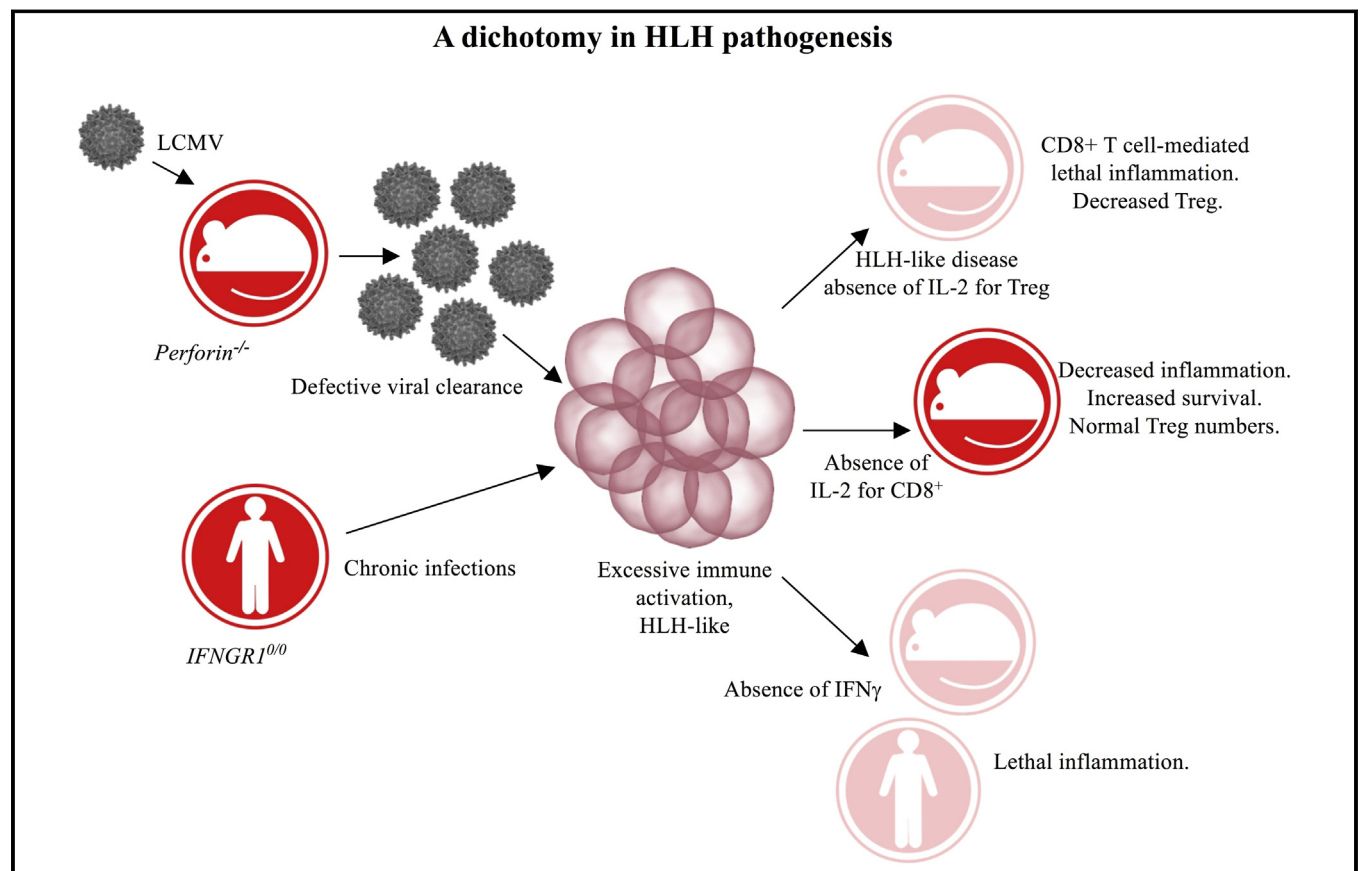


IFN- γ and CD25 drive distinct pathologic features during hemophagocytic lymphohistiocytosis

Stéphanie Humblet-Baron, MD, PhD,^{a,b,*} Dean Franckaert, PhD,^{a,b,*} James Dooley, PhD,^{a,b} Fatima Ailal, MD,^c Aziz Bousfiha, MD,^c Caroline Deswarte, MSc,^{d,e} Carmen Oleaga-Quintas, MSc,^{d,e} Jean-Laurent Casanova, MD, PhD,^{d,e,f,g,h} Jacinta Bustamante, MD, PhD,^{d,e,f,i} and Adrian Liston, PhD^{a,b}
Leuven, Belgium, Casablanca, Morocco, Paris, France, and New York, NY

GRAPHICAL ABSTRACT



From ^athe VIB Center for Brain & Disease Research, Leuven; ^bKU Leuven–University of Leuven, Department of Microbiology and Immunology, Leuven; ^cthe Clinical Immunology Unit, Casablanca Children's Hospital, Ibn Rochd Laboratoire LICIA d'Immunologie Clinique, Inflammation et Allergie, Medical School, Hassan II University, Casablanca; ^dthe Laboratory of Human Genetics of Infectious Diseases, Necker Branch, INSERM U1163, Imagine Institute, Necker Hospital for Sick Children, Paris; ^eParis Descartes University, Paris; ^fthe St Giles Laboratory of Human Genetics of Infectious Diseases, Rockefeller Branch, Rockefeller University, New York; ^gHoward Hughes Medical Institute, New York; and ^hthe Pediatric Hematology-Immunology Unit and ⁱthe Center for the Study of Primary Immunodeficiencies, Assistance Publique-Hôpitaux de Paris AP-HP, Necker Hospital for Sick Children, Paris.

*These authors contributed equally to this work as first authors.

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Corresponding author: Adrian Liston, PhD, Autoimmune Genetics Laboratory, VIB, Leuven, Belgium, and the Department of Microbiology and Immunology, University of Leuven, Herestraat 49, 3000 Leuven, Belgium. E-mail: adrian.liston@gmail.com. 0091-6749/\$36.00

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Background: Inflammatory activation of CD8⁺ T cells can, when left unchecked, drive severe immunopathology. Hyperstimulation of CD8⁺ T cells through a broad set of triggering signals can precipitate hemophagocytic lymphohistiocytosis (HLH), a life-threatening systemic inflammatory disorder.

Objective: The mechanism linking CD8⁺ T-cell hyperactivation to pathology is controversial, with excessive production of IFN- γ and, more recently, excessive consumption of IL-2, which are proposed as competing hypotheses. We formally tested the proximal mechanistic events of each pathway in a mouse model of HLH.

Methods: In addition to reporting a complete autosomal recessive IFN- γ receptor 1-deficient patient with multiple aspects of HLH pathology, we used the mouse model of perforin (*Prf1*)^{KO} mice infected with lymphocytic choriomeningitis virus to genetically eliminate either IFN- γ production or CD25 expression and assess the immunologic, hematologic, and physiologic disease measurement.

Results: We found a striking dichotomy between the mechanistic basis of the hematologic and inflammatory components of CD8⁺ T cell-mediated pathology. The hematologic features of HLH were completely dependent on IFN- γ production, with complete correction after loss of IFN- γ production without any role for CD8⁺ T cell-mediated IL-2 consumption. By contrast, the mechanistic contribution of the immunologic features was reversed, with no role for IFN- γ production but substantial correction after reduction of IL-2 consumption by hyperactivated CD8⁺ T cells. These results were complemented by the characterization of an IFN- γ receptor 1-deficient patients with HLH-like disease, in whom multiple aspects of HLH pathology were observed in the absence of IFN- γ signaling.

Conclusion: These results synthesize the competing mechanistic models of HLH pathology into a dichotomous pathogenesis driven through discrete pathways. A holistic model provides a new paradigm for understanding HLH and, more broadly, the consequences of CD8⁺ T-cell hyperactivation, thereby paving the way for clinical intervention based on the features of HLH in individual patients. (J Allergy Clin Immunol 2019;■■■:■■■-■■■.)

Key words: IFN- γ , CD25, CD8⁺ T-cell hyperactivation, hemophagocytic lymphohistiocytosis

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening systemic inflammatory disorder. The inflammation in this disease is characterized by excessive activation of the immune system (mainly cytotoxic T cells and macrophages) and a cytokine storm (including IFN- γ , IL-6, and TNF- α). Clinical manifestations include prolonged fever, cytopenia, hepatosplenomegaly, hepatitis, and, in some patients, neurologic symptoms. The classical presentation can lead to patient death caused by uncontrolled inflammation, multiorgan failure, or secondary severe infection, if left untreated.¹⁻³ Successful treatment requires drastic clinical intervention through steroids, etoposide, and immunosuppressive reagents, with only a 50% to 60% survival rate.⁴⁻⁶

HLH is a broad clinical term encompassing a diverse set of inflammatory contexts mediated by CD8⁺ T-cell hyperactivation.

Abbreviations used

AR:	Autosomal recessive
DKO:	Double knockout
EBV-B cell:	EBV-transformed B lymphocyte
HLH:	Hemophagocytic lymphohistiocytosis
IFN- γ R1:	IFN- γ receptor 1
JAK:	Janus kinase
LCMV:	Lymphocytic choriomeningitis virus
NK:	Natural killer
sCD25:	Soluble CD25
STAT:	Signal transducer and activator of transcription
Treg:	Regulatory T

Primary HLH refers to patients with severe inherited defects in genes related to the cytotoxic pathways of T and natural killer (NK) cells or in genes involved in EBV clearance, with viral infection frequently serving as a trigger for clinical manifestation.⁷ When a genetic cause is established, allogeneic hematopoietic stem cell transplantation is the only curative treatment. Secondary or acquired HLH encompasses patients who have the syndrome as a consequence of escalating CD8⁺ T-cell responses against infections (mainly after EBV and cytomegalovirus but also after bacterial, fungal, or parasitic infections), malignancies (mainly lymphomas), or autoimmune targets, such as occurs in patients with systemic juvenile idiopathic arthritis or systemic lupus erythematosus.^{1,3,5,8} Thus HLH serves as the archetype for understanding the pathology mediated by hyperactivation of CD8⁺ T cells within a general clinical context. The poor prognosis of patients with HLH, even when given the best current treatment, indicates that our understanding of the molecular basis of this disease is unsatisfactory.

HLH is a complex disease comprising 2 main features with both immunologic manifestations, including cytotoxic T-cell activation and cytokine storm, and hematologic manifestations, including severe anemia. In addition, hemophagocytosis describes the presence of activated macrophages able to engulf blood cells, and its presence has been reported to be a direct contributor to anemia.⁹ Whether the immunologic and hematologic aspects of the disease share a common mechanistic basis or independently arise from distinct molecular mechanisms has not been explored to date.

