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Etoposide Selectively Ablates Activated T Cells To Control the Immunoregulatory Disorder Hemophagocytic Lymphohistiocytosis

Theodore S. Johnson,* Catherine E. Terrell,† Scott H. Millen,‡ Jonathan D. Katz,† David A. Hildeman,† and Michael B. Jordan†‡

Hemophagocytic lymphohistiocytosis (HLH) is an inborn disorder of immune regulation caused by mutations affecting perforin-dependent cytotoxicity. Defects in this pathway impair negative feedback between cytotoxic lymphocytes and APCs, leading to prolonged and pathologic activation of T cells. Etoposide, a widely used chemotherapeutic drug that inhibits topoisomerase II, is the mainstay of treatment for HLH, although its therapeutic mechanism remains unknown. We used a murine model of HLH, involving lymphocytic choriomeningitis virus infection of perforin-deficient mice, to study the activity and mechanism of etoposide for treating HLH and found that it substantially alleviated all symptoms of murine HLH and allowed prolonged survival. This therapeutic effect was relatively unique among chemotherapeutic agents tested, suggesting distinctive effects on the immune response. We found that the therapeutic mechanism of etoposide in this model system involved potent deletion of activated T cells and efficient suppression of inflammatory cytokine production. This effect was remarkably selective; etoposide did not exert a direct anti-inflammatory effect on macrophages or dendritic cells, and it did not cause deletion of quiescent naive or memory T cells. Finally, etoposide’s immunomodulatory effects were similar in wild-type and perforin-deficient animals. Thus, etoposide treats HLH by selectively eliminating pathologic, activated T cells and may have usefulness as a novel immune modulator in a broad array of immunopathologic disorders. The Journal of Immunology, 2014, 192: 84–91.

Although immunity against pathogens is necessary for survival, the specificity and magnitude of immune responses must be tightly regulated to avoid dangerous immunopathology. Hemophagocytic lymphohistiocytosis (HLH) is a unique inborn disorder of immune regulation in which immune responses are appropriately directed (not autoimmune) but may be rapidly fatal as the result of inefficient control of T cell activation. Mutations affecting prf1 (perforin) and related genes were found to be causal in most affected families. Studies in animal models established a chain of pathogenesis in which defective perforin-mediated cytotoxic feedback allows prolonged/excessive Ag presentation by dendritic cells (DCs), which drives abnormal CD8⁺ T cell activation, IFN-γ production, and subsequent macrophage-mediated immunopathology. Although this pathologic immune activation may have varied manifestations, a constellation of features typifies patients with HLH: fever, splenomegaly, severe cytopenias, elevated ferritin (often extreme), depressed levels of fibrinogen (paradoxical, in the context of inflammation), elevated markers of T cell and macrophage activation (e.g., soluble CD25 and CD163), and hemophagocytosis (the appearance of macrophages engulfing blood and marrow cells) in various tissues (6, 7).

Although HLH is rapidly fatal without specific therapy, treatment with the chemotherapeutic agent etoposide (ETOP) was empirically discovered to be life-saving >30 years ago (8). International follow-up studies established ETOP-based regimens as the standard of care (9, 10). Despite this extensive clinical study, it remains unclear how ETOP treats HLH or why it has succeeded while other chemotherapeutic or immunosuppressive agents tried sporadically in the 1970s and 1980s were largely unsuccessful (11). The only study (12) to examine this question analyzed ETOP-driven apoptosis of lymphocytes from patients with HLH and found that they responded in a similar fashion as did those from normal individuals. The question of how a chemotherapeutic agent treats an intrinsic immune regulatory disorder is also broadly relevant to the fields of autoimmunity and transplantation, in which DNA-damaging chemotherapeutic agents are used to treat rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis, as well as to prevent graft-versus-host disease.

