# IL-2 consumption by highly activated CD8 T cells induces regulatory T-cell dysfunction in patients with hemophagocytic lymphohistiocytosis

Stéphanie Humblet-Baron, MD, PhD,[a,b]* Dean Franckaert, MSc,[a,b]* James Dooley, MSc,[a,b] Simon Bornschein, MSc,[a,b] Bénédicte Cauwe, PhD,[a,b] Susann Schönefeldt, MSc,[a,b] Xavier Bossuyt, MD, PhD,[b,c] Patrick Matthys, PhD,[e] Frédéric Baron, MD, PhD,[f] Carine Wouters, MD, PhD,[c,d] and Adrian Liston, PhD[a,b] *Leuven and Liege, Belgium*

**Background: Hemophagocytic lymphohistiocytosis (HLH) is a severe inflammatory condition driven by excessive CD8$^+$ T-cell activation. HLH occurs as both acquired and familial hemophagocytic lymphohistiocytosis (FHL) forms. In both conditions, a sterile or infectious trigger is required for disease initiation, which then becomes self-sustaining and life-threatening. Recent studies have attributed the key distal event to excessive IFN-γ production; however, the proximal events driving immune dysregulation have remained undefined.**
**Objective: We sought to investigate the role of regulatory T (Treg) cells in the pathophysiology of experimental FHL.**
**Methods: Because mutation in perforin is a common cause of FHL, we used an experimental FHL mouse model in which disease in perforin-deficient mice is triggered by lymphocytic choriomeningitis virus (LCMV). We assessed Treg and CD8$^+$ T-cell homeostasis and activation during the changing systemic conditions in the mice. In addition, human blood samples were collected and analyzed during the HLH episode.**
**Results: We found no primary Treg cell defects in perforin-deficient mice. However, Treg cell numbers collapsed after LCMV inoculation. The collapse of Treg cell numbers in LCMV-triggered perforin-deficient, but not wild-type, mice was accompanied by the combination of lower IL-2 secretion by conventional CD4$^+$ T cells, increased IL-2 consumption by activated CD8$^+$ T cells, and secretion of competitive soluble CD25. Moreover low Treg cell numbers were observed in untreated patients experiencing HLH flares.**
**Conclusion: These results demonstrate that excessive CD8$^+$ T-cell activation rewires the IL-2 homeostatic network away from Treg cell maintenance and toward feed-forward inflammation. These results also provide a potential mechanistic pathway for the progression of infectious inflammation to persistent inflammation in patients with HLH. (J Allergy Clin Immunol 2016;138:200-9.)**

***Key words:*** *Regulatory T cells, familial hemophagocytic lymphohistiocytosis, IL-2, immune homeostasis, perforin, lymphocytic choriomeningitis virus*

Hemophagocytic lymphohistiocytosis (HLH) is a severe inflammatory immune syndrome characterized by prolonged fever with hepatosplenomegaly, cytopenia, hepatitis, and neurologic manifestations.[1,2] The principal immunologic features of this syndrome are uncontrolled expansion of CD8$^+$ cytotoxic T cells, activation of antigen-presenting cells/macrophages (histiocytes), natural killer cell dysfunction, and florid cytokine storm, including high amounts of IFN-γ and TNF-α. Clinical manifestations are a consequence of hypercytokinemia and infiltration by activated lymphocytes and histiocytes of the bone marrow, spleen, liver, and central nervous system.[1,2]

Familial hemophagocytic lymphohistiocytosis (FHL) is caused by genetic deficiency in the cytotoxic pathways of T and natural killer cells, with mutations in the genes encoding perforin *(Prf1)* or the perforin secretion components Munc13-4, Munc18-2, or Syntaxin 11 accounting for most cases.[2] A secondary form of acquired HLH can also arise after a broad variety of initiators, including infections, malignancies, or autoimmune diseases, with a specific condition called macrophage activation syndrome (MAS) mainly diagnosed in patients with systemic juvenile idiopathic arthritis.[1,2]