One of the key developments in the understanding of HLH has been the development of a mouse model that recapitulates the key features of patients. The mouse model uses perforin (*Prf1*)^{KO} mice, replicating the frequent *PRF1* mutation in patients with primary HLH. *Prf1*^{KO} mice are healthy in the baseline state; however, when infected with lymphocytic choriomeningitis virus (LCMV), *Prf1*^{KO} mice have many of the same hematologic and immunologic characteristics of HLH, with severe anemia and fatal inflammation.^{10,11} Analysis of patients with HLH and the HLH mouse model has been used to develop a working model for HLH pathogenesis. In this model defects in cytotoxicity result in greater viral titers and an inability to clear antigen-presenting cells, leading to extended immune stimulation and increased activation of CD8⁺ T cells.¹² These CD8⁺ T cells then have amplified expression of cytokines that further precipitate systemic inflammation, including activation of other immune cells, especially macrophages, creating a cytokine storm.¹¹

IFN- γ is thought to be the most critical cytokine in HLH pathology, with both the hematologic and inflammatory aspects of the disease attributed to its excessive production.^{9,11,13,14} However, the role of IFN- γ has been recently challenged, with the findings of HLH in patients with genetic defects in the IFN- γ pathway.^{15–17} Specifically, 3 patients have been reported to have HLH disease and an IFN- γ R defect, although among these, only 1 patient received a definitive diagnosis of a complete absence of IFN- γ signaling through IFN- γ R2, whereas the other 2 patients had either a partial or implied defect of IFN- γ receptor 1 (IFN- γ R1).^{15,16} Supporting the IFN- γ independence of disease, development of systemic juvenile idiopathic arthritis–like inflammation has been described in *Irfng* knockout mice.^{18,19} Finally, gene expression profiling of PBMCs from children with untreated HLH did not identify differential expression of IFN- γ or IFN- γ -regulated genes as an HLH signature.²⁰

Recently, we proposed an additional alternative model of HLH pathogenesis.²¹ CD8⁺ T cells from LCMV-infected *Prf1*^{KO} mice not only exhibit hyperproduction of IFN- γ but also manifest extreme expression levels of IL-2 receptor α (CD25). Enhanced consumption of IL-2 by activated CD8⁺ T cells is accompanied by a collapse in regulatory T (Treg) cell numbers,²¹ a feature that could account for the feed-forward inflammatory loop observed in patients with HLH. Although these models are not mutually exclusive, resolution of this controversy is of great importance because a clinical trial using anti-IFN- γ antibody in patients with HLH is currently ongoing.²²

Here we sought to formally test the competing hypotheses for the pathologic mechanisms of CD8⁺ T-cell hyperstimulation in patients with HLH. In addition to reporting a complete autosomal recessive (AR) IFN- γ R1–deficient patient with multiple aspects of HLH pathology, we used the mouse model of *Prf1*^{KO} mice infected with LCMV to genetically eliminate either IFN- γ production or CD25 expression and assess immunologic, hematologic, and physiologic disease measurement. We found a striking dichotomy between the hematologic and inflammatory components of HLH pathology. IFN- γ production during HLH was essential for the hematologic features, with loss of IFN- γ completely correcting anemia but having no effect on immune activation, wasting, and survival. By contrast, restricting IL-2 consumption by CD8⁺ T cells had no effect on the hematologic features of HLH but restored Treg cell numbers, suppressed the hyperinflammatory state, and extended the lifespan. These results provide a paradigm shift for understanding the consequences of CD8⁺ T-cell hyperactivation, with the 2 sets of symptoms (inflammatory and hematologic) driven through dichotomous pathways that together drive the HLH clinical presentation.

METHODS

Patients

Clinical data were obtained from a patient with HLH symptoms during clinical care in Casablanca, Morocco. Further diagnostic investigation was performed at the Laboratory of Human Genetics of Infectious Diseases, INSERM U1163, Imagine Institute, in accordance with local regulations, and with the approval of the Institutional Review Board of Necker Hospital for Sick Children, Paris, France. Written informed consent was obtained from the patient's family.

Mice

Perforin-deficient (C57BL/6-*Prf1*^{tm1Sdz/J}),²³ IFN- γ -deficient (C57BL/6-*Irfng*^{tm1Ts}),²⁴ and CD8-Cre transgenic (C57BL/6-Tg[Cd8a-cre]1Itan/J)²⁵ mice were obtained from the Jackson Laboratory (Bar Harbor, Me) and used on the C57BL/6 background. *CD25*^{KO} mice were generated from EU-COMM embryonic stem cells, with production through blastocyst injection. Transgenic founders were transiently intercrossed with Flpase mice (FLPe)²⁶ to excise the neomycin resistance cassette and convert the allele to a floxed status. The resulting *CD25*^{fl/fl} mice were backcrossed to the C57BL/6 background for more than 8 generations before intercrossing with CD8-Cre mice. All mice were maintained in specific pathogen-free facilities of the University of Leuven. All experiments were approved by the University of Leuven Animal Ethics Committee. All mice were used at 8 to 15 weeks of age.

Virus and infection

LCMV-Armstrong was produced and titrated, as previously described.²⁷ Briefly, the LCMV strain was propagated in baby hamster kidney cells and titrated on Vero African green monkey kidney cells, as previously described.²⁸ At 8 to 12 weeks of age, mice were infected with 10⁵ plaque-forming units of LCMV-Armstrong intraperitoneally (day 0) and monitored and analyzed at day 10 after infection for immunologic and hematologic parameters.

Blood and plasma analysis

Mouse blood samples were analyzed on Cell-Dyn 3700 (Abbott, Lake Forest, Ill) to determine red blood cell and platelet counts, as well as hematocrit and hemoglobin contents. Plasma samples were analyzed with the mouse IL-2R α DuoSet ELISA (R&D Systems, Minneapolis, Minn) and the MSD inflammatory cytokine panel (Meso Scale Diagnostics, Rockville, Md), according to the manufacturers' protocol.

Genomic DNA and whole-exome sequencing

Genomic DNA was extracted from whole blood, and 3 μ g was sent for whole-exome sequencing. Briefly, an adapter-ligated library was prepared with the Paired-End Genomic DNA Sample Prep kit V1 (Illumina, San Diego, Calif). The SureSelect Human All Exon kit (Agilent Technologies, Santa Clara, Calif) was then used for exome capture. Exome capture was performed with the 71Mb SureSelect Human All Exon kit (Agilent Technologies).

In vitro patient cell line analysis

EBV-transformed B lymphocytes (EBV-B cells) were generated from freshly collected PBMCs. IFN- γ R1 expression (phycoerythrin–labeled mAbs specific human or isotype-matched controls; BD Biosciences, San Jose, Calif) on the surfaces of EBV-B cells was determined by means of flow cytometric analysis, as previously described.²⁹ EBV-B cells were stimulated for 30 minutes with IFN- γ (10⁵ IU/mL) or IFN- α (10⁵ IU/mL), and phosphorylated signal transducer and activator of transcription (STAT) 1 (anti-Y701–pSTAT1–Alexa Fluor 468, 612132; BD Transduction Laboratory) was detected by using flow cytometric analysis. Staining was assessed on a Galios cytometer (Beckman Coulter, Fullerton, Calif), and the results were analyzed with FlowJo software (version 10; TreeStar, Ashland, Ore).

Mutation testing

The wild-type *IFNGR1* allele was inserted into V5-topo-pcDNA3.1 (Invitrogen, Carlsbad, Calif). The p.W99R and c.131del mutants were generated by using site-directed mutagenesis (PfuUltra II Fusion HS DNA Polymerase; Agilent Technologies). IFN- γ R1–deficient primary human fibroblasts immortalized with SV40T antigen (SV40 fibroblasts) were transfected with an insertless V5-tagged pcDNA3.1 plasmid (empty plasmid [EV]) and one of the *IFNGR1* V5-tagged pcDNA3.1 plasmids in the presence of X-treme GENE9 transfection reagent (Sigma-Aldrich, St Louis, Mo). Total proteins

were solubilized in extraction buffer, as previously described.³⁰ Samples were run on SDS-PAGE in 10% polyacrylamide gels (Bio-Rad Laboratories, Hercules, Calif), and the resulting bands were transferred onto fluorescence polyvinylidene difluoride membranes (Bio-Rad Laboratories) with the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). Immunoblotting was performed with antibodies against glyceraldehyde-3-phosphate dehydrogenase (14C10; Cell Signaling Technology, Danvers, Mass) and the V5 tag (Thermo Fisher Scientific, Waltham, Mass). Bound antibody was detected with ECL Western blotting detection reagents (Bio-Rad Laboratories) on Chemidoc.

The electrophoretic mobility shift assay was carried out, as previously described.³⁰ Briefly, cells were stimulated for 20 minutes with IFN- γ at 10^4 IU/mL. We then obtained a nuclear extract from these cells and incubated 5 to 10 μ g of this nuclear extract with the IRDye 700 gamma-activating sequence probe, which corresponds to the Fc- γ R1 promoter; subjected the mixture to electrophoresis in a polyacrylamide gel; and read it with the LI-COR Clx machine (LI-COR Biosciences, Lincoln, Neb).

Degranulation assay

Degranulation of human NK cells and CD8⁺ T cells was performed as follows: 3×10^5 PBMCs were incubated for 3 hours *in vitro* alone in the presence of 1:1 K562 cells or with phorbol 12-myristate 13-acetate (50 ng/mL; Sigma-Aldrich) and ionomycin (250 ng/mL; Sigma-Aldrich). Cells were then stained for surface staining, including CD3 (SK7), CD8 (RPA-T8), CD56 (MEM188), CD16 (CB16), CD107a (LAMP-1), CD107b (H4B4), CD94 (HP-3D9) (all from Invitrogen, Thermo Fisher), and CD14 (M5E2; from BioLegend, San Diego, Calif) and analyzed with the FACSCanto II flow cytometer (BD Biosciences).

Histology

Tissues were fixed in 4% paraformaldehyde and continued to trimming and processing for hematoxylin and eosin staining. Images were evaluated on a Nikon Labophot-2 microscope and captured with a Nikon D5000 digital microscopic camera (Nikon, Shinagawa, Japan). Histopathologic analysis was performed blinded and subjectively by BioGenetics Research Laboratories (Greenbank, Wash), as previously described.^{31,32} Briefly, severity score 1 was used to include pathologic findings that showed no significant lesions and normal morphology. Severity score 2 was assigned to all tissues showing minimal (minor and infrequent, <10% of defined area) and mild (noticeable microscopic change but no overt feature, 11% to 20% of defined area involved in case of focal, multifocal, or diffuse effects) findings. Moderate findings, including prominent changes (21% to 40% of defined area for focal, multifocal, or diffuse effects), were assigned severity score 3. Overwhelming microscopic features (41% to 100% for focal, multifocal, or diffuse effects in defined area) were assigned severity grade 4.

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 5×10^5 cells/well in 96-well tissue-culture plates in complete RPMI containing phorbol 12-myristate 13-acetate (50 ng/mL; Sigma-Aldrich), ionomycin (250 ng/mL; Sigma-Aldrich), and monensin (1/1500; BD Biosciences) for 4 hours at 37°C. All cells were fixed with BD Cytotfix (BD Biosciences) or fixed and permeabilized with the eBioscience Foxp3 staining kit (eBioscience, San Diego, Calif). Anti-murine antibodies included anti-CD4 (RM4-5), anti-CD8a (53-6.7), anti-forkhead box P3 (FJK-16s), anti-CD25 (PC61.5), anti-CD127 (A7R34), anti-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), anti-KLRG1 (2F1), anti-programmed cell death protein 1 (J43), anti-pSTAT5 (SRBCZX), and anti-Ki67 (SolA15) from eBioscience. Data were collected on the FACSCanto II (BD Biosciences) and analyzed by using FlowJo software for Macintosh (version 9.6).