In the current study, we used a well-described murine model of HLH, involving lymphocytic choriomeningitis virus (LCMV) infection of perforin-deficient (prf⁻/⁻) mice, to study the in vivo therapeutic mechanism of action for ETOP. We found that ETOP has clear therapeutic activity in this model, alleviating all features of HLH. When compared with a broad array of chemotherapeutic agents, we found that it was relatively unique. Because ETOP is...
often observed to have rapid and dramatic effects in clinical HLH, it has been commonly speculated that it may act directly on inflamed macrophages. Contrary to this expectation, we found no direct beneficial effect on either macrophage activation or Ag presentation by DCs. Instead, we found that ETOP strongly suppressed IFN-γ levels. This, in turn, was due to near complete ablation of pathologically activated, virus-specific T cells. Notably, this depletion was also seen in wild-type (WT) animals and was extremely specific in vivo; quiescent naïve and memory cells were largely spared. Because of its potency and selectivity, we conclude that ETOP has unique immunomodulating qualities in HLH and may have broader usefulness as an immunosuppressive agent.

Materials and Methods  
**Mice, viruses, and in vivo treatments**  
C57BL/6, prf−/−, and IFN-γ−/− mice were obtained from The Jackson Laboratory. TCR-transgenic P14, SMARTA, and OT1 mice were a gift from P. Marrack (University of Colorado/Howard Hughes Medical Institute, Denver, CO). Mice were housed in a specific pathogen–free facility, and animal experiments were approved by the Institutional Animal Care and Use Committee of Cincinnati Children’s Hospital Medical Center. LCMV-WE viral stocks were generated and titered as described (13). Mice were infected via i.p. injection of 200 PFU. Vaccinia-ova was obtained from the Cincinnati Children’s Hospital Medical Center. Mice were examined longitudinally, typically three times/wk, for development of signs of HLH-like disease using a clinical scoring system to gauge mortality (1, 28, 29). We found that prf−/− mice treated with ETOP survived long-term after LCMV infection, whereas those treated with drug vehicle died within 3 wk of infection (Fig. 1A). A dose titration revealed that ETOP’s effects on survival and immune activation (see below) were titratable (Supplemental Fig. 1). To more accurately measure global disease severity in LCMV-infected mice, we developed a clinical scoring system, ranging from asymptomatic (0) to dead (12), based on weight loss, stance, coordination, skin tenting, conjunctivitis, ascites, and mortality (Supplemental Table I). As expected, LCMV-infected prf−/− mice displayed much higher clinical scores, whereas LCMV-infected WT mice experienced mild clinical symptoms and generally recovered by day 20 of illness (Fig. 1B). ETOP-treated prf−/− mice had higher initial scores than did WT mice, but they recovered and displayed prolonged survival (Fig. 1A). ETOP-treated WT mice displayed no clear benefit or worsening using this scale compared with WT controls (data not shown).

We also examined HLH disease-related biomarkers and cytopenias in these mice. In human HLH, elevations in plasma-soluble CD25 (IL-2R, α-chain) and ferritin levels are thought to correlate with systemic T cell activation and acute-phase inflammation, respectively, whereas variable decreases in plasma fibrinogen levels are less well understood (7). These biomarker profiles are recapitulated in LCMV-infected prf−/− mice relative to infected WT controls, but they were efficiently reversed with ETOP administration (Fig. 1C–E). Furthermore, ETOP treatment rescued LCMV-infected prf−/− mice from the development of cytopenias (Fig. 1F–H). Finally, we examined multiple tissues for evidence of histologic improvement and found that ETOP preserved normal splenic architecture (which is largely effaced in prf−/− mice after LCMV infection), decreased inflammatory liver infiltrates, and, ironically, increased marrow cellularity (Fig. 1I). Thus, ETOP treatment leads to improvements in disease severity, mortality, disease-related biomarkers, cytopenias, and histology in LCMV-infected prf−/− mice.

**Statistics**  
All statistical tests were performed using NCSS2007 statistical analysis software. Where appropriate, results are given as the mean ± SEM, with statistical significance determined by a two-tailed t test.

