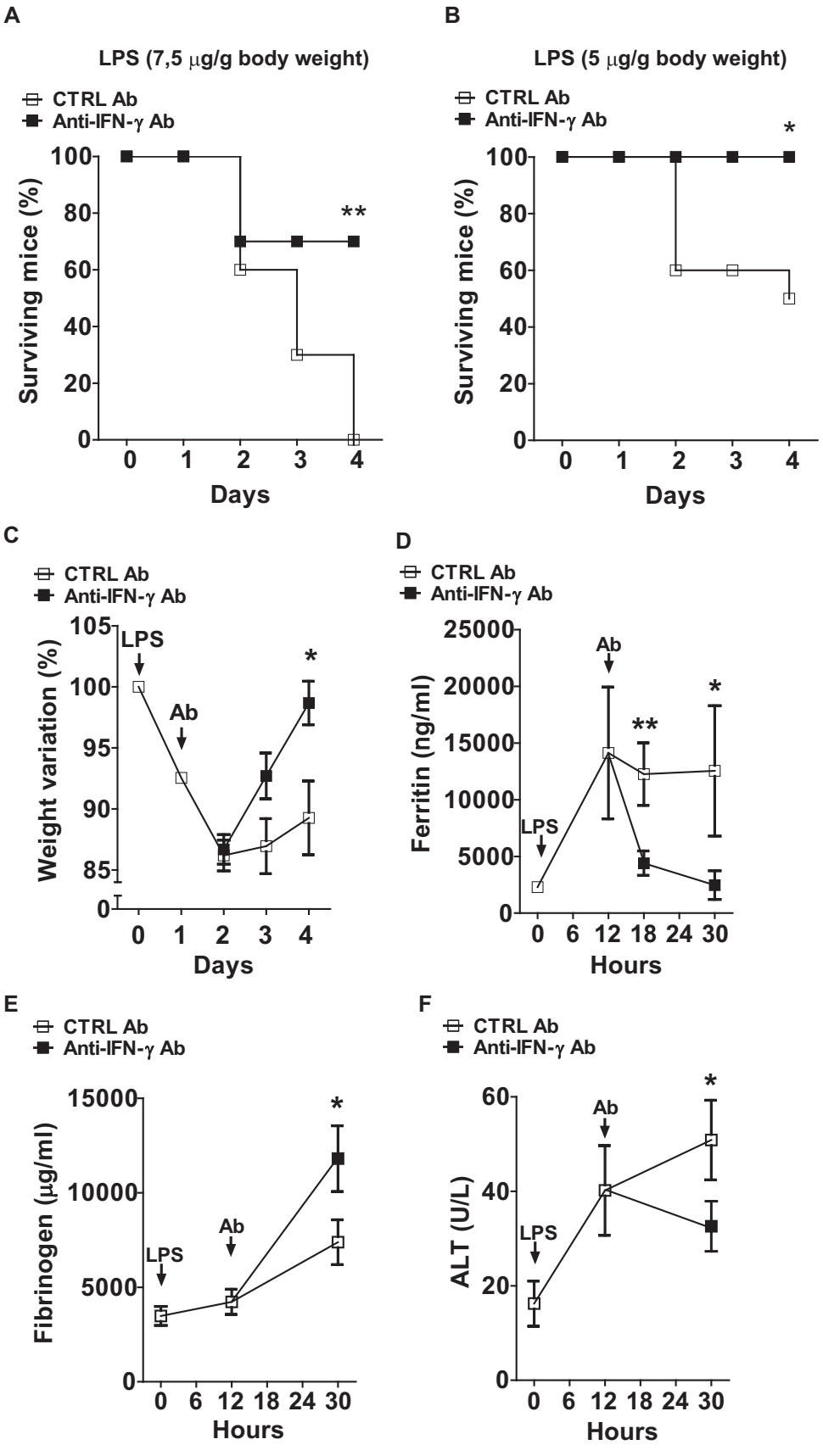
production and has been demonstrated to act upstream to induce IFN-γ in an animal model of fulminant MAS,[27] we assessed mRNA expression of the *Il12a* and *Il12b* genes, encoding for the 2 heterologous chains of IL-12 p35 and p40, respectively. We found that these genes were strongly upregulated in livers from LPS-challenged IL-6TG mice and significantly higher in both the liver and spleen in IL-6TG mice compared with WT mice (Fig 1, *E* and *F*). In IL-6TG mouse livers, strong positive correlations between *Il12a* and *Il12b* and *Ifng* mRNA expression were also found ($r = 0.93$, $P = .001$ and $r = 0.083$, $P = .007$, respectively), further suggesting a possible role for IL-12 in inducing IFN-γ production in the tissue.