Statistics

Statistical analysis was performed with 1-way ANOVA with the Tukey multiple comparisons test. All statistical analyses were carried out with GraphPad Prism software (version 7; GraphPad Software, La Jolla, Calif).

RESULTS

IFN- γ production drives the hematologic, but not immunologic, components of HLH pathology

LCMV infection of *Prf1*^{KO} mice constitutes a robust mouse model of HLH, with hematologic abnormalities, exuberant inflammation, and early mortality.¹¹ Previous studies using genetic deficiency or antibody-mediated neutralization have highlighted the role of IFN- γ in the anemia occurring in LCMV-infected *Prf1*^{KO} mice^{9,11,13,14}; however, the other aspects of HLH pathology have not been assessed. We backcrossed *Prf1*^{KO} mice with *Ifng*^{KO} mice, creating *Prf1*^{KO}*Ifng*^{KO} double-knockout (DKO) mice. LCMV infection of wild-type, *Prf1*^{KO}, and *Prf1*^{KO}*Ifng*^{KO} DKO mice produced a severe disruption of the erythroid cell compartment in *Prf1*^{KO} mice, which was completely corrected in DKO mice (Fig 1, A-C). *Ifng*^{KO} mice, with wild-type perforin, did not exhibit any hematologic abnormalities (Fig 1, A-C). Intriguingly, although the anemia was prevented by IFN- γ deficiency, DKO mice still exhibited liver hemophagocytosis (Fig 1, D and E). These data demonstrate that low red cell counts and hemoglobin and hematocrit values are IFN- γ -dependent pathologies.

Having established the IFN- γ dependency of the hematologic signature of HLH, we next assessed the physiologic and immunologic components of the disease. Unlike wild-type mice, *Prf1*^{KO} mice present with distressed behavior during the late stages of LCMV infection coupled with severe wasting necessitating death at day 10 (Fig 2, A). Despite a complete recovery of hematologic abnormalities, *Prf1*^{KO}*Ifng*^{KO} DKO mice experienced the same distressed behavior and wasting, also requiring death at day 10 (Fig 2, A).

Prf1^{KO} mice demonstrated an enlarged spleen and liver damage after LCMV infection, which was also unaffected by IFN- γ genotype (see Fig E1 in this article's Online Repository at www.jacionline.org). In patients with HLH, one of the primary pathologic events is the infiltration and destruction of tissues, leading to multiorgan failure.^{3,5} Although wild-type and *Ifng*^{KO} mice recovered from LCMV infection without widespread liver damage, *Prf1*^{KO} and *Prf1*^{KO}*Ifng*^{KO} DKO mice both experienced extensive reticuloendothelial cell infiltration (Fig 2, B). Therefore the physiologic manifestations of HLH pathology were not dependent on excessive IFN- γ production.

Previous publications from our group and others have demonstrated a clear correlation between serious weight loss and critical inflammation in the mouse HLH model.^{21,33} Therefore we performed a detailed scrutiny of the immune compartment. First, numbers of CD8⁺ T cells, which are known to be responsible for disease severity,¹¹ were increased in the lymph nodes of *Prf1*^{KO}*Ifng*^{KO} DKO mice to an even greater degree than *Prf1*^{KO} mice (Fig 2, C). In *Prf1*^{KO} mice infected with LCMV, there is not only an increase in the size of the CD8⁺ T-cell compartment but also a marked shift toward an activated phenotype, with high percentages of effector memory subsets (CD8⁺CD44⁺CD62L⁻) and short-lived effector cells (CD8⁺CD44⁺KLRG1⁺CD127⁻), which are both hallmarks of HLH inflammatory conditions

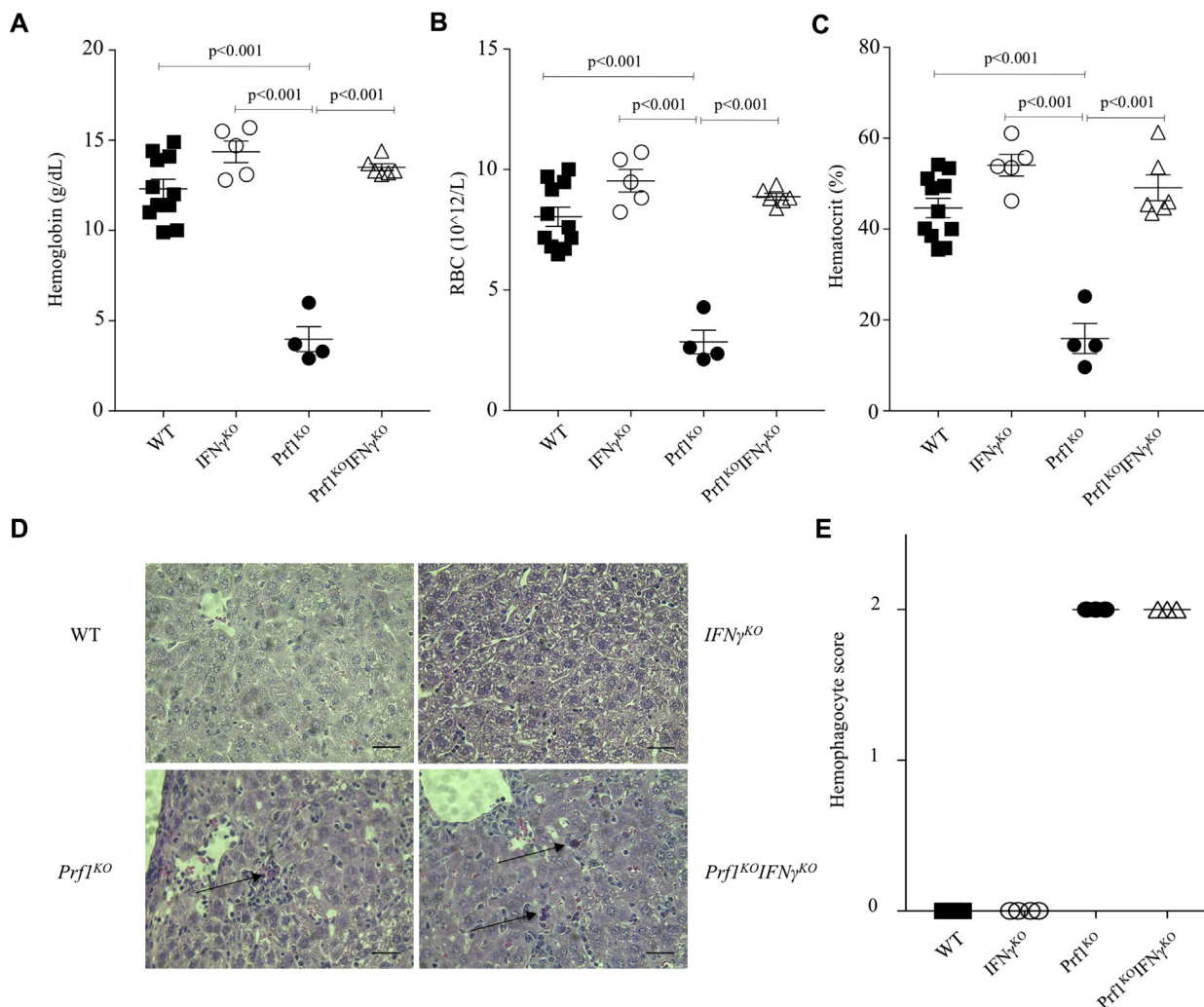


FIG 1. The hematologic component of murine HLH is dependent on excessive IFN- γ production. Wild-type (WT), *Ifng*^{KO}, *Prf1*^{KO}, and *Prf1*^{KO}*Ifng*^{KO} mice were infected with LCMV-Armstrong. **A–C**, On day 10, blood samples from each strain were assessed for hemoglobin values (Fig 1, A), red blood cell counts (Fig 1, B), and hematocrit values (Fig 1, C; n = 11, 5, 4, and 6). Results were pooled from 4 experiments. **D** and **E**, Representative histology from the liver (Fig 1, D) and histologic scoring for hemophagocyte presence in the liver (Fig 1, E) on hematoxylin and eosin-stained sections on day 10 (n = 3, 4, 3, and 3). Arrows indicate hemophagocytes. Scale bar = 50 μ m. Analysis was done with 1-way ANOVA with the Tukey multiple comparisons test; SEMs and individual data points are shown.

(Fig 2, D). *Prf1*^{KO}*Ifng*^{KO} DKO mice, despite the absence of IFN- γ , exhibited the same escalation of these inflammatory mediators as *Prf1*^{KO} mice, well above the levels normally associated with LCMV infection in wild-type or *Ifng*^{KO} mice (Fig 2, D).

Recently, we added exuberant CD25 expression on CD8⁺ T cells to the characteristic hallmarks of murine HLH, a condition associated with Treg cell collapse.²¹ This immunologic manifestation was reproduced here in *Prf1*^{KO} mice, with an identical presentation in *Prf1*^{KO}*Ifng*^{KO} DKO mice. Specifically, CD25 expression as both a percentage (Fig 2, E) and intensity (Fig 2, F–G) were greater on CD8⁺ T cells from LCMV-infected *Prf1*^{KO} and *Prf1*^{KO}*Ifng*^{KO} DKO mice than on LCMV-infected wild-type or *Ifng*^{KO} control mice. Moreover, high levels of soluble CD25 (sCD25) in serum has been used as a diagnostic marker for HLH,^{1,5} and when analyzed in our cohort, sCD25 was found at the highest level in *Prf1*^{KO} and *Prf1*^{KO}*Ifng*^{KO} DKO mice, clearly

emphasizing the presence of systemic inflammation (Fig 2, H). Similar results were seen with CD4⁺ T cells, with increased activation in *Prf1*^{KO} mice that was not ameliorated by the additional loss of IFN- γ (see Fig E2 in this article's Online Repository at www.jacionline.org). These data clearly demonstrate that the immunologic features of HLH arise independent of the expression of IFN- γ , with hyperactivated CD8⁺ T cells still possessing deleterious effects on normal mouse physiology.