Despite progress in our understanding of HLH pathogenesis, treatment of HLH has remained unsatisfactory. In addition to treating the trigger event, current treatments for HLH are based on highly immunosuppressive drugs and chemotherapy, whereas allogeneic hematopoietic stem cell transplantation can be considered in patients with FHL and severe forms of HLH.[3]

Preclinical models for studying FHL have been developed using lymphocytic choriomeningitis virus (LCMV)–triggering inflammation in perforin-deficient mice.[4,5] These murine models recapitulate the key components of human disease, including life-threatening inflammation, cytopenia, cytokine storm, and

From [a]the Translational Immunology Laboratory, VIB, Leuven; [b]the Department of Microbiology and Immunology and [e]the Rega Institute, KUL–University of Leuven; [c]University Hospitals Leuven; [d]Pediatric Immunology, KUL–University of Leuven; and [f]GIGA I$^3$ and the Department of Hematology, University of Liège.
*These authors contributed equally to this work.
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Corresponding author: Adrian Liston, PhD, Autoimmune Genetics Laboratory, VIB and Department of Microbiology and Immunology, University of Leuven, Leuven, Belgium. E-mail: adrian.liston@vib.be.

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*Abbreviations used*
CTLA4: Cytotoxic T lymphocyte–associated antigen 4
FHL: Familial hemophagocytic lymphohistiocytosis
Foxp3: Forkhead box protein 3
HLH: Hemophagocytic lymphohistiocytosis
IPEX: Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
LCMV: Lymphocytic choriomeningitis virus
MAS: Macrophage activation syndrome
*Prf1*: Perforin gene
sCD25: Soluble CD25
STAT5: Signal transducer and activator of transcription 5
Treg: Regulatory T

immune cell infiltration in different organs, including the liver and central nervous system. Specifically, these studies helped to uncover the pathophysiology of FHL, demonstrating that both $CD8^+$ T cells and IFN-γ are the principal terminal effectors of the disease. In addition, sustained antigen presentation has been reported to be essential to trigger $CD8^+$ activation,[6] and most patients with FHL have a history of viral infections acting as triggers. However, current models do not explain one of the most perplexing aspects of the clinical presentation of HLH, namely that some patients are given a diagnosis after the (presumably) viral trigger has been cleared, with self-perpetuating sterile inflammation developing. In addition, *in utero* FHL diseases have been described,[7,8] again indicating the capacity for the disease to be noncontingent on persistent infection. This observation indicates that we are currently still missing a key step in the pathogenesis of HLH, namely how excessive IFN-γ production and $CD8^+$ T-cell activation during a viral infection can continue and progress into a persistent hyperinflammatory state independent of viral burden. This process suggests that excessive $CD8^+$ T-cell activation might require additional proximal events to develop into the relentless inflammatory state of HLH.

One potential player in the pathogenic process of HLH might be regulatory T (Treg) cells.[9] Treg cells have a unique suppressive function in the immune system, a function imparted by the transcription factor forkhead box protein P3 (Foxp3). Mutations in *FOXP3* cause a fatal autoimmune and inflammatory disorder in both human subjects (immune dysregulation, polyendocrinopathy, enteropathy, X-linked [IPEX] syndrome) and mice (Scurfy mice). In addition, defects in Treg cell homeostasis promote autoimmunity and inflammation in patients with multiple other disorders,[10,11] demonstrating the vital role of Treg cells in preventing systemic inflammation. Here, we sought to determine whether Treg cells were involved in the malfunction of immune suppression that allows defective antiviral immunity to progress to fatal systemic inflammation in patients with FHL. Using LCMV-driven inflammation in perforin-deficient mice, we demonstrate that excessive activation of $CD8^+$ T cells during FHL creates an IL-2–limiting environment and reverses the IL-2 consumption hierarchy. These processes drive a collapse of the Treg cell population in both mice and patients and provide potential mechanistic explanation for the progression of infectious inflammation into the persistent systemic inflammation characteristic of HLH.