When we measured circulating levels of IFN-γ, low levels (in the picogram per milliliter range) were detected, with a trend for higher levels in LPS-challenged IL-6TG mice compared with WT mice (Fig 2). These results are consistent with recent observations demonstrating that production of IFN-γ typically occurs in peripheral tissues.[28] In this respect circulating levels of the IFN-γ–inducible chemokines CXCL9 and CXCL10 have been suggested as possible markers of tissue IFN-γ production. Indeed in LPS-challenged IL-6TG mice, high levels of CXCL9 and CXCL10 were found in plasma (Fig 2).

Interestingly, we found also that circulating levels of CXCL9 were significantly correlated with liver mRNA levels (18 hours after LPS administration) of *Ifng* ($r = 0.83$, $P = .029$) and *Cxcl9* ($r = 0.89$, $P = .017$). Similarly, circulating CXCL10 levels were significantly correlated to liver mRNA levels of *Ifng* ($r = 0.82$, $P = .030$) and *Cxcl10* ($r = 0.89$, $P = .017$). We also found a tight correlation between plasma CXCL9 levels and spleen *Cxcl9* mRNA levels ($r = 1.0$, $P = .001$). The same analyses were performed in WT mice, and no correlations were observed. Furthermore, and consistent with previously published data at 6 hours from LPS challenge,[20] at 18 hours, levels of proinflammatory cytokines, including IL-1β, TNF-α, and IL-6, were markedly higher in LPS-challenged IL-6TG mice compared with WT mice (Fig 2).

Altogether, our data show that in the mouse model of MAS, *Ifng* expression is increased, the STAT1-mediated IFN-γ pathway is upregulated, and the IFN-γ–inducible chemokines are upregulated locally and systemically. The significant correlation between *Ifng* and chemokine transcript levels in peripheral tissues and chemokine levels in the blood supports the conclusion that circulating levels of IFN-γ–inducible chemokines reflect tissue activation of the IFN-γ pathway, as well as tissue production of IFN-γ–inducible chemokines.

Incidentally, liver hematoxylin and eosin staining did not reveal major abnormalities in hepatocytes. However, dilated sinusoids populated by mononucleated cells were observed in LPS-challenged IL-6TG mice but not in WT mice (Fig 3, *A*). Macrophages are most probably key early cells in the pathogenesis of MAS; indeed, immunohistochemical analyses showed a markedly higher number of CD68 (a panmacrophage


A
LPS (7,5 μg/g body weight)
CTRL Ab
Anti-IFN-γ Ab
Surviving mice (%)
Days
**
B
LPS (5 μg/g body weight)
CTRL Ab
Anti-IFN-γ Ab
Surviving mice (%)
Days
*
C
CTRL Ab
Anti-IFN-γ Ab
Weight variation (%)
LPS
Ab
Days
*
D
CTRL Ab
Anti-IFN-γ Ab
Ferritin (ng/ml)
Ab
LPS
**
*
Hours
E
CTRL Ab
Anti-IFN-γ Ab
Fibrinogen (μg/ml)
LPS
Ab
*
Hours
F
CTRL Ab
Anti-IFN-γ Ab
ALT (U/L)
Ab
LPS
*
Hours

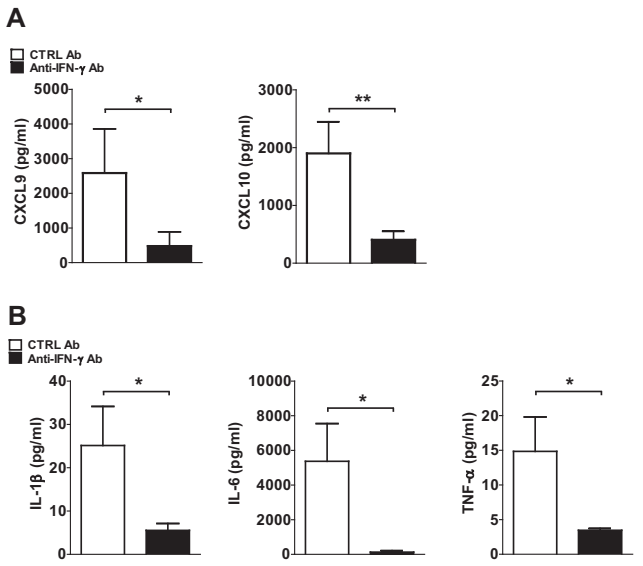


FIG 5. In mice treated with the anti–IFN-γ antibody *(Ab)*, circulating levels of proinflammatory cytokines are downmodulated. IL-6TG mice were challenged with LPS (5 μg/g body weight), and 12 hours after the challenge, mice were treated with the IFN-γ neutralizing antibody XMG2.1 (100 μg/g body weight) or with the mAb35 control antibody. Eighteen hours after treatment with antibodies, mice were killed, and levels of CXCL9 and CXCL10 **(A)** and proinflammatory cytokines **(B)**, including IL-1β, IL-6, and TNF-α, were assayed in plasma samples collected at the time of death. Data (means ± SEMs) are representative of at least 2 independent experiments with at least 3 mice in each group. Statistical analyses were performed with the 1-tailed Mann-Whitney *U* test: $*P < .05$ and $**P < .01$.

marker)–positive cells in livers from IL-6TG mice compared with WT mice (Fig 3, *B*), suggesting a role for macrophages in the development of the disease in IL-6TG mice.