Development of HLH in a patient with an absence of IFN- γ signaling

We identified a patient affected by a complete AR IFN- γ R1 deficiency who had multiple HLH diagnostic criteria secondary to severe mycobacterial infection (Fig 3, A). A 2-month-old girl from first-degree consanguineous parents from Morocco was

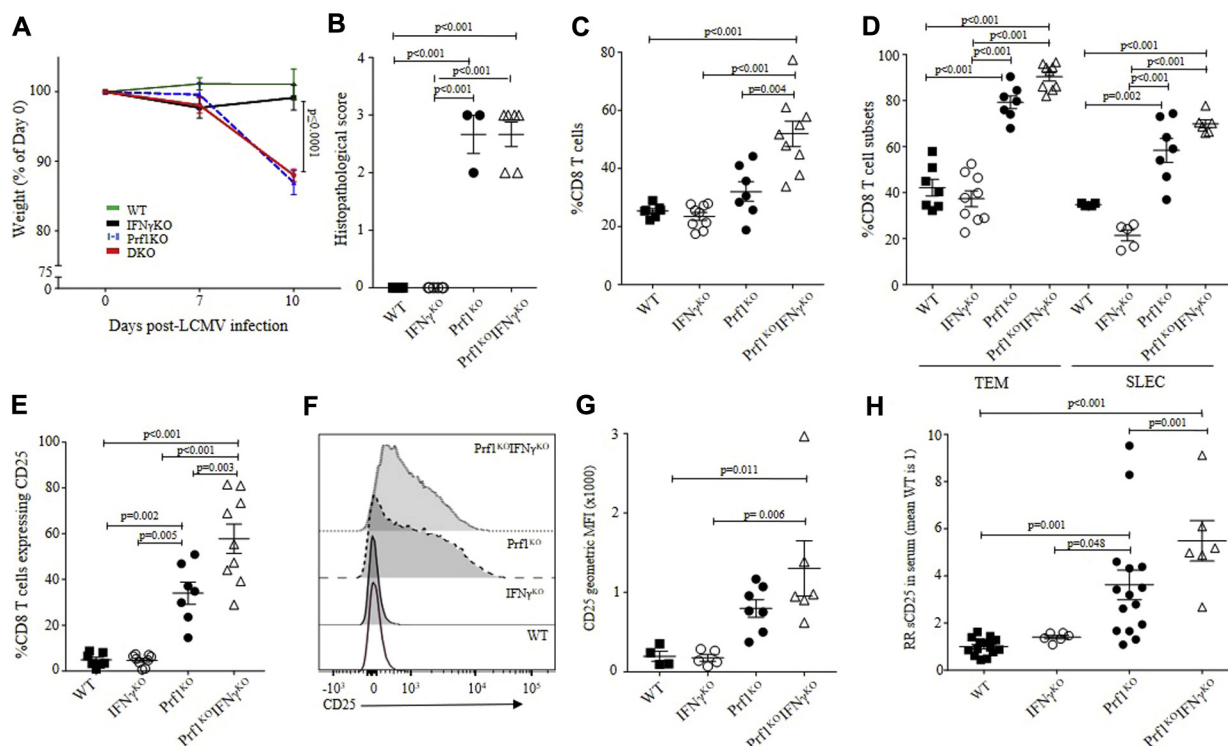


FIG 2. Inflammatory and physiologic aspects of murine HLH arise independent of IFN- γ . Wild-type (WT), *Ifng*^{KO}, *Prf1*^{KO}, and *Prf1*^{KO}/*Ifng*^{KO} mice were infected with LCMV-Armstrong. **A**, Mouse weights on days 0, 7, and 10 after LCMV infection ($n = 7, 9, 7$, and 9). **B**, Histologic scoring for immune cell infiltrates in the liver on hematoxylin and eosin-stained sections on day 10 ($n = 5, 6, 3$, and 6). **C**, Percentage of CD8⁺ T cells in lymph nodes ($n = 7, 9, 7$, and 9). **D**, Percentage of CD8⁺ T cells in CD8⁺CD62L⁺CD44⁺ effector memory (TEM; $n = 7, 9, 7$, and 9) and percentage of activated CD8⁺ T cells in the CD8⁺CD127⁺KLRG1^{high} (short-lived effector cell) subsets ($n = 4, 5, 7$, and 6). **E**, Percentage of CD8⁺ T cells expressing CD25⁺ cells ($n = 7, 9, 7$, and 9). **F**, Representative CD25 staining on CD8⁺ T cells. **G**, CD25 geometric mean fluorescence intensity on total CD8⁺ T cells ($n = 4, 5, 7$, and 6). **H**, Relative ratio of sCD25 in serum (mean WT is 1). Analysis was done with 1-way ANOVA with the Tukey multiple comparisons test; SEMs and individual data points are shown. Results were pooled from 4 experiments (3 experiments for sCD25 measurements).

given a diagnosis of BCG vaccine reactivation with the presence of homolateral lymphadenopathy. There was no improvement despite antibiotherapy. Two months later, her clinical status deteriorated further, and an HLH diagnosis was made in the presence of splenomegaly, high fever, pancytopenia (anemia and thrombocytopenia), hyperferritinemia, and hypertriglyceridemia (see Table E1 in this article's Online Repository at www.jacionline.org). Although her blood showed normal NK cells (in terms of both numbers and percentages according to the patients' age), NK cell function was impaired (see Fig E3 in this article's Online Repository at www.jacionline.org), and samples for sCD25 assessment were not available. Hemophagocytosis was not found in her bone marrow. The patient received antibiotics (ciprofloxacin, amikacin, clarithromycin, rifampicin, isoniazid, and ethambutol), steroids, and cyclosporine and blood transfusions. The patient stabilized under this regimen. Further clinical investigation of the patient was not possible.

With a severe mycobacterial infection at a young age, further investigation regarding the genetic origin of the disease was performed, with suspicion of Mendelian susceptibility to mycobacterial disease (MSMD).³⁴ A rare homozygotic missense mutation, p.W99R, within the *IFNGR1* gene was detected by using whole-exome sequencing. This mutation was confirmed by means of Sanger sequencing. The mother was heterozygous for

this mutation. No DNA sample was available from the father. No mutation in the genes involved in primary HLH was detected (Fig 3, B). Although IFN- γ R1 protein was detected at a level comparable with that in healthy control subjects (Fig 3, C), a further *in vitro* assay demonstrated a complete deficiency in receptor signaling, with an absence of STAT1-Y701 phosphorylation in the patient's EBV-B cells after IFN- γ stimulation *in vitro*, whereas stimulation after IFN- α promoted normal STAT1 phosphorylation (Fig 3, D).

To formally characterize the *IFNGR1* allele, we used an exogenous expression system. IFN- γ R1-deficient SV40 fibroblasts were transiently transfected with wild-type, p.W99R, and c.131del (defined loss of expression allele, responsible of complete AR IFN- γ R1 deficiency) *IFNGR1* cDNA.

As with the analysis of primary cells from patients, the p.W99R allele in this transfection system demonstrated normal levels of protein expression (Fig 3, E). Cells were stimulated with IFN- γ , and gamma-activated factor DNA-binding activity was evaluated by using the electrophoretic mobility shift assay with a gamma-activating sequence probe. SV40 fibroblasts transfected with p.W99R had an abolished response to IFN- γ , similar to negative control values (Fig 3, F). These findings demonstrate that the p.W99R allele is a loss of signaling function allele with normal protein expression. In the patient a high level of IFN- γ on plasma,

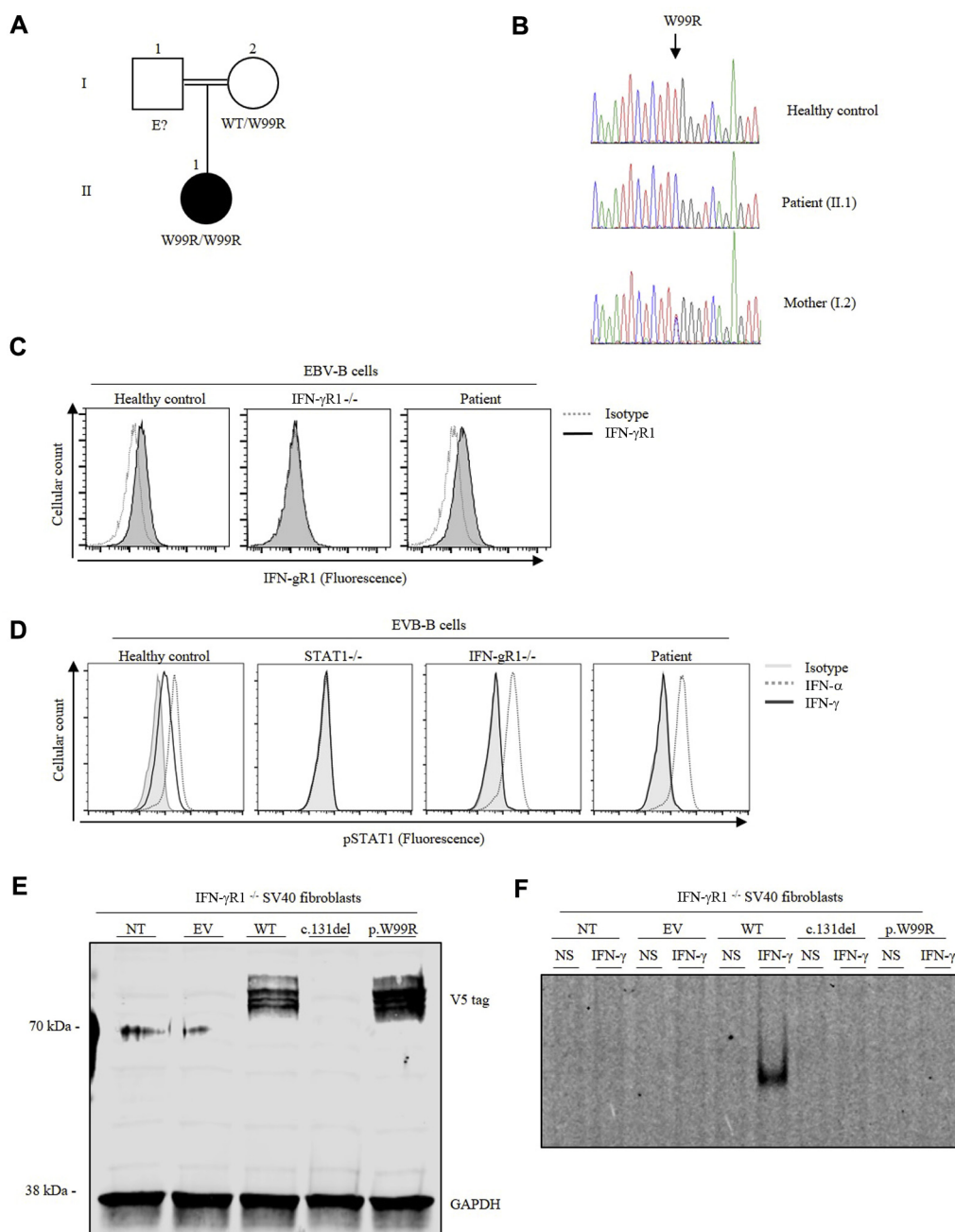


FIG 3. IFN- γ R1 deficiency in a patient with HLH-like disease. **A**, Pedigree of kindred showing the index case and parents; each generation is designated by a Roman numeral (*I* and *II*). No genomic DNA was available for testing for the father (*E?*). **B**, Electropherogram (healthy control subject, the patient [II,1], and her mother) showing the position of the mutation in the *IFNGR1* gene. **C**, Flow cytometric analysis of IFN- γ R1 expression on the surfaces of EBV-B cells from a healthy control subject, the IFN- γ R1-deficient patient, and patient II,1 with W99R mutation. Histograms represent expression of IFN- γ R1 (gray shading) and the isotype control (dashed line). **D**, pSTAT1 response of EBV-B cells to IFN- γ (black line) or IFN- α (dashed line) stimulation in the healthy control subject, the IFN- γ R1-deficient patient, and patient II,1. **E**, IFN- γ R1^{-/-} SV40 fibroblasts were left untransfected (NT) or were transiently transfected with empty vector (EV), WT, c.131del (negative control), or p.W99R mutant *IFNGR1* plasmids. Total lysis and Western blots were performed with an anti-V5 antibody, with glyceraldehyde-3-phosphate dehydrogenase as the loading control. **F**, IFN- γ R1^{-/-} SV40 fibroblasts were left untransfected (NT) or were transiently transfected with EV, WT, c.131del (negative control), and p.W99R mutant *IFNGR1* plasmids and were either left nonstimulated (NS) or stimulated with 10⁵ IU/mL IFN- γ . DNA-binding activity was then analyzed by using the electrophoretic mobility shift assay with a gamma-activating sequence probe. WT, Wild-type.