**Results**  
**ETOP treatment rescues LCMV-infected prf−/− mice from HLH-like disease**  
LCMV-infected prf−/− mice were previously characterized by our group and validated as a robust murine model for human HLH (2). These mice develop a fatal HLH-like syndrome within weeks following LCMV infection, displaying all of the diagnostic and typical features seen in patients with HLH. We tested the effects of ETOP on HLH-like disease development in this model by giving a single i.p. injection (50 mg/kg) at 5 d post-LCMV infection. We chose this time point because it is around the time that immune responses in WT and prf−/− mice begin to diverge, and we chose this dose because it is within the range of xenograft studies and is the murine equivalent of what is used clinically for treating HLH (1, 28, 29). We found that prf−/− mice treated with ETOP survived long-term after LCMV infection, whereas those treated with drug vehicle died within 3 wk of infection (Fig. 1A). A dose titration revealed that ETOP’s effects on survival and immune activation (see below) were titratable (Supplemental Fig. 1). To more accurately measure global disease severity in LCMV-infected mice, we developed a clinical scoring system, ranging from asymptomatic (0) to dead (12), based on weight loss, stance, coordination, skin tenting, conjunctivitis, ascites, and mortality (Supplemental Table I). As expected, LCMV-infected prf−/− mice displayed much higher clinical scores, whereas LCMV-infected WT mice experienced mild clinical symptoms and generally recovered by day 20 of illness (Fig. 1B). ETOP-treated prf−/− mice had higher initial scores than did WT mice, but they recovered and displayed prolonged survival (Fig. 1A). ETOP-treated WT mice displayed no clear benefit or worsening using this scale compared with WT controls (data not shown).

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**Flow cytometry and microscopy**  
All Abs were obtained from BioLegend or eBioscience. Tetrameric MHC reagents were produced in SP9 insect cells as described (26). For quantification of T cells producing IFN-γ in vivo, mice were injected with brefedlin A prior to sacrifice, and spleens were crushed in PBS/1% paraformaldehyde as described (3). For measuring phosphatidylserine (PS) exposure, cells were stained with recombinant Alexa Fluor 647–labeled MFG-E8-D89E, a high affinity PS-binding protein (27). For microscopy, tissues were fixed in 10% buffered formalin and embedded in paraffin, followed by sectioning and staining with H&E. Images were obtained with an Evos XL core microscope using a 10× or 20× objective.
and blood hemoglobin levels (Fig. 2C) for LCMV-infected prf<sup>−/−</sup> mice given a single i.p. injection of various chemotherapy drugs 5 d postinfection. LCMV-infected WT mice treated with carrier 5 d postinfection are included for comparison. Mice were monitored longitudinally for survival (A) and disease severity using a clinical scoring system described in Materials and Methods (B). Plasma was assessed by ELISA at day 8 postinfection for soluble CD25 (C) and at day 12 for ferritin (D), and fibrinogen (E). Blood was drawn on day 15 d after LCMV infection for assessment of blood counts, including hemoglobin (F), platelet (G), and neutrophil (H) levels. I) Tissues were obtained 12 d postinfection and stained with H&E, revealing the preservation of normal splenic architecture, decreased liver infiltrates, and improved marrow cellularity with ETOP treatment. Scale bar, 100 μm. Data are mean ± SEM and are compiled from 8–23 mice/group assayed across three or more experiments. *p < 0.005.
FIGURE 2. Only select chemotherapeutic agents have efficacy in treating murine HLH. LCMV-infected prf−/− mice were treated with CPM, ETOP, MTX, cladribine (2CDA), CDDP, CLO, DOXO, FLU, 5-fluorouracil (5FU), L-asparaginase (L-ASP), vinblastine (VBL), or carrier control (Prf−/− CON) by i.p. injection 5 d postinfection. LCMV-infected WT mice treated with carrier control (WT CON) 5 d postinfection are included for comparison. Mice were monitored serially for disease severity using a clinical scoring system to measure therapeutic effect (A) or lack thereof (B). (C) Blood was drawn on day 15 post-LCMV infection to assess hemoglobin levels. Data are mean ± SEM of three or four mice/group, representative of three or more independent experiments. *: All mice in treatment group deceased. *p < 0.004.

not alter the disease course (data not shown). Thus, ETOP appears relatively unique among a range of agents tested for its ability to alleviate murine HLH. The two exceptions to this finding, CPM and MTX, are notable for their uses as immune suppressive agents in a variety of clinical contexts or for intrathecal treatment of HLH-associated inflammation of the CNS.