## METHODS

### Patients

Diagnostic data were obtained from patients with FHL/MAS during routine clinical care at UZ Leuven. Neonatal patients were excluded from analysis. Of the 7 patients identified during active FHL/MAS, 3 had not received any treatment related to an FHL/MAS episode at the time of the analysis, whereas 4 other patients had already started corticosteroids, cyclosporine treatment, or both. Written informed consent was obtained from each patient. The Ethics Committee of the University of Leuven and University Hospital approved the consent form and the current research study.

### Mice

Perforin-deficient (C57BL/6-*Prf1*$^{tm1Sdz}$/J, Jackson Laboratory, Bar Harbor, Me) and C57BL/6 controls (Charles River, Wilmington, Mass) were analyzed at 8 to 12 weeks of age. Perforin-deficient mice were backcrossed to the *Cd127*$^{Cre/wt}$*Mcl1*$^{fl/wt}$ background[12,13] for use of the huCD4 reporter. Mice were maintained in specific pathogen-free facilities at the University of Leuven. All experiments were approved by the University of Leuven and the University of Liège Animal Ethics Committee.

### Virus and infection

LCMV-Armstrong was produced and titrated, as previously described.[14] Mice were infected with $10^5$ plaque-forming units of LCMV-Armstrong intraperitoneally on day 0 and were monitored and analyzed at day 10 after infection, unless otherwise specified. A subset of mice were administered murine IL-2/anti–IL-2 immune complex (1.5 μg/15 μg per mouse, JES6-1A12 clone; eBioscience, San Diego, Calif) intraperitoneally every other day starting on day 0 with LCMV injection.

### Mouse blood analysis

Mouse blood samples were analyzed with the CELL-DYN 3700 (Abbott, Abbott Park, Ill) to determine red cell and platelet counts, as well as hematocrit and hemoglobin contents.

### Flow cytometry

Single-cell suspensions were prepared from mouse spleens and pooled lymph nodes (cervical, inguinal, mesenteric, axillary, and brachial). For intracellular cytokine staining, lymphocytes were plated at $5 \times 10^5$ cells/well in 96-well tissue-culture plates in complete RPMI containing phorbol 12-myristate 13-acetate (50 ng/mL; Sigma-Aldrich, St Louis, Mo), ionomycin (250 ng/mL, Sigma-Aldrich), and monensin (1:1500; BD Bioscience, San Jose, Calif) for 4 hours at 37°C. All cells were fixed with BD Cytofix (BD Biosciences) or fixed and permeabilized with the eBioscience Foxp3 staining kit (eBioscience). For phospho–signal transducer and activator of transcription 5 (STAT5) staining, lymphocytes were plated at $5 \times 10^6$ cells/well in 96-well tissue-culture plates in complete RPMI containing murine IL-2 (100 ng/mL, eBioscience) and stimulated for 30 minutes before fixation with IC Fixation Buffer (eBioscience) and methanol. Anti-murine antibodies included anti-CD4 (RM4-5), anti-CD8a (53-6.7), anti-FoxP3 (FJK-16s), anti-CD25 (PC61.5), anti–cytotoxic T lymphocyte–associated antigen 4 (CTLA4; UC10-4B9), anti-CD69 (H1.2F3), anti-CD103 (2E7), anti-CD44 (IM7), anti-CD62L (MEL-14), anti–IL-2 (JES6-5H4), anti–IFN-γ (XMG1.2), and anti–phospho-STAT5 (SRBCZX) from eBioscience and anti-Ki67 (B56) from BD Biosciences. Anti-human CD4 staining was performed with BV-421 (OKT4; BioLegend, San Diego, Calif). Human peripheral blood was stained with anti-human antibodies, including anti-CD4 (5K3), anti-CD25 (2A3), and anti-CD127 (hIL-7R-M21). Data were collected on a BD FACSCanto II (BD Biosciences) and analyzed with FlowJo software for Mac, version 9.6 (TreeStar, Ashland, Ore).

### $CD8^+$ *in vitro* stimulation

Purified $CD8^+$ T cells were isolated from spleens and lymph nodes of wild-type and IFN-γ–deficient mice (Jackson Laboratory) by using untouched