Neutralization of IFN-γ improves survival, body weight recovery, and laboratory parameters in LPS-challenged IL-6TG mice

Having demonstrated that the IFN-γ pathway is upregulated in murine MAS induced by LPS challenge in IL-6TG animals, the effect of neutralization of IFN-γ was tested by administering an anti–IFN-γ antibody. After control antibody administration, all mice challenged with a dose of LPS known to be lethal in 100% of the animals (7.5 μg/g body weight) died within 4 days, whereas 70% of mice treated with the anti–IFN-γ antibody survived (Fig 4, *A*). Similarly, after control antibody administration, 5 of 10 mice challenged with a dose of LPS known to be lethal in 50% of animals (5 μg/g body weight) died within 4 days, whereas all mice treated with the anti–IFN-γ antibody survived (Fig 4, *B*). No additional deaths occurred in the following days in both groups of mice and in both experiments (data not shown).

In mice injected with 5 μg/g LPS, anti–IFN-γ treatment significantly improved body weight recovery, with animals reaching their baseline weight within 3 days (Fig 4, *C*). After 12 hours from LPS administration, a marked increase in ferritin levels was observed, which is consistent with previous data.[20] Although ferritin levels did not change in control antibody–treated IL-6TG mice, a sharp decrease was observed in mice treated with the anti–IFN-γ antibody (Fig 4, *D*). After LPS administration, levels of plasma fibrinogen, an additional laboratory feature of MAS, were significantly lower in IL-6TG mice compared with those in WT mice (mean ± SD, 4229 ± 1885 vs 6654 ± 1526 μg/mL, $P = .01$), suggesting increased intravascular coagulation activation in this animal model. We found that in LPS-challenged IL-6TG mice fibrinogen levels were significantly higher in mice treated with the anti–IFN-γ antibody compared with mice treated with the control antibody (Fig 4, *E*).

To biochemically evaluate liver involvement, we measured ALT levels. As previously demonstrated, we did not observe a significant difference in circulating ALT levels between LPS-challenged IL-6TG and WT mice both at baseline and 12 hours after LPS (mean ± SD: basal levels, 13.58 ± 6.8 vs 12.2 ± 10.07 U/L; 12 hours after LPS administration, 40.2 ± 21.24 vs 38.7 ± 15.30 U/L). In IL-6TG mice after administration of the anti–IFN-γ antibody, ALT levels were significantly reduced compared with those in control-treated animals (Fig 4, *F*).

IFN-γ–inducible chemokines and proinflammatory cytokines are downregulated in mice treated with the anti–IFN-γ antibody and are related to changes in ferritin levels during effective treatment

In anti–IFN-γ antibody–treated IL-6TG mice plasma levels of CXCL9 and CXCL10 were markedly lower than those of mice treated with control antibody (Fig 5, *A*). Moreover, plasma levels of IL-1β, IL-6, and TNF-α, 3 classical proinflammatory cytokines, were significantly lower in anti–IFN-γ antibody–treated than in control antibody–treated mice (Fig 5, *B*). Finally, in LPS-challenged mice treated with the control and the anti–IFN-γ antibody, lower circulating levels of CXCL9 and CXCL10 were significantly associated with lower levels of IL-6, IL-1β, and TNF-α (Fig 6, *A*), as well as with lower serum ferritin levels (Fig 6, *B* and *C*). These results support the conclusion that a decrease in IFN-γ activity, as estimated by the decrease in CXCL9 and CXCL10 levels, is related to downstream events, such as inflammatory cytokine levels and clinical parameters of disease.

FIG 4. Treatment with a neutralizing anti–IFN-γ antibody *(Ab)* improves survival and clinical and laboratory parameters of MAS in LPS-challenged IL-6TG mice. IL-6TG mice were challenged with 7.5 μg/g body weight of LPS **(A)** or with 5 μg/g body weight of LPS **(B)** at day 0, and 12 hours after the challenge, mice were treated with the IFN-γ neutralizing antibody XMG2.1 (100 μg/g body weight) or with the control antibody mAb35 and were monitored for survival. Survival rates were determined every day for 4 days (n = 10 mice per group). The Fisher exact test was performed. **C-F,** IL-6TG mice were challenged with LPS (5 μg/g body weight) at day 0 and treated, as previously described, with the XMG2.1 or mAb35 control antibody, and clinical and laboratory parameters were evaluated: body weight variation (Fig 4, *C*) and serum ferritin (Fig 4, *D*), plasma fibrinogen (Fig 4, *E*), and plasma ALT (Fig 4, *F*) levels. In Fig 4, *A*, average body weight loss data are presented with last observation carried forward for mice that succumbed to LPS challenge. Data (means ± SEMs) are representative of at least 2 independent experiments with at least 3 mice in each group. Statistical analyses were performed with the 1-tailed Mann-Whitney *U* test: $*P < .05$ and $**P < .01$.