ETOP treatment does not directly decrease macrophage activation or Ag presentation by DCs in LCMV-infected prf−/− mice

Based on clinical observations and more recent experimental data (4), it is widely believed that systemic activation of macrophages plays a prominent role in the development of HLH. This fact, combined with the prompt clinical response observed in some patients after starting ETOP, suggested to many that ETOP exerts its therapeutic effect by suppressing macrophage activation and/or Ag presentation (30). To assess this potential mechanism of action, we examined macrophage phenotypes after ETOP treatment of LCMV-infected prf−/− mice. When we examined tissues 1–2 wk after ETOP treatment, we observed decreased macrophage infiltration by histology and flow cytometry (F4/80+ cells decreased from ∼13 to 9%) (Fig. 1, data not shown). However, because disease development was completely reversed in these animals, it was not clear whether this was merely a secondary effect. In contrast, when we examined tissues on day 7, after treating with ETOP on day 5, we found that the highly activated phenotype of splenic macrophages (assessing CD40, CD80, CD86, and MHC class II on F4/80+ cells in spleen, marrow, and liver) was unchanged (Fig. 3A, 3B, data not shown). Furthermore, the numbers of macrophages and DCs, as a percentage of splenocytes, were not decreased after ETOP treatment (∼13% F4/80+ and 2% CD11c+/MHC II+; with or without treatment). In addition to surface phenotype and numbers, we sought to assess the function of APCs after ETOP. We recently reported that abnormalities of DC function, rather than DC numbers, are a critical determinant of immune dysregulation in prf−/− mice (5). To investigate this, we isolated DCs from ETOP- or carrier-treated, LCMV-infected prf−/− mice (treatment on day 5, DC isolation on day 7) and cultured them with LCMV-specific CD8+ and CD4+ T cells. As expected, we found that DCs from prf−/− mice presented endogenously derived viral Ag much more potently than did those from WT mice (Fig. 3C, 3D). Notably, DCs from ETOP-treated prf−/− mice revealed no decrease in Ag-presentation potency. In fact, in some experiments, Ag presentation was actually increased by ETOP treatment (Fig. 3C, data not shown). This finding was similar to prior observations that in vivo T cell deple- tion leads to increases in Ag presentation by DCs (5). Thus, ETOP does not appear to have a direct suppressive effect on macrophage activation or Ag presentation by DCs.

ETOP alleviates murine HLH by suppressing IFN-γ levels in LCMV-infected prf−/− mice

Prior studies (2) indicated that hyperproduction of IFN-γ is necessary for development of HLH in LCMV-infected prf−/− mice. Therefore, we hypothesized that the therapeutic effect of ETOP in these mice is due to a reduction in IFN-γ levels. Accordingly, we observed that ETOP treatment on day 5 led to a dramatic suppression of serum IFN-γ levels in prf−/− mice (Fig. 4A). Further- more, we predicted that if suppression of IFN-γ levels is a component of ETOP’s therapeutic mechanism, then drug treatment after the in vivo peak of cytokine production would have little efficacy, because macrophages have already been exposed to high levels of IFN-γ, and ETOP does not appear to act directly on macrophages (2, 4). In contrast, if ETOP directly affected macrophage activation, then treatment would still be therapeutic at later time points. To test our prediction, we treated LCMV-infected prf−/− mice with ETOP at day 10, a time point after peak cytokines levels but before significant anemia has developed (4). In this case, neither the disease course (Fig. 4B) nor the disease-related anemia (Fig. 4C) was ameliorated by late ETOP therapy. In contrast, mice deficient for both perforin and IFN-γ (double knockout [DKO]) were protected from anemia and fatal disease (Fig. 4B, 4C).