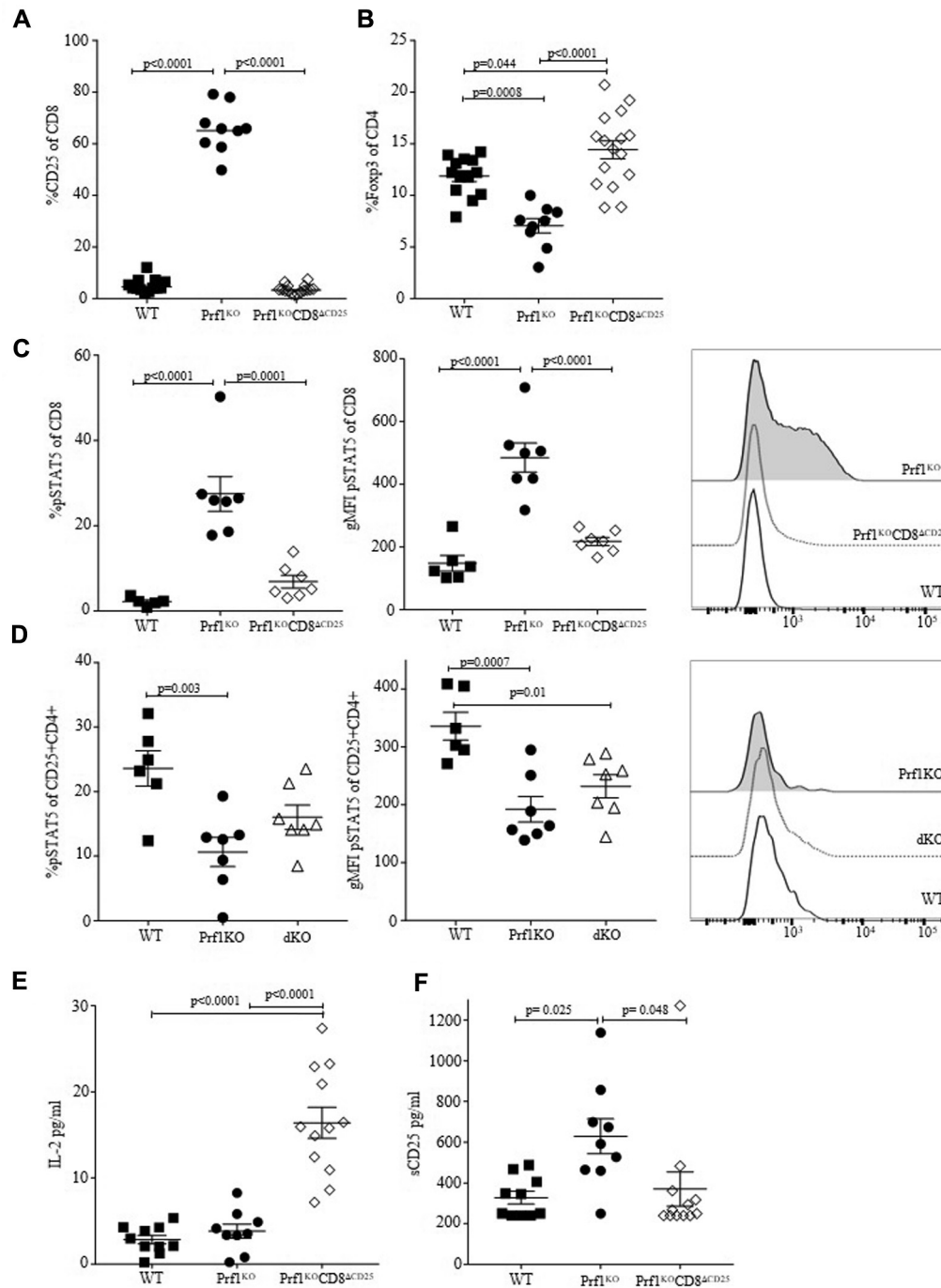


FIG 4. Removal of CD25 expression on CD8⁺ T cells prevents rewiring of IL-2 consumption and the collapse of Treg cells during HLH. Wild-type (WT), Prf1^{KO}, and Prf1^{KO}CD8^ΔCD25 mice were infected with LCMV-Armstrong and assessed on day 10. **A**, Percentage of CD8⁺ T cells expressing CD25 (n = 13, 9, and 16). **B**, Percentage of Treg cells within the CD4⁺ T-cell population (n = 13, 9, and 16). **C**, *left*, Percentage of cells positive for pSTAT5 staining in CD8⁺ T cells; *middle*, geometric mean fluorescence intensity for pSTAT5 staining on CD8⁺ cells; *right*, representative pSTAT5 staining on CD8⁺ T cells (n = 5, 7, and 7). **D**, *left*, Percentage of cells positive for pSTAT5 staining in CD4⁺CD25⁺ Treg cells within the CD4⁺ T-cell population; *middle*, geometric mean fluorescence intensity of pSTAT5 staining on CD4⁺CD25⁺ Treg cells; *right*, representative pSTAT5 staining on CD4⁺Foxp3⁺ Treg cells (n = 6, 7, and 7). **E**, IL-2 concentration in serum (n = 10, 9, and 12). **F**, sCD25 levels in serum (n = 10, 9, and 12). Analysis was done with 1-way ANOVA with the Tukey multiple comparisons test; SEMs and individual data points shown. Results were pooled from 4 experiments (3 experiments for pSTAT5 measurements).

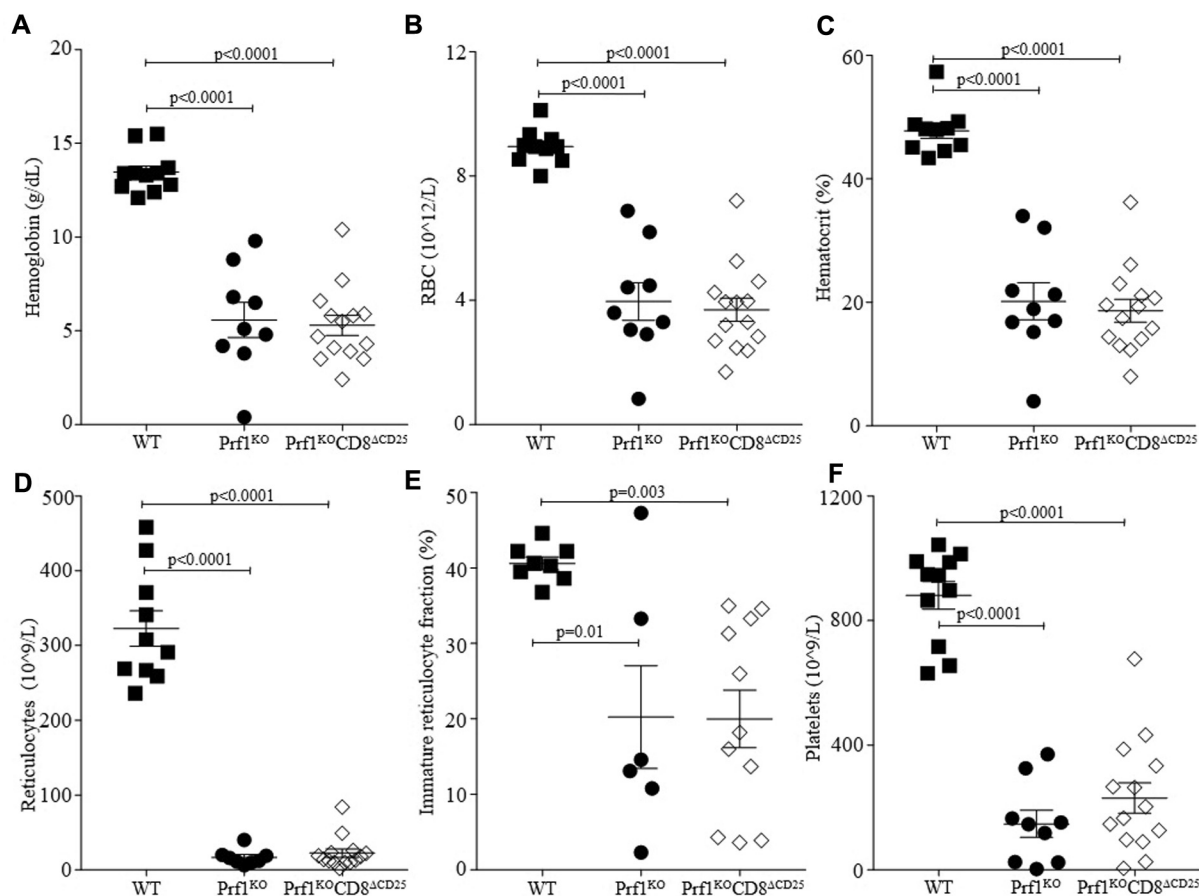


FIG 5. Correction of the IL-2 consumption phenotype of hyperactivated *CD8*⁺ T cells does not affect the hematologic features of patients with HLH. Wild-type (WT), *Prf1*^{KO}, and *Prf1*^{KO} *CD8*^{ΔCD25} mice were infected with LCMV-Armstrong. On day 10, blood samples from each strain were assessed for hemoglobin values (**A**), red blood cell counts (**B**) (n = 10, 9, and 14), hematocrit values (**C**) (n = 11, 9, and 14), reticulocyte counts (**D**) (n = 10, 8, and 14), immature reticulocyte fraction (**E**) (n = 8, 6, and 11), and platelet counts (**F**) (n = 11, 9, and 14). Analysis was done with 1-way ANOVA with the Tukey multiple comparisons test; SEMs and individual data points are shown. Results were pooled from 4 experiments.

absence of IL-12p70 production (after BCG⁺ IFN- γ activation), and normal IFN- γ production (after BCG⁺ IL-12p40 activation) in whole blood assay were also detected (data not shown), as found in other patients with complete AR IFN- γ R1 deficiency.³⁴

Building on previously reported HLH cases with impaired IFN- γ ,¹⁵⁻¹⁷ this patient demonstrates that multiple aspects of HLH pathology can arise with a complete absence of IFN- γ signaling. Furthermore, the absence of hemophagocytosis in an IFN- γ R1-deficient patient with HLH-like disease complements the mouse data (above) in that hematologic aspects of HLH might be IFN- γ dependent, even though overall disease progression is not.