A

	IL-6	IL-1β	TNF-α	CXCL10
CXCL9	r=0.92 p<0.0001	r=0.73 p=0.006	r=0.88 p=0.0002	r=0.92 p<0.0001
CXCL10	r=0.95 p<0.0001	r=0.68 p=0.01	r=0.83 p=0.008	

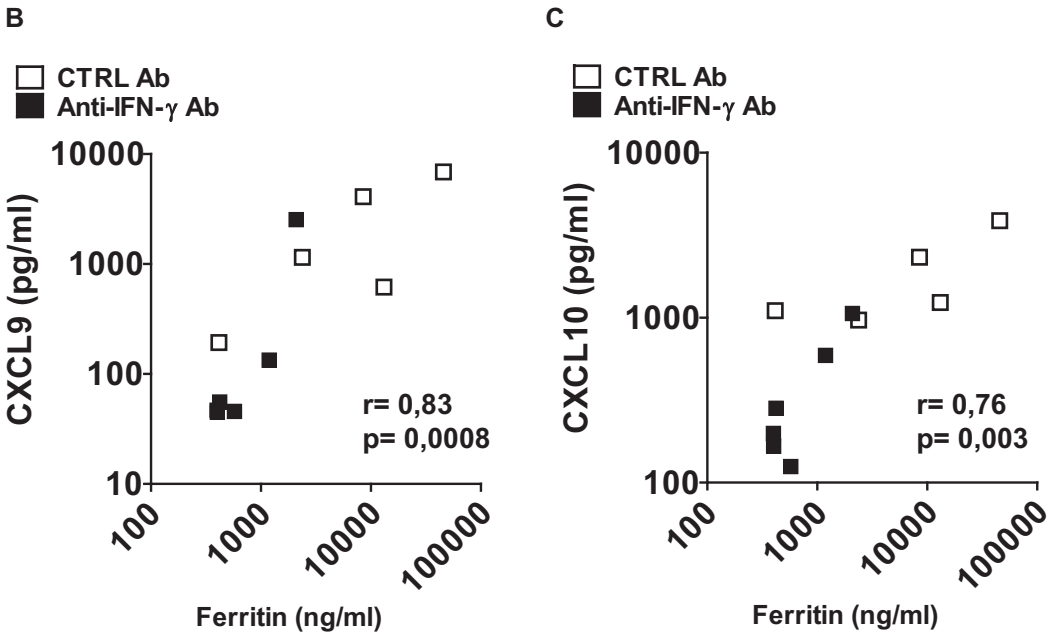


FIG 6. Circulating levels of the IFN-γ–inducible chemokines CXCL9 and CXCL10 are correlated to changes in proinflammatory cytokine and ferritin levels during effective treatment in IL-6TG mice. IL-6TG mice were challenged with LPS (5 μg/g body weight), and 12 hours after the challenge, mice were treated with the IFN-γ neutralizing antibody XMG2.1 (100 μg/g body weight; *solid squares*) or with the mAb35 control antibody *(open squares)*. Eighteen hours after treatment with antibodies, mice were killed, and levels of CXCL9 and CXCL10 were measured in plasma and related to circulating levels of the proinflammatory cytokines IL-6, IL-1β, and TNF-α **(A)**, as well as to serum ferritin levels (**B** and **C**). The Spearman rank correlation coefficient was calculated *(r)* to assess correlations between variables.

DISCUSSION

Although several observations in murine models and indirect evidence in patients point to a pivotal pathogenic role of IFN-γ in patients with pHLH,[17,18] limited data are available on its role in pathogenesis of the different forms of secondary HLH. In this study we used a murine model of MAS in which clinical and laboratory features of MAS are induced by administration of a TLR ligand in IL-6 transgenic mice.[20] We found that IL-6TG mice challenged with LPS display upregulation of the IFN-γ pathway, as shown by higher mRNA expression levels of *Ifng*, higher levels of phospho-STAT1 and the IFN-γ–inducible genes *Cxcl9* and *Cxcl10* in the liver and spleen, and higher levels of circulating CXCL9 and CXCL10. Moreover, we found that treatment of IL-6TG mice with an anti–IFN-γ antibody improved survival, body weight, and laboratory parameters. Finally, neutralization of IFN-γ led also to a marked decrease in circulating levels of CXCL9 and CXCL10, chemokines typically induced by IFN-γ, as well as of the downstream inflammatory cytokines TNF-α, IL-6, and IL-1β.