If decreasing inflammatory cytokine production is an important aspect of ETOP’s mechanism in HLH, then “adding back” IFN-γ to ETOP-treated prf−/− mice would abolish its therapeutic benefit. To test this prediction, we used s.c. osmotic pumps to deliver IFN-γ or saline to LCMV-infected prf−/− mice treated with ETOP on day 5. We implanted pumps shortly after administering ETOP or carrier, titrated to maintain serum IFN-γ levels of 3–6 ng/ml for up to 7 d. This level is somewhat lower than that seen in control LCMV-infected prf−/− mice but much higher than that seen in ETOP-treated or WT mice. Consistent with previous results, mice treated with ETOP and infused with saline showed improvement in disease symptoms and survival (Fig. 4D, 4E). However, when mice were given exogenous IFN-γ, the benefit of ETOP therapy...
was completely abrogated (Fig. 4D, 4E). Thus, suppression of excessive IFN-γ production is a key aspect of ETOP’s mechanism of action in murine HLH. ETOP acts via selective destruction of activated T cells in LCMV-infected prf−/− mice

Because we demonstrated previously that most detectable IFN-γ in LCMV-infected prf−/− mice is derived from highly activated CD8+ T cells (2), we hypothesized that ETOP was suppressing excessive IFN-γ production by depleting or potentially deactivating these cells. To test this hypothesis directly, we measured the number of CD8+ T cells producing IFN-γ in vivo using a previously described technique (3). We found that ETOP decreased the number of IFN-γ–producing T cells to levels that were similar to (or lower than) those seen in WT mice (Fig. 5A). Next, we directly assessed depletion of virus-specific T cells by staining with a peptide-MHC tetrameric staining reagent (H-2Db-Gp33) incorporating an immunodominant LCMV epitope (Gp33–41). We found that ETOP treatment led to near complete ablation of LCMV-specific CD8+ T cells in all animals (Fig. 5B). The absolute number of Db-Gp33+ CD8+ T cells decreased from ~2–4 million cells/spleen to ,50,000 in most animals, with almost one third of ETOP-treated animals displaying no Db-Gp33–specific T cells above staining background. To account for the practical limits of detection using MHC-tetrameric reagents, we (conservatively) scored animals in which no Ag-specific T cells could be detected as “100-fold” depleted. When we compiled data from multiple experiments involving a large number of animals (>20/group), we found that ETOP treatment did not directly decrease macrophage activation or the quality of Ag presentation by DCs in LCMV-infected prf−/− mice. LCMV-infected prf−/− mice were treated with ETOP or carrier at 5 d postinfection. LCMV-infected WT mice treated with carrier are included for comparison. Surface phenotype of splenic macrophages (F4/80+) was assessed 2 d later by flow cytometry, including CD80 (A) and MHC class II (B) levels. DCs (CD11c+/MHC II+) were magnetically sorted from collagenase-treated spleens (pooled, four animals/group) at 7 d postinfection and plated with LCMV-specific transgenic CD8+ (C) or CD4+ (D) T cells (P14 or SMARTA) to assess MHC class I and class II–restricted presentation of endogenously acquired viral Ags. Ag presentation to T cells was quantitated by IFN-γ production after overnight culture. No IFN-γ was detected when T cells of irrelevant specificity were cultured with DCs (data not shown). Data are mean ± SEM of three or four animals/group, representative of three independent experiments. *p < 0.05.
that, in both prf−/− and WT animals, ETOP treatment caused a nearly 100-fold depletion (compared with carrier-treated mice in the same experiment) of virus-specific CD8+ T cells (Fig. 5C). Thus, ETOP was suppressing IFN-γ production in prf−/− mice by potently ablating (not “deactivating”) pathologically activated, virus-specific T cells. In contrast to this profound depletion of LCMV-specific T cells, naive T cells (defined as CD44lo) and pre-existing (quiescent) memory T cells (see Materials and Methods) were largely spared. A similar depletional effect of ETOP was seen on CD4+ T cells, where virus-specific cells (those staining with IAb-GP61 tetramer) were largely ablated, whereas naive CD4+ T cells were spared (Fig. 5D). Although the role of CD4+ T cells in driving HLH pathogenesis is not as clear as that of CD8+ T cells, ETOP’s ability to selectively affect activated CD8+ and CD4+ T cells in both WT and prf−/− animals suggests that it could be useful for immunomodulation in a broad range of contexts.