IL-2 consumption by hyperactivated *CD8*⁺ T cells contributes to the immunologic and pathophysiologic processes of HLH without altering hematologic features

To test the role of IL-2 consumption by *CD8*⁺ T cells during hyperstimulation, we generated *CD25*^{fllox} mice from

EUCOMM embryonic stem cells (see Fig E4 in this article's Online Repository at www.jacionline.org), deleted the lacZ and neo with a FLPe deleter strain,²⁶ and intercrossed them with mice bearing the *CD8*⁺ peripheral enhancer-driven Cre transgene.²⁵ The system preserves expression of CD25 on Treg cells and allows *CD8*⁺ T cells access to IL-2 consumption through the constitutively expressed low-affinity dimeric IL-2 receptor but prevents activation-induced expression of the high-affinity trimeric IL-2 receptor. *CD8*^{ΔCD25} mice were then backcrossed to the *Prf1*^{KO} background, allowing the comparison of wild-type, *Prf1*^{KO}, and *Prf1*^{KO} *CD8*^{ΔCD25} mice during LCMV infection.

The IL-2 consumption model is based on the observation that *CD8*⁺ T cells from LCMV-infected *Prf1*^{KO} mice express CD25 levels greater than those observed on Treg cells, resulting in a rewiring of IL-2 consumption from Treg cells to *CD8*⁺ T cells and a collapse in Treg cell numbers.²¹ Therefore we first checked whether the *CD8*^{ΔCD25} strategy prevented rewiring of IL-2 consumption. Few *CD8*⁺ T cells express CD25 in wild-type mice, even 10 days after LCMV infection (Fig 4, A). *Prf1*^{KO} mice, by

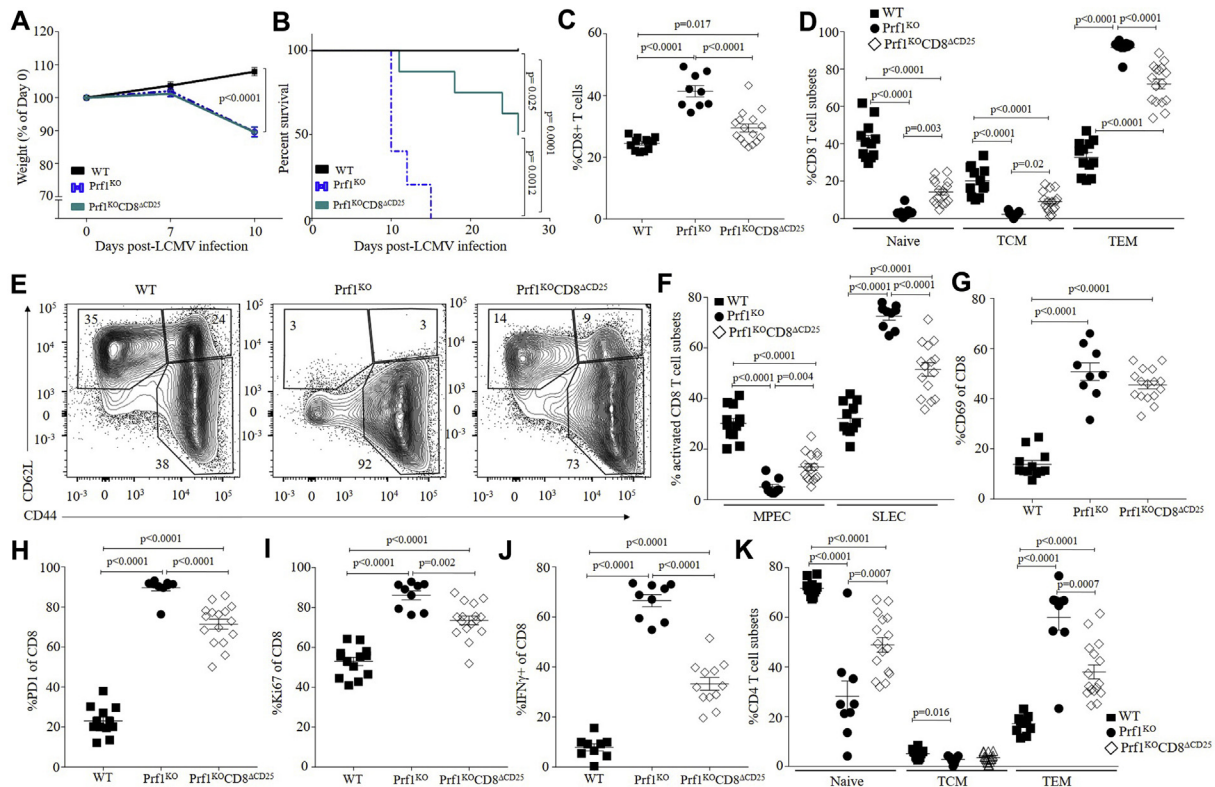


FIG 6. Immunologic and pathophysiologic processes of HLH are dependent on CD25 expression by CD8⁺ T cells. Wild-type (WT), *Prf1*^{KO}, and *Prf1*^{KO}CD8^ΔCD25 mice were infected with LCMV-Armstrong and assessed for immunologic features on day 10. **A**, Mouse weights on days 0, 7, and 10 after LCMV infection (n = 10, 7, and 13). **B**, Survival after LCMV infection (n = 8, 5, and 8). **C**, Percentage of CD8⁺ T cells in lymph nodes (n = 12, 9, and 16). **D** and **E**, Percentage (Fig 6, D) and representative flow cytometry (Fig 6, E) for CD8⁺ T cells in the naive (CD62L⁺CD44⁻), central memory (TCM; CD62L⁺CD44⁺), and effector memory (TEM; CD62L⁻CD44⁺) class (n = 12, 9, and 16). **F**, Percentage of activated CD8⁺ T cells in the CD8⁺CD127⁺KLRG1^{low} (memory precursor effector cell [MPEC]; n = 12, 9, and 16) and CD8⁺CD127⁺KLRG1^{high} (short-lived effector cell [SLEC]) subsets (n = 11, 9, and 15). **G–J**, Percentage of CD8⁺ T cells expressing CD69 (n = 12, 9, and 16; Fig 6, G), programmed cell death protein 1 (PD-1; n = 12, 9, and 16; Fig 6, H), Ki67 (n = 13, 9, and 16; Fig 6, I), and IFN-γ (n = 9, 9, and 12; Fig 6, J). **K**, Percentage of CD4⁺ T cells in the naive (CD62L⁺CD44⁻), central memory (TCM; CD62L⁺CD44⁺), and effector memory (TEM; CD62L⁻CD44⁺) class (n = 12, 9, and 16). Analysis was done with 1-way ANOVA with the Tukey multiple comparisons test; SEMs and individual data points are shown. Results were pooled from 3 to 4 experiments.

contrast, show a dramatic increase, with CD25 expressed by greater than 50% of CD8⁺ T cells. This increase was absent in *Prf1*^{KO}CD8^ΔCD25 mice, which, akin to wild-type mice, showed no prominent CD25⁺ population (Fig 4, A). The effect of CD25 expression on IL-2 consumption (as measured through phosphorylation of STAT5) was dramatic: wild-type mice demonstrated no notable pSTAT5 in CD8⁺ T cells (Fig 4, C) and a high level in Treg cells (Fig 4, B), whereas *Prf1*^{KO} mice exhibited a dramatic increase in pSTAT5 in CD8⁺ T cells and a concomitant decrease in Treg cell numbers (Fig 4, B and C). *Prf1*^{KO}CD8^ΔCD25 mice showed an almost complete rescue of this IL-2 consumption rewiring, with pSTAT5 patterns recapitulating the wild-type situation (Fig 4, C and D). Notably, this system did not completely eliminate CD8⁺ T-cell access to IL-2 signaling, with pSTAT5 levels marginally greater than those in wild-type mice (Fig 4, C); rather, it just corrected the exuberant rewiring occurring in *Prf1*^{KO} mice. We previously hypothesized that this “IL-2 theft” was responsible for the observed reduction in Treg cell numbers²¹; our results formally demonstrate this, with an increase

in IL-2 availability in serum (Fig 4, E) and a rescue of Treg cell numbers in *Prf1*^{KO}CD8^ΔCD25 mice (Fig 4, B). In addition, while evaluating the presence of sCD25, a hallmark diagnostic marker of HLH, we found that *Prf1*^{KO}CD8^ΔCD25 mice presented with very low amounts of sCD25 in their serum, definitively identifying CD8⁺ T cells as the source of sCD25 during the severe HLH inflammation (Fig 4, F). Overall, these data validate the *Prf1*^{KO}CD8^ΔCD25 system for functional testing of the role of CD8⁺ T cell-mediated IL-2 consumption on the pathogenesis of HLH.

To determine the role of IL-2 consumption on the hematologic features of HLH, we infected wild-type, *Prf1*^{KO}, and *Prf1*^{KO}CD8^ΔCD25 mice with LCMV and measured hemoglobin values, red cell counts, hematocrit values, and numbers of reticulocytes and platelets at day 10. Unlike IFN-γ deficiency, which completely rescued the anemic features of *Prf1*^{KO} mice (Fig 1), loss of CD25 expression on CD8⁺ T cells had no effect on any parameter, with hematologic deficiencies equivalent to those of LCMV-infected *Prf1*^{KO} mice in each case (Fig 5). These results

clearly establish the hematologic manifestations of HLH as IFN- γ dependent and CD25 independent.

Despite the severe anemia of *Prf1*^{KO}CD8^{ΔCD25} mice after LCMV infection, the physiologic response to infection was markedly improved over that of *Prf1*^{KO} mice. *Prf1*^{KO}CD8^{ΔCD25} mice did not show the distressed behavior observed in *Prf1*^{KO} mice at day 10 (data not shown); however, they experienced the same reduction in weight (Fig 6, A). More critically with regard to pathology, the *Prf1*^{KO}CD8^{ΔCD25} mice experienced a substantial extension in survival (Fig 6, B). This correction of inflammatory pathologies was accompanied by a (at least partial) correction of most of the critical immunological parameters. The CD8⁺ T-cell expansion observed in *Prf1*^{KO} mice was largely abrogated (Fig 6, C), with the shift toward an effector memory T and short-lived effector cell phenotype intermediate in *Prf1*^{KO}CD8^{ΔCD25} mice compared with *Prf1*^{KO} and wild-type mice (Fig 6, D-F). With the exception of CD69, partial corrections were also observed in programmed cell death protein 1, Ki67, and, importantly, IFN- γ expression by CD8⁺ T cells (Fig 6, G-J). A near-complete correction was also observed in the CD4⁺ T-cell population, with the shift from naive to effector memory T cells in *Prf1*^{KO} mice effectively quenched by negation of IL-2 consumption by CD8⁺ T cells (Fig 6, K). By contrast, neither the liver damage observed in LCMV-infected *Prf1*^{KO} mice (see Fig E5 in this article's Online Repository at www.jacionline.org) nor the cytokine storm (see Fig E6 in this article's Online Repository at www.jacionline.org) were corrected. Together, these data demonstrate that escalated CD25 expression and the resulting "IL-2 theft" is one of the major mechanistic drivers of the immunologic and pathophysiologic features of HLH.