Despite the evidence that in normal mice systemic overexposure to high levels of IFN-γ is sufficient to drive cytopenias and hemophagocytosis in a STAT1-dependent manner,[29] data on the role of IFN-γ in models of secondary HLH are scarce and in part contradictory. In a model of HLH secondary to infections induced by TLR9-repeated stimulation, on a normal genetic background and in the absence of underlying disease, an important pathogenic role for IFN-γ has been reported.[19] Moreover, by using IFN-γ–overexpressing mice, IFN-γ has been identified as a mediator of systemic inflammatory disease, supporting the hypothesis that there is a critical threshold of IFN-γ that, when achieved either locally in tissues or systemically, drives the development of an autoinflammatory-like disease, interestingly with high ferritin levels.[30] In apparent contrast, immune stimulation of IFN-γ knockout mice with Freund complete adjuvant produces a systemic inflammatory disease that includes features of sJIA, as well as MAS, such as anemia, increased numbers of immature blood cells, increased serum levels of IL-6, hemophagocytosis, and defective natural killer cell cytotoxicity; it is noteworthy that cytopenias and hyperferritenemia were not demonstrated in this model.[31] Canna et al[27] demonstrated that in mice treated with an IL-10 receptor blocking antibody and a TLR9 agonist, fulminant MAS and

hemophagocytosis arise. When these mice were knocked out for IFN-γ, they had immunopathology and hemophagocytosis comparable with that seen in WT mice but did not become anemic and had greater numbers of splenic erythroid precursors.[27] Similarly, in a mouse model of HLH associated with cytomegalovirus-related sepsis, IFN-γ knockout mice display a more severe spectrum of the disease,[32] therefore suggesting a protective role for IFN-γ. The interpretation of these results, which appear to be at least in part contradictory, might be particularly difficult because of compensatory mechanisms secondary to a lack of or increased expression of IFN-γ since prenatal age in these animal models.

Although these approaches provide a wealth of information on the pathophysiology of hyperinflammation, these compensatory mechanisms make it difficult to directly extrapolate the findings into a therapeutic approach in subjects with a "normal" immune response. Indeed, recently, 2 unrelated patients with novel mutations in IFN-γ receptors, which is highly suggestive of functional receptor deficiency, have been described with a clinical presentation that closely resembled that of HLH in the context of overwhelming mycobacterial infections (with associated herpes virus infections).[33] This observation has suggested that IFN-γ–independent pathways can contribute to the development of the clinical syndrome HLH or at least to some of the characteristic features.

As previously mentioned, animal models of pHLH caused by deletion of the genes involved in pHLH on an otherwise normal immune response have unequivocally demonstrated a pivotal pathogenic role of IFN-γ. We have used a model in which MAS clinical and laboratory features are triggered in animals with high levels of circulating human IL-6 since early phases of life (not prenatally), with a normally regulated expression of IFN-γ.[20] Our model has similarities with MAS development in patients with sJIA, where MAS is typically triggered by acute infections during an underlying case of active sJIA, which is typically characterized by increased IL-6 levels.

Our data show high mRNA expression of IFN-γ associated with high *Il12a* and *Il12b* mRNA expression in the liver and spleen, suggesting that IL-12 might be at least one of the factors involved in increased production of IFN-γ in the presence of high *in vivo* levels of IL-6. Overexpression of IFN-γ is biologically relevant, as shown by the prolonged phosphorylation of STAT1 in the liver and spleen and by the marked upregulation of liver and spleen expression and plasma levels of IFN-γ–inducible chemokines. These observations in the liver and spleen, which are target tissues in the setting of MAS, appear to complement observations in patients with secondary HLH[34] and MAS. Billiau et al[35] showed the presence of IFN-γ–producing activated $CD8^+$ lymphocytes in 4 liver biopsy specimens of patients with secondary HLH, including 1 patient with MAS. More recently, high levels of IFN-γ and CXCL10 in the sera of 5 patients with HLH, 3 of them with MAS, have also been reported.[36] Recently, we found high circulating levels of IFN-γ and IFN-γ–inducible chemokines in 20 patients with active MAS but not in patients with active sJIA without MAS.[37] It should be noted that gene expression profile studies of PBMCs of patients with active sJIA with or without MAS did not show an IFN-γ signature.[38-40] However, although gene expression profile studies on PBMCs reveal events that occur in peripheral blood, our animal data in the liver and spleen support the hypothesis that, in patients with MAS, marked production of IFN-γ and IFN-γ–inducible proteins occurs first in diseased tissues, after which proteins leak into peripheral blood. Supporting this hypothesis, we found that in LPS-challenged IL-6TG mice there was a significant correlation of circulating levels of CXCL9 and CXCL10 with liver/spleen mRNA levels of *Cxcl9* and *Cxcl10* and, more importantly, with tissue mRNA levels of *Ifng*. Notably, Put et al[36] reported evident expression of the IFN-γ–inducible proteins indoleamine 2,3-dioxygenase and CXCL10 in 1 lymph node biopsy specimen from 1 patient with sJIA with MAS. Furthermore, some of us demonstrated in the model of HLH secondary to infection induced by repeated TLR9 stimulation that total IFN-γ levels produced in tissues are 500- to 2000-fold higher than those measured in blood and identified the spleen and liver as major sites of IFN-γ production.[28] These results are consistent with our finding of a trend for increased circulating levels of IFN-γ in our mice with MAS. Together, these observations in patients with MAS and in MAS animal models support the hypothesis that IFN-γ production is markedly increased, that high IFN-γ production is biologically relevant, and that overactivation of the IFN-γ pathway appears to occur in peripheral tissues, which are typically involved by the disease, such as the liver and spleen.