ETOP directly and preferentially induces apoptosis of activated T cells

To determine whether ETOP was acting directly on activated T cells, we cultured spleen cells from LCMV-infected or uninfected prf−/− mice with a titration of drug and measured apoptosis by assessing cell permeability and PS exposure. We found that ETOP induced apoptosis of activated T cells at physiologically relevant concentrations after overnight culture (Fig. 6). We observed similar results with highly purified, in vitro–activated T cells, further confirming a direct effect (data not shown). When we assessed the kinetics of in vivo therapy, we found that ETOP-induced T cell ablation was evident within 24 h of treatment, suggesting a similar mechanism (Supplemental Fig. 2). Finally, we observed that activated CD8+ T cells from LCMV-infected mice (CD44hi, day 6) were significantly more sensitive to the apoptosis-inducing effects of ETOP than were naive T cells (CD44lo) from uninfected animals (Fig. 6B). Thus, ETOP acts directly on CD8+ T cells to induce apoptosis, and activation potentiates this effect.

Discussion

Although ETOP is the foundation of modern HLH therapy, there has been little understanding of how it reverses life-threatening inflammation in patients with HLH or why it has succeeded...
over other chemotherapeutic or anti-inflammatory agents. In the current study, we found that ETOP’s therapeutic activity can be robustly reproduced in a well-established murine model of HLH. This observation gave us a unique window to dissect its mechanism of action in a disease-relevant context. Consistent with very sparse clinical data, we found that ETOP has a relatively unique ability to suppress HLH-like immunopathology. Furthermore, we found that ETOP’s effects on this pathologic immune response are highly specific for activated T cells and largely spare quiescent T cells and innate immune cells. This high degree of selectivity suggests that ETOP may have unique immunomodulatory qualities in a wider array of immunopathologic conditions. Our findings also underscore the potential usefulness of studying HLH as a prototype for T cell–driven disorders.

Our findings have several implications related to the pathophysiology and treatment of HLH. First, it is notable that DEX, a broadly immunosuppressive agent, had minimal therapeutic effects compared with ETOP (Fig. 1). In additional studies (data not shown), we found that the combination of ETOP and DEX has additive effects, but these benefits are modest. Because HLH appears to entail abnormal regulation of Ag presentation (5), we interpret this finding to suggest that pharmacologic suppression of T cell activation (such as with corticosteroids or calcineurin inhibitors) may be largely futile in the presence of ongoing and/or strong antigenic stimuli. Instead, deletion of the offending T cells appears to be the most effective approach. Indeed, the main clinical alternative to ETOP for treating HLH is the use of T cell–depleting Abs (antithymocyte globulin or alemtuzumab) (1). Second, although an indirect consequence of ETOP-mediated T cell deletion, the key role of IFN-γ suppression underscores the importance of this inflammatory mediator in HLH and provides additional impetus for translational efforts testing IFN-γ blockade. One caveat: the relatively narrow time window that we observed for effective ETOP treatment suggests that kinetics or other aspects (perhaps IFN-γ related) of this model diverge from clinical HLH, in which ETOP may be effective even after prolonged illness. Third, our findings underscore the importance of T cells as therapeutic targets in HLH, reinforcing existing clinical and experimental data.

First synthesized in 1966, ETOP exhibits antineoplastic activity against acute myeloid leukemia, lymphomas, and a variety of solid-tumor cancers (32). ETOP inhibits topoisomerase II, leading to dsDNA breaks (32), although it is not precisely understood how this specific genotoxic lesion exerts a selective effect on activated T cells. Ongoing studies on this topic may reveal new aspects of T cell biology and new therapeutic targets for immunologic diseases.