DISCUSSION

Together, these data provide a novel comprehensive model for the pathogenesis of HLH. A disparate group of disease triggers are possible, each of which initiates the upstream events of CD8⁺ T-cell hyperstimulation. In the case of primary HLH, the initiation event is typically an infectious trigger, with defective elimination of infected cells caused by impaired cytotoxicity by CD8⁺ T cells and NK cells and an induced numeric loss of NK cells.²¹ This in turn drives excessive antigen presentation to CD8⁺ T cells,⁷ likely because of a combination of increased viral titers and prolonged antigen stimulation (defective elimination of antigen-presenting cells).¹² In patients with secondary HLH, the initiation trigger varies but still results in hyperstimulation of CD8⁺ T cells against viral antigen, tumoral antigens, or self-antigens.^{1,5,8} The CD8⁺ T-cell hyperstimulation leads to excessive activation and proliferation of CD8⁺ T cells, creating a hyperactivated state.¹¹ The downstream events directly proximal to pathology here diverge: excessive production of IFN- γ leads to hematologic features, whereas excessive consumption of IL-2 contributes to immunologic features and ultimately to death caused by severe inflammation.

The IFN- γ and CD25 pathogenic pathways act independent of each other, with correction of one pathway leaving the other intact. In the mouse model CD25 deficiency in CD8⁺ T cells resulted in partial recovery of the inflammatory components of HLH, demonstrating that CD25 expression, although central, is complemented by additional inflammatory pathways. The remaining mortality in CD25-deficient mice with HLH might be due to these residual inflammatory pathways or to the pathologic

consequences of the IFN- γ -dependent hematologic components. In a translational context, this incomplete recovery is consistent with the incomplete response to cyclosporin A treatment.

This synthesized model paints a more complex picture for the role of IFN- γ in patients with HLH. Although studies focusing on hematologic features have found IFN- γ inhibition to be an effective approach,^{9,13,14} our study reveals that it does not prevent the lethal inflammatory signature of HLH in both patients and mouse models. Indeed, if anything, IFN- γ exhibited regulatory properties over the inflammatory aspects of disease, with enhanced inflammation after genetic removal of IFN- γ in mice with HLH. Rather than acting as an inflammatory mediator, it is likely that the key effects of IFN- γ are hematologic. The mechanistic pathway for the anemia is thought to be macrophage-mediated destruction of red cells in the spleen,³⁵ as observed in HLH models.^{9,11,13,14} Because we also observed a low reticulocyte count (Fig 5), IFN- γ might also function through disrupting bone marrow support of erythroid lineage differentiation.³⁶ Thus although the ongoing clinical trials of anti-IFN- γ antibody in patients with HLH are likely to reverse anemia, they might not be effective as a monotherapy, with the inflammatory aspects requiring a distinct approach. This adjunct therapy will need to target the consumption of IL-2 by CD8⁺ T cells, the collapse of Treg cells, or the downstream (IFN- γ -independent) inflammatory cascade that drives the immune aspects of the disease.

Interestingly, inhibition of the universal cytokine receptor signaling Janus kinase (JAK) pathway has been very recently used with success in the same preclinical model of HLH used here.^{33,37} The use of ruxolitinib, a JAK1/JAK2 inhibitor, has been proved to be efficient at controlling CD8⁺ T-cell expansion and dampening the cytokine storm in patients with HLH.³³ Although this activity has been attributed to IFN- γ inhibition (and, indeed, hematologic correction should be attributed to this function), the inhibitor also strongly affects other cytokines, including IL-2, which potentially explains correction of the inflammatory aspects.^{33,37} TNF- α in particular is an interesting candidate responsible for the inflammatory cascade downstream of Treg cell loss because early experiments have previously demonstrated a long-term survival after infection with LCMV in double-knockout mice for perforin and TNF receptor 1,³⁸ and TNF- α levels are reduced in ruxolitinib-treated mice.^{33,37} However, because the TNF- α pathway does not depend on JAK1/2, it is likely that a complex milieu of other cytokines participate upstream.

Finally, we would note that these results have implications beyond HLH. Although primary HLH is caused by a rare and specific set of genetic lesions, the diverse causes of secondary HLH demonstrate these principles to be more generic outcomes of excessive hyperstimulation of CD8⁺ T cells. Indeed, the same pathways (IFN- γ production and IL-2 consumption) that drive clinical pathology in the extreme context during HLH appear to operate at a subclinical level during "normal" CD8⁺ activation events. Thus even wild-type mice infected with LCMV exhibit transient anemia and a limited temporal decrease in Treg cell numbers,³⁹ a feature observed in other strong infection responses.⁴⁰ Thus these deleterious outcomes can perhaps be considered the tolerable costs of mounting an effective CD8⁺ T-cell response, which only rarely escalate into frank immunopathology.

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Key messages

- In patients with HLH, hematologic features are strictly dependent on IFN- γ . However, in both patients and the mouse model, absence of IFN- γ signaling does not prevent the inflammatory aspect of the disease.
- IL-2 signaling plays a key role in excessive CD8⁺ T-cell activation during HLH-related inflammation, providing a potential therapeutic target for HLH treatment.

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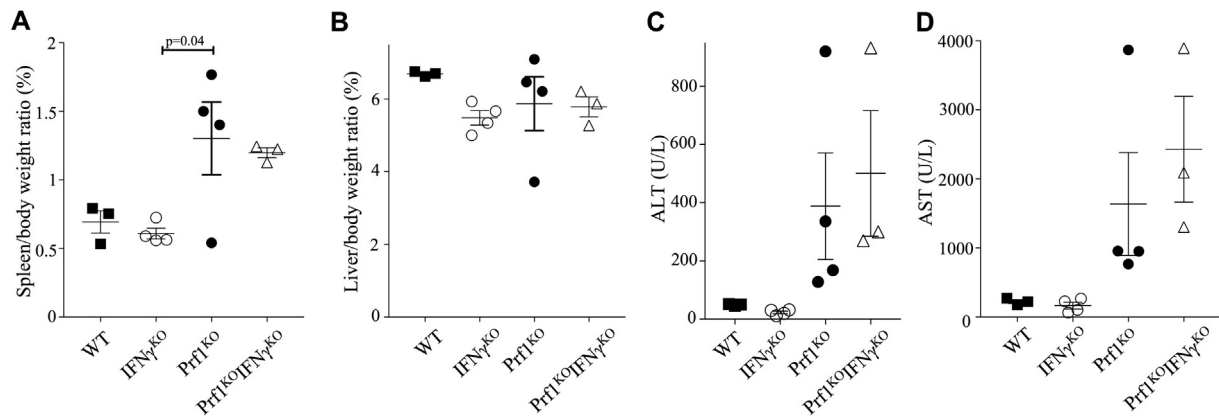


FIG E1. Splenomegaly and liver damage in murine HLH arise independent of IFN- γ . Wild-type (WT), *Ifng*^{KO}, *Prf1*^{KO} (and *Prf1*^{KO}/*Ifng*^{KO/WT} littermate control animals), and *Prf1*^{KO}/*Ifng*^{KO} mice were infected with LCMV-Armstrong (n = 3, 4, 4, and 3). **A**, Spleen weight (day 10)/total body weight (day 0) ratio (as a percentage). **B**, Liver weight (day 10)/total body weight (day 0) ratio (as a percentage). **C** and **D**, Serum liver enzyme levels for alanine transaminase (ALT; Fig E1, C) and aspartate aminotransferase (AST; Fig E1, D) on day 10. Analysis was done with 1-way ANOVA with the Tukey multiple comparisons test; SEMs and individual data points are shown.

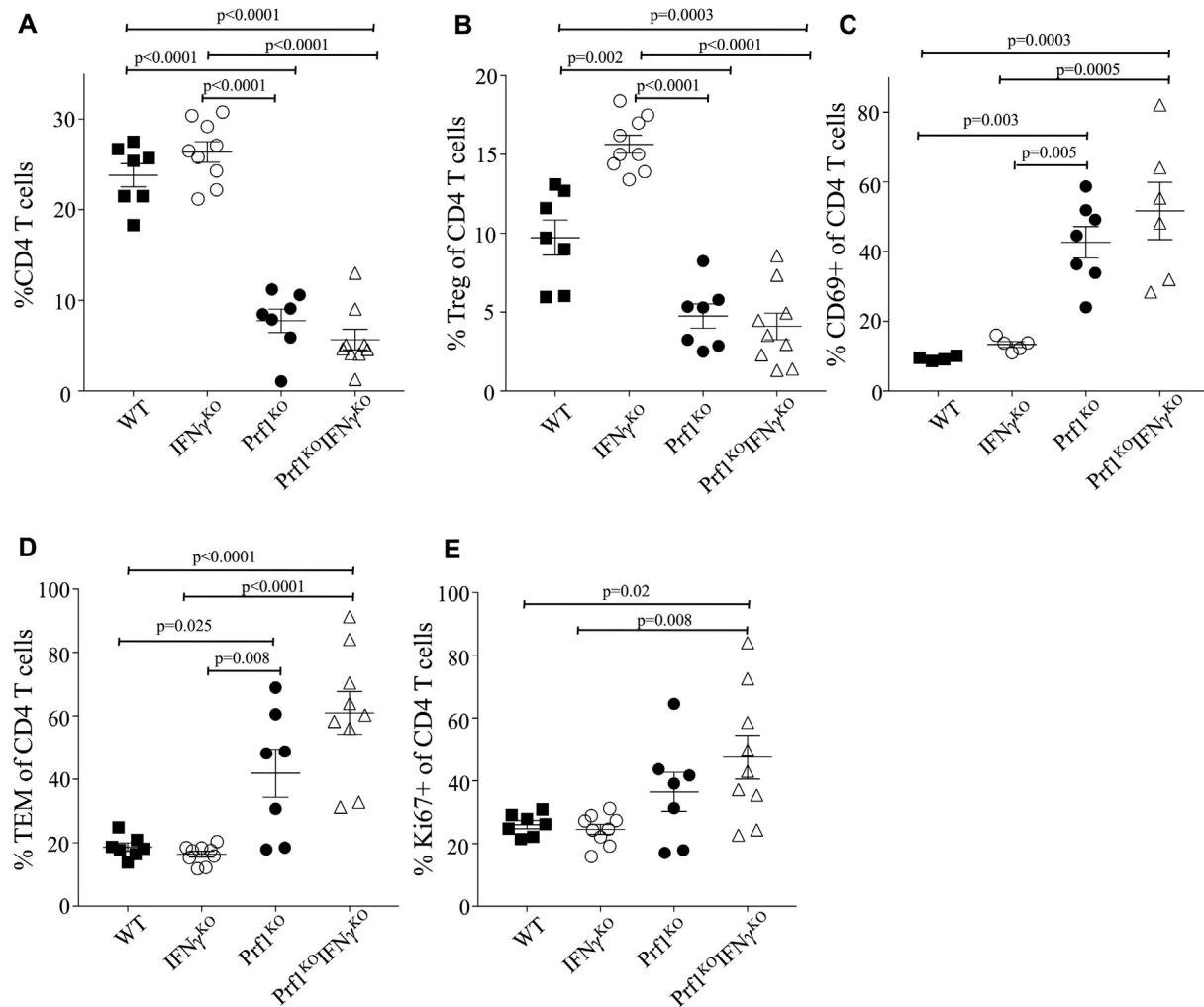


FIG E2. Inflammatory and physiologic aspects of murine HLH arise independent of IFN- γ . Wild-type (WT), *Ifng*^{KO}, *Prf1*^{KO}, and *Prf1*^{KO}/*Ifng*^{KO} mice were infected with LCMV-Armstrong. **A**, Percentage of CD4⁺ T cells in lymph nodes ($n = 7, 9, 7$, and 9). **B**, Percentage of Treg cells of CD4⁺ T cells in lymph nodes ($n = 7, 9, 7$, and 9). **C**, Percentage of CD4⁺ T cells expressing CD69 ($n = 4, 5, 7$, and 6). **D**, Percentage of CD4⁺ T cells in CD4⁺CD62L⁻CD44⁺ effector memory (TEM) cells ($n = 7, 9, 7$, and 9). **E**, Percentage of CD4⁺ T cells expressing Ki67 ($n = 7, 9, 7$, and 9). Analysis was done with 1-way ANOVA with the Tukey multiple comparisons test; SEMs and individual data points are shown. Results were pooled from 4 experiments (3 experiments for CD69 measurements).