We also demonstrated the pathogenic role of IFN-γ in the murine MAS model. Treatment with an anti–IFN-γ antibody led to a significant increase in survival and reverted clinical and laboratory features of MAS, including body weight loss and ferritin, ALT, and fibrinogen levels. Furthermore, neutralization of IFN-γ led to a significant decrease in levels of classical downstream proinflammatory cytokines. Interestingly, we found that in LPS-challenged mice circulating CXCL9 and CXCL10 levels were significantly related to IL-1β, IL-6, TNF-α, and ferritin levels. Further supporting the relevance of IFN-γ neutralization, we showed that the extent of change in upstream events (ie, CXCL9 and CXCL10 levels) is related to the extent of the decrease in downstream events, such as levels of proinflammatory cytokines, or even more so, levels of ferritin, a classical laboratory parameter of disease activity in clinical practice. Consistent with these data in mice, we found that in patients with MAS sampled longitudinally during effective traditional treatment, circulating levels of both CXCL9 and CXCL10 paralleled the decrease in ferritin levels during the progressive clinical improvement (data not shown). Data in animals and human subjects suggest also that serum levels of CXCL9 and possibly CXCL10, because they reflect tissue production of IFN-γ, might represent additional biomarkers of disease activity in the setting of MAS. Their relative value compared with standard biochemical parameters, such as ferritin levels, in identifying patients in the early stages of MAS before it progresses to its life-threatening stage should be investigated in a larger number of patients, possibly in a multicenter study.

Our experimental model of MAS has the limitation of having short duration (death occurs in 2-4 days after LPS administration), therefore representing an acute model of the disease useful to study early stages (ie, induction mechanisms) of the disease. In contrast, we cannot evaluate improvement in late events, such as tissue infiltration and damage (fibrosis/collagen deposition). Nevertheless, this MAS model has allowed us to deepen our understanding of the early events that are upstream of the disease development. Indeed, we showed that in livers of mice with MAS, the number of $CD68^+$ macrophages and expression of IL-12, mainly produced by M1 macrophages, was increased compared with that in WT mice.

In conclusion, in this study we found a role for high levels of IL-6 in inducing increased macrophage infiltration and upregulation *of Il12* expression in the liver and demonstrated the pivotal role of IFN-γ in a murine model of MAS. Our data, together with those obtained in animal models of pHLH and of HLH secondary to infections,[17-19,28] support the hypothesis that HLH forms of different origin share a common inflammatory effector pathway involving IFN-γ. Our results also have significant implications for the treatment of patients with MAS. Encouraging preliminary efficacy and safety data of the phase 2 clinical trial in patients with pHLH with the anti–IFN-γ mAb NI-0501 have been reported recently.[41]

Key messages

- **In a murine model of MAS, the IFN-γ pathway is markedly activated in the liver and spleen in the early phase of the disease.**
- **IFN-γ neutralization ameliorates survival and clinical and laboratory features of MAS.**
- **Tissue and circulating levels of the IFN-γ–inducible chemokines CXCL9 and CXCL10 reflect activation of the pathway and might represent biomarkers of disease in human patients with MAS.**