Of the agents tested, three chemotherapeutic drugs were capable of attenuating murine HLH disease pathology: ETOP, CPM, and MTX. Although these agents are biochemically diverse (a topoisomerase inhibitor, DNA alkylator, and an antimetabolite, respectively), they share clinical usefulness as immunosuppressive drugs. Although ETOP is the basis of modern HLH therapy, MTX is used as intrathecal therapy for CNS inflammation in HLH (6, 33), whereas CPM rarely has been reported as monotherapy (34) or as part of combination chemotherapy (35) of HLH. Although ETOP has not been studied in the context of auto- or alloimmunity, both MTX and CPM are used in a variety of severe autoimmune conditions and for the prevention/treatment of graft-versus-host disease (36, 37). Surprisingly, FLU, a purine analog with strong antilymphocyte qualities (38), was not therapeutic in our system, despite multiple dosing strategies. Therefore, it appears that mechanistic differences between these agents are relevant to their immunomodulatory qualities and will require further study.

One of the most notable findings of the current study is the remarkable selectivity that we observed for ETOP as a T cell–depleting and immunomodulating agent. It ablated activated, antiviral T cells nearly 100-fold and (indirectly) suppressed inflammatory cytokine levels, while essentially sparing quiescent naive and memory T cells. Our findings are consistent with prior reports noting that ETOP causes apoptosis of activated, but not resting, lymphocytes in vitro (39–41). Notably, we observed similar potency and selectivity in prf−/− and WT animals (Fig. 5, data not shown). Together, these findings suggest that ETOP could be used to efficiently eliminate unwanted or pathologic T cells in a wider variety of contexts, based on their activation status. Indeed, as detailed by McNally et al. (42) in this issue, etoposide appears to have similarly immune selective therapeutic effect in experimental autoimmune encephalitis. Sparing of quiescent T cells would preserve protective memory responses and would not impaire responses to newly encountered pathogens. However, additional studies are needed because other investigators observed temporary suppressive effects on T cells surviving ETOP exposure (43). Despite potential limits, a selective deletional approach could compare very favorably with nonselective T cell–depleting agents (such as alemtuzumab) or broadly immunosuppressive drugs (such as corticosteroids). However, additional preclinical studies will be needed to assess the potential efficacy of ETOP in immune disorders beyond HLH.

The principal adverse effects of ETOP and other chemotherapeutic agents relate to off-target genotoxicity, including dose-dependent suppression of hematopoiesis and risk for secondary leukemia. In general, therapy-related leukemia has been reported after treatment with prolonged or high-intensity combination regimens incorporating ETOP and other genotoxic agents, although patients with HLH receiving unusually prolonged courses of ETOP monotherapy have developed leukemias (44, 45). Paradoxically, we found that ETOP prevented the development of cytopenas and marrow hypocellularity in treated animals, presumably because its desirable effects on T cells were more potent than its off-target effects on marrow cells. Although such adverse effects may be managed clinically, further studies into the mechanistic basis of immune modulation, genotoxicity, and marrow suppression may help to further improve the therapeutic index of ETOP and related drugs for the treatment of HLH and other immunologic disorders.

Acknowledgments
We thank Dr. Erin Zoller for technical assistance in osmotic pump placement and Dr. Robert Thacker for assistance with the ex vivo Ag–stimulation assays. In addition, we appreciate the gracious support of the Cincinnati Children’s Hospital Medical Center clinical pharmacy.

Disclosures
The authors have no financial conflicts of interest.