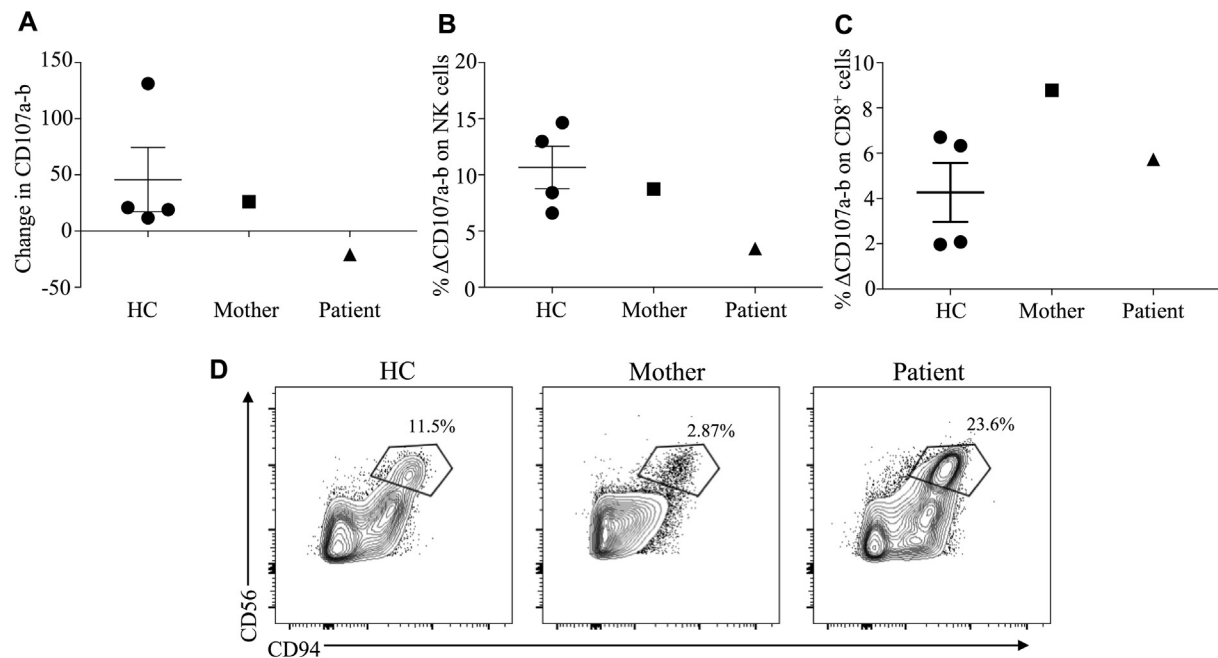


FIG E3. Defective NK cell function in IFN- γ R1-deficient patient. PBMCs from the patient, her mother, and healthy control subjects (HC) were stimulated *in vitro* for 3 hours and assessed for NK cell degranulation by using surface expression of CD107a and CD107b after and before stimulation (Δ CD107a-b). **A**, CD107a and CD107b expression on NK cells after suboptimal K562 stimulation. **B**, CD107a and CD107b expression on NK cells after phorbol 12-myristate 13-acetate and ionomycin stimulation. **C**, CD8⁺ T-cell CD107a and CD107b expression after phorbol 12-myristate 13-acetate and ionomycin stimulation. **D**, Expression of CD56 and CD94 on gated NK cells *ex vivo*. The CD56^{bright}CD94⁺ noncytotoxic subset is gated. HC, Healthy control subjects.

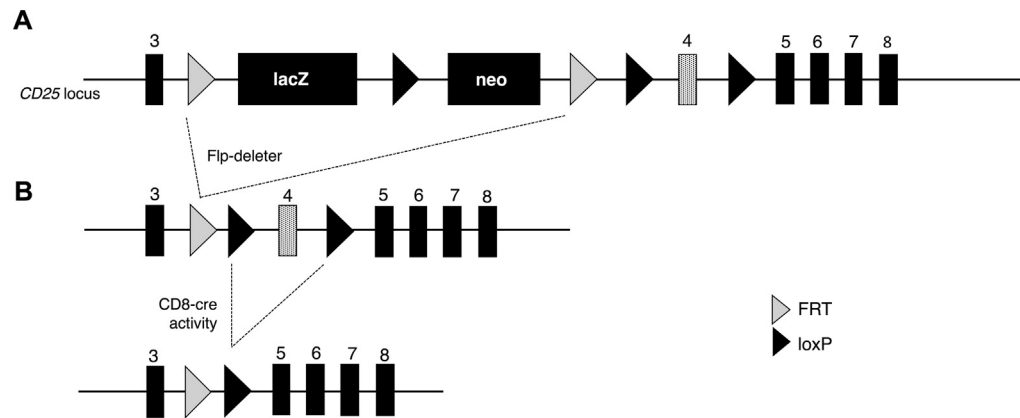


FIG E4. Generation of *CD25^{fllox}* mice. **A**, Design of the *CD25* locus in original EUComm embryonic stem cells. **B**, Design of the *CD25* locus in *CD25^{fllox}* mice after transient intercrossing with a ubiquitous Flpase.

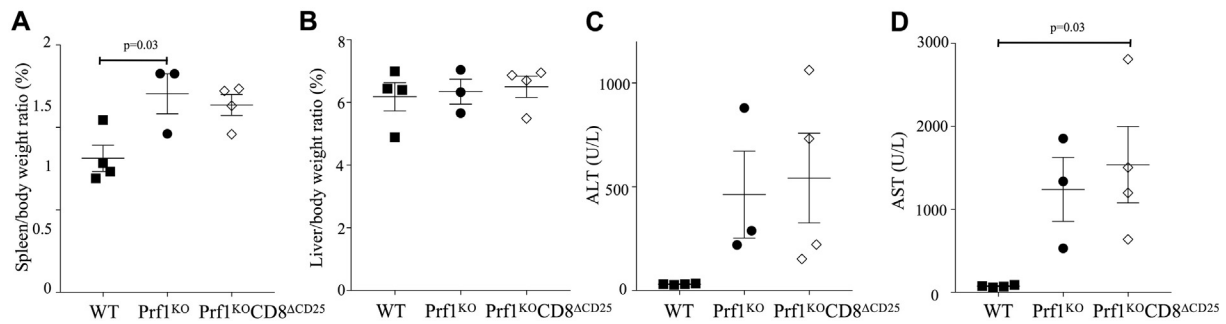


FIG E5. Splenomegaly and liver damage in murine HLH arise independent of CD25 expression. Wild-type (*WT*), *Prf1*^{KO}, and *Prf1*^{KO}*CD8*^{ΔCD25} mice were infected with LCMV-Armstrong (*n* = 4, 3, and 4). **A**, Spleen weight (day 10)/total body weight (day 0) ratio (as a percentage). **B**, Liver weight (day 10)/total body weight (day 0) ratio (as a percentage). **C** and **D**, Serum liver enzyme levels for alanine transaminase (*ALT*; Fig E5, *C*) and aspartate aminotransferase (*AST*; Fig E5, *D*) on day 10. Analysis was done with 1-way ANOVA with the Tukey multiple comparisons test; SEMs and individual data points are shown.

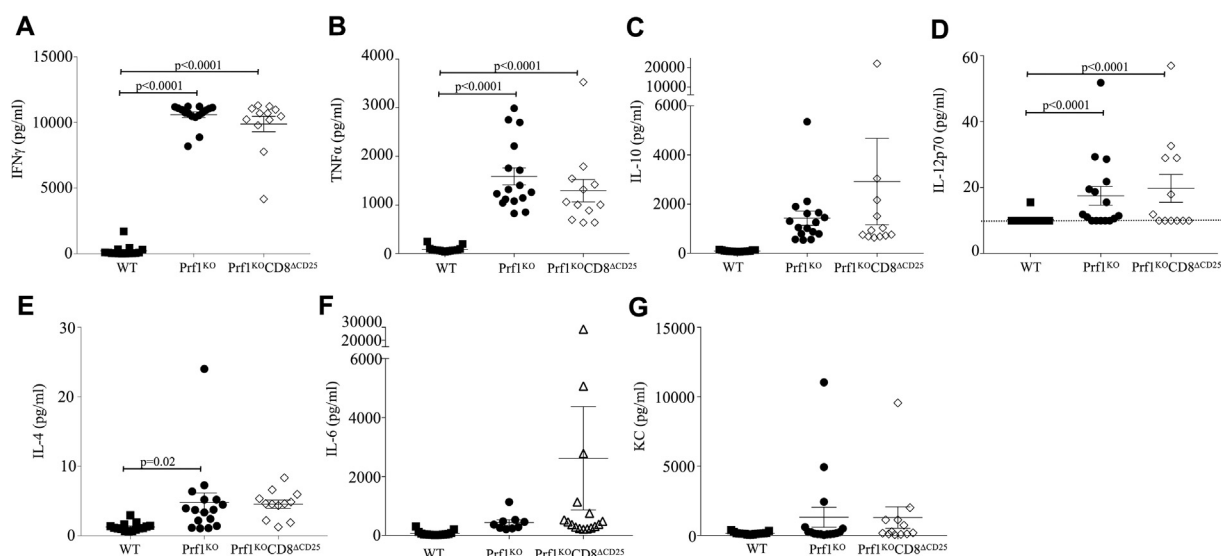


FIG E6. Removal of CD25 expression on CD8⁺ T cells does not prevent the cytokine storm during HLH. Wild-type (WT), *Prf1*^{KO}, and *Prf1*^{KO}CD8^{ΔCD25} mice were infected with LCMV-Armstrong and assessed on day 10 (n = 15, 16, and 12). Serum concentrations of IFN-γ (A), TNF-α (B), IL-10 (C), IL-12p70 (D), IL-4 (E), IL-6 (F), and keratinocytes (KC; G) are shown. Analysis was done with 1-way ANOVA with the Tukey multiple comparisons test; SEMs and individual data points are shown.

TABLE E1. Clinical and biological parameters in the patient according to HLH criteria

Criteria	Patient
1. Fever	Observed
2. Splenomegaly	Observed
3. Cytopenia	Hemoglobin, 5 g/dL Platelets, 30,000/mm ³ Normal leukocytes
4. Increased triglyceride level and decreased fibrinogen levels	Hypertriglyceridemia, 2.11 g/L Fibrinogen, normal (4.2 g/L)
5. Ferritin >500 ng/mL	1043 ng/mL
6. Hemophagocytosis	Not found in bone marrow
7. Low or absent NK cell activity	Normal NK cell numbers (332 cells/mm ³), decreased activity
8. sCD25	Not assessed