REFERENCES

1. Ravelli A, Grom AA, Behrens EM, Cron RQ. Macrophage activation syndrome as part of systemic juvenile idiopathic arthritis: diagnosis, genetics, pathophysiology and treatment. Genes Immun 2012;13:289-98.
2. Grom AA, Horne A, De Benedetti F. Macrophage activation syndrome in the era of biologic therapy. Nat Rev Rheumatol 2016;12:259-68.
3. Schulert GS, Grom AA. Macrophage activation syndrome and cytokine-directed therapies. Best Pract Res Clin Rheumatol 2014;28:277-92.
4. Freeman HR, Ramanan AV. Review of haemophagocytic lymphohistiocytosis. Arch Dis Child 2011;96:688-93.
5. Schulert GS, Grom AA. Pathogenesis of macrophage activation syndrome and potential for cytokine- directed therapies. Annu Rev Med 2015;66:145-59.
6. Vastert SJ, Prakken BJ. Paediatric rheumatic disease: Diagnosing macrophage activation syndrome in systemic JIA. Nat Rev Rheumatol 2014;10:640-2.
7. Risma K, Jordan MB. Hemophagocytic lymphohistiocytosis: updates and evolving concepts. Curr Opin Pediatr 2012;24:9-15.
8. Strippoli R, Caiello I, De Benedetti F. Reaching the threshold: a multilayer pathogenesis of macrophage activation syndrome. J Rheumatol 2013; 40:761-7.
9. Grom AA, Villanueva J, Lee S, Goldmuntz EA, Passo MH, Filipovich A. Natural killer cell dysfunction in patients with systemic-onset juvenile rheumatoid arthritis and macrophage activation syndrome. J Pediatr 2003;142:292-6.
10. Wulffraat NM, Rijkers GT, Elst E, Brooimans R, Kuis W. Reduced perforin expression in systemic juvenile idiopathic arthritis is restored by autologous stem-cell transplantation. Rheumatology 2003;42:375-9.
11. Zhang M, Behrens EM, Atkinson TP, Shakoory B, Grom AA, Cron RQ. Genetic defects in cytolysis in macrophage activation syndrome. Curr Rheumatol Rep 2014;16:439.
12. Kaufman KM, Linghu B, Szustakowski JD, Husami A, Yang F, Zhang K, et al. Whole-exome sequencing reveals overlap between macrophage activation syndrome in systemic juvenile idiopathic arthritis and familial hemophagocytic lymphohistiocytosis. Arthritis Rheumatol 2014;66:3486-95.
13. Vastert SJ, van Wijk R, D'Urbano LE, de Vooght KM, de Jager W, Ravelli A, et al. Mutations in the perforin gene can be linked to macrophage activation syndrome in patients with systemic onset juvenile idiopathic arthritis. Rheumatology 2010;49: 441-9.
14. Zhang K, Biroschak J, Glass DN, Thompson SD, Finkel T, Passo MH, et al. Macrophage activation syndrome in patients with systemic juvenile idiopathic arthritis is associated with MUNC13-4 polymorphisms. Arthritis Rheum 2008; 58:2892-6.
15. Sepulveda FE, Garrigue A, Maschalidi S, Garfa-Traore M, Menasche G, Fischer A, et al. Polygenic mutations in the cytotoxicity pathway increase susceptibility to develop HLH immunopathology in mice. Blood 2016;127:2113-21.
16. Xu XJ, Tang YM, Song H, Yang SL, Xu WQ, Zhao N, et al. Diagnostic accuracy of a specific cytokine pattern in hemophagocytic lymphohistiocytosis in children. J Pediatr 2012;160:984-90.e1.
17. Jordan MB, Hildeman D, Kappler J, Marrack P. An animal model of hemophagocytic lymphohistiocytosis (HLH): CD8+ T cells and interferon gamma are essential for the disorder. Blood 2004;104:735-43.
18. Pachlopnik Schmid J, Ho CH, Chretien F, Lefebvre JM, Pivert G, Kosco-Vilbois M, et al. Neutralization of IFNgamma defeats haemophagocytosis in LCMV-infected perforin- and Rab27a-deficient mice. EMBO Mol Med 2009;1:112-24.
19. Behrens EM, Canna SW, Slade K, Rao S, Kreiger PA, Paessler M, et al. Repeated TLR9 stimulation results in macrophage activation syndrome-like disease in mice. J Clin Invest 2011;121:2264-77.
20. Strippoli R, Carvello F, Scianaro R, De Pasquale L, Vivarelli M, Petrini S, et al. Amplification of the response to Toll-like receptor ligands by prolonged exposure to interleukin-6 in mice: implication for the pathogenesis of macrophage activation syndrome. Arthritis Rheum 2012;64:1680-8.
21. De Benedetti F, Brunner HI, Ruperto N, Kenwright A, Wright S, Calvo I, et al. Randomized trial of tocilizumab in systemic juvenile idiopathic arthritis. N Engl J Med 2012;367:2385-95.
22. de Benedetti F, Martini A. Targeting the interleukin-6 receptor: a new treatment for systemic juvenile idiopathic arthritis? Arthritis Rheum 2005;52:687-93.
23. De Benedetti F, Alonzi T, Moretta A, Lazzaro D, Costa P, Poli V, et al. Interleukin 6 causes growth impairment in transgenic mice through a decrease in insulin-like growth factor-I. A model for stunted growth in children with chronic inflammation. J Clin Invest 1997;99:643-50.
24. Hu X, Ivashkiv LB. Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases. Immunity 2009;31: 539-50.
25. Schindler C, Levy DE, Decker T. JAK-STAT signaling: from interferons to cytokines. J Biol Chem 2007;282:20059-63.
26. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 2003;3:133-46.
27. Canna SW, Wrobel J, Chu N, Kreiger PA, Paessler M, Behrens EM. Interferon-gamma mediates anemia but is dispensable for fulminant toll-like receptor 9-induced macrophage activation syndrome and hemophagocytosis in mice. Arthritis Rheum 2013;65:1764-75.
28. Buatois V, Chatel L, Cons L, Lory S, Richard F, Guilhot F, et al. Use of a mouse model to identify a blood biomarker for IFNgamma activity in pediatric secondary hemophagocytic lymphohistiocytosis. Transl Res 2017;180:37-52.e2.
29. Zoller EE, Lykens JE, Terrell CE, Aliberti J, Filipovich AH, Henson PM, et al. Hemophagocytosis causes a consumptive anemia of inflammation. J Exp Med 2011;208:1203-14.
30. Reinhardt RL, Liang HE, Bao K, Price AE, Mohrs M, Kelly BL, et al. A novel model for IFN-gamma-mediated autoinflammatory syndromes. J Immunol 2015; 194:2358-68.
31. Avau A, Mitera T, Put S, Put K, Brisse E, Filtjens J, et al. Systemic juvenile idiopathic arthritis-like syndrome in mice following stimulation of the immune system with Freund's complete adjuvant: regulation by interferon-gamma. Arthritis Rheumatol 2014;66:1340-51.
32. Brisse E, Imbrechts M, Put K, Avau A, Mitera T, Berghmans N, et al. Mouse cytomegalovirus infection in BALB/c mice resembles virus-associated secondary hemophagocytic lymphohistiocytosis and shows a pathogenesis distinct from primary hemophagocytic lymphohistiocytosis. J Immunol 2016; 196:3124-34.
33. Tesi B, Sieni E, Neves C, Romano F, Cetica V, Cordeiro AI, et al. Hemophagocytic lymphohistiocytosis in 2 patients with underlying IFN-gamma receptor deficiency. J Allergy Clin Immunol 2015;135:1638-41.
34. Takada H, Takahata Y, Nomura A, Ohga S, Mizuno Y, Hara T. Increased serum levels of interferon-gamma-inducible protein 10 and monokine induced by gamma interferon in patients with haemophagocytic lymphohistiocytosis. Clin Exp Immunol 2003;133:448-53.
35. Billiau AD, Roskams T, Van Damme-Lombaerts R, Matthys P, Wouters C. Macrophage activation syndrome: characteristic findings on liver biopsy illustrating the key role of activated, IFN-gamma-producing lymphocytes and IL-6- and TNF-alpha-producing macrophages. Blood 2005;105:1648-51.
36. Put K, Avau A, Brisse E, Mitera T, Put S, Proost P, et al. Cytokines in systemic juvenile idiopathic arthritis and haemophagocytic lymphohistiocytosis: tipping the balance between interleukin-18 and interferon-gamma. Rheumatology 2015; 54:1507-17.
37. Bracaglia C, de Graaf K, Pires Marafon D, Guilhot F, Ferlin W, Prencipe G, et al. Elevated circulating levels of interferon-gamma and interferon-gamma-induced