References


**Supplemental Table 1: Murine HLH Disease Severity Scoring System**

In order to more accurately measure disease severity in LCMV-infected mice, we have developed a clinical scoring system ranging from 0 (asymptomatic) to 15 based on weight loss (0-3), stance (0-3), coordination (0-3), skin tenting (0-2), conjunctivitis (0-2), ascites (0-2), and mortality (cumulative score 12). LCMV-infected prf-/ mice experience exaggerated symptomaticity over time yielding high clinical scores before becoming moribund, defined as a clinical score 11 or greater. In contrast, LCMV-infected wild-type C57BL/6 mice (WT) experience mild weight loss and clinical symptoms, generally recovering by day 15 of illness.

<table>
<thead>
<tr>
<th>SCORE</th>
<th>Consistent Findings</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>&lt; 5% weight loss</td>
</tr>
<tr>
<td>1</td>
<td>5% - 10% weight loss</td>
</tr>
<tr>
<td>2</td>
<td>10% - 15% weight loss</td>
</tr>
<tr>
<td>3</td>
<td>&gt;15% weight loss</td>
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**Weight loss (0-3)**

**Stance (0-3)**

**Coordination (0-3)** Assessed by twirl test (twirl mouse 3X over cage and gently drop mouse in center of cage). Sometimes it is necessary to repeat or make sure there is at least 1 companion mouse in the “drop in” cage to provide a visual cue. Should not allow more than 2 companion mice in “drop in” cage to minimize crowding effect.

<table>
<thead>
<tr>
<th>SCORE</th>
<th>Consistent Findings</th>
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<tbody>
<tr>
<td>0</td>
<td>Moves quickly to corner without direction change</td>
</tr>
<tr>
<td>1</td>
<td>Hesitation, but goes to corner without direction change</td>
</tr>
<tr>
<td>2</td>
<td>May hesitate; Goes to a corner within 5 seconds, but changes direction on the way</td>
</tr>
<tr>
<td>3</td>
<td>Does not make it to a corner within 5 seconds; usually huddled, shaking or walking in circles</td>
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**Skin tenting (0-2)** Assessed by pinch test (pinch mouse skin on chest and observe skin tenting)

<table>
<thead>
<tr>
<th>SCORE</th>
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<tbody>
<tr>
<td>0</td>
<td>None; skin snaps back into place</td>
</tr>
<tr>
<td>1</td>
<td>Skin initially tents, but returns to normal by 3 seconds</td>
</tr>
<tr>
<td>2</td>
<td>Skin remains tented after 3 seconds</td>
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</tbody>
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**Conjunctivitis (0-2)**

<table>
<thead>
<tr>
<th>SCORE</th>
<th>Consistent Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Crusting of 1 eye</td>
</tr>
<tr>
<td>2</td>
<td>Crusting of 2 eyes (Sacrifice mouse)</td>
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**Ascites (0-2)**

<table>
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<tr>
<th>SCORE</th>
<th>Consistent Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None; abdomen remains depressed after palpation</td>
</tr>
<tr>
<td>1</td>
<td>Full abdomen that may represent ascites</td>
</tr>
<tr>
<td>2</td>
<td>Definitive and/or massive ascites</td>
</tr>
</tbody>
</table>

**Moribund (cumulative score 11)**

Mice achieving a cumulative score of 11 on 2 sequential daily assessments are considered moribund and **sacrificed**. Any mouse unable to move adequately so that it cannot eat or drink, or with weight loss >20% is considered moribund and is sacrificed without a second assessment.

**Mortality (cumulative score 12)**

Dead mice are assigned a cumulative score of 12 for analysis purposes.
Supplemental Figure 1. Dose:response relationship for etoposide as a therapeutic agent in murine HLH.  
A. Prf-/- mice were treated on day 5 of LCMV infection with etoposide at the indicated doses. Day 8 serum IFN-γ levels were determined by ELISA. WT mice are shown for comparison. 
B. Survival in this cohort was determined.
Supplemental Figure 2. Timing and efficacy of etoposide for ablation of activated T cells. LCMV-infected prf-/- mice were treated with etoposide (50mg/kg ip) at the indicated day post infection. LCMV-specific CD8+ T cells (D\(^b\)-GP33 tetramer+) were enumerated in the spleen on day 8 after infection. The fold change compared to carrier treated controls is plotted.