chemokines characterise patients with macrophage activation syndrome complicating systemic juvenile idiopathic arthritis. Ann Rheum Dis 2017;76:166-72.
38. Fall N, Barnes M, Thornton S, Luyrink L, Olson J, Ilowite NT, et al. Gene expression profiling of peripheral blood from patients with untreated new-onset systemic juvenile idiopathic arthritis reveals molecular heterogeneity that may predict macrophage activation syndrome. Arthritis Rheum 2007;56:3793-804.
39. Sikora KA, Fall N, Thornton S, Grom AA. The limited role of interferon-gamma in systemic juvenile idiopathic arthritis cannot be explained by cellular hyporesponsiveness. Arthritis Rheum 2012;64:3799-808.
40. Ogilvie EM, Khan A, Hubank M, Kellam P, Woo P. Specific gene expression profiles in systemic juvenile idiopathic arthritis. Arthritis Rheum 2007;56:1954-65.
41. Jordan M, Allen C, De Benedetti F, Grom AA, Ballabio M, Ferlin WG, et al. A novel targeted approach to the treatment of hemophagocytic lymphohistiocytosis (HLH) with an anti-interferon gamma (IFNγ) monoclonal antibody (mAb), NI-0501: First Results from a Pilot Phase 2 Study in Children with Primary HLH. Presented at: 57th ASH Annual Meeting & Exposition LBA-3; Orlando, FL, December 5-8, 2015.