Research Article

Trigger-dependent differences determine therapeutic outcome in murine primary hemophagocytic lymphohistiocytosis

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Familial hemophagocytic lymphohistiocytosis (FHL) is a hyperinflammatory syndrome affecting patients with genetic cytotoxicity defects. Perforin-deficient (PKO) mice recapitulate the full clinical picture of FHL after infection with lymphocytic choriomeningitis virus (LCMV). Hyperactivated CD8 T cells and IFN-γ have been identified as the key drivers of FHL and represent targets for therapeutic interventions. However, the response of patients is variable. This could be due to trigger-dependent differences in pathogenesis, which is difficult to address in FHL patients, since the trigger frequently escapes detection. We established an alternative FHL model using intravenous infection of PKO mice with murine CMV (MCMV)Smith. PKO mice developed acute FHL after both infections and fulfilled HLH diagnostic criteria accompanied by excessive IFN-γ production by disease-inducing T cells, that enrich in the BM. However, direct comparison of the two infection models disclosed trigger-dependence of FHL progression and revealed a higher contribution of CD4 T cells and NK cells to IFN-γ production after MCMV infection. Importantly, therapeutic intervention by IFN-γ neutralization or CD8 T-cell depletion had less benefit in MCMV-triggered FHL compared to LCMV-triggered FHL, likely due to MCMV-induced cytopathology. Thus, the context of the specific triggering viral infection can impact the success of targeted immunotherapeutic HLH control.

Keywords: hemophagocytic lymphohistiocytosis · immunodeficiency · immunotherapy · lymphocyte cytotoxicity · viral infection

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a hyperinflammatory disease characterized by uncontrolled lymphocyte and macrophage activation [1,2]. Familial (primary) forms of HLH (FHL) are caused by genetic defects in lymphocyte cytotoxicity [3–7]. The clinical diagnosis of primary HLH rests on the fulfilment of at least five out of eight clinical and laboratory criteria that occur in patients with a defect in a gene related to lymphocyte cytotoxicity. Current understanding of primary HLH pathogenesis is mostly based on murine models and suggests that disease manifestation is triggered by infections [8,9]. Infection of perforin-deficient (PKO) mice with lymphocytic choriomeningitis virus (LCMV) reproduces all symptoms of the human disease [10–13]. Impaired virus control in the absence of perforin [14], also accounting for CD8\(^+\) T cell failure to kill antigen-presenting cells [15], leads to their continuous stimulation. Excessive cytokine production by these activated CD8\(^+\) T cells, in particular IFN-\(\gamma\) [10,11], promotes macrophage activation causing additional cytokine production [16]. In line with this concept, CD8\(^+\) T cell depletion or IFN-\(\gamma\) blockade can attenuate LCMV induced HLH in PKO mice [10,11,17]. Accordingly, T cell-directed ATG therapy has been used successfully for human primary HLH [18] and anti-IFN-\(\gamma\) therapy is currently being evaluated [19].

Not all FHL patients respond equally to these targeted immunotherapies, raising the question whether the pathogenesis of primary HLH follows a uniform pattern or whether it varies, e.g., depending on the disease trigger. This question is difficult to address in patients since they are diagnosed at different disease stages and parallel immunosuppressive and anti-infective therapies are rapidly initiated. Thus, infectious triggers are only found in less than a third of HLH patients [20]. In mice, LCMV is non-cytopathic [21], implying that infection itself does not cause cellular damage. However, LCMV is a potent inducer of CD8\(^+\) T cell expansion and IFN-\(\gamma\) secretion [22,23]. This can lead to immune-mediated pathology, in particular if virus control is impaired by a cytotoxicity defect [12]. However, the most commonly identified infectious agents in HLH patients are potentially cytopathic herpesviruses, EBV, and CMV. It is unclear whether viruses with higher cytopathogenicity and different patterns of immune cell activation elicit primary HLH with a similar pathogenesis. Although perforin-deficient mice have been infected with numerous pathogens [24], apart from LCMV none have elicited the full diagnostic HLH criteria. The most detailed studies were performed with murine CMV (MCMV) [25–27]. The control of this cytopathic virus in C57BL/6 mice is critically dependent on NK cells, perforin, and IFN-\(\gamma\) [28,29]. MCMV can induce clinical symptoms of “secondary” HLH (occurring in the absence of a genetic cytotoxicity defect) in WT BALB/c mice [26] and some features of primary HLH have been observed in perforin-deficient C57BL/6 mice [30–32]. However, there is debate to which extent the reported pathology reflects cytopathic effects rather than consequences of hyperinflammation, characteristic for HLH [25,30–32].

Here, we established that intravenous MCMV infection of C57BL/6 PKO mice induces primary HLH according to human HLH criteria. We performed a side-by-side comparison of primary HLH pathophysiology induced by LCMV or MCMV using PKO-IFN-\(\gamma\) reporter mice [33] to analyze whether CD8\(^+\) T cells and IFN-\(\gamma\) similarly contribute to disease and whether the hyperactive T cells target the same organs with similar kinetics. We show that independent of the triggering infection, impaired cytotoxicity favors an IFN-\(\gamma\) dominated immune response, with enrichment of IFN-\(\gamma\) producing lymphocytes in the BM. However, the cellular sources of IFN-\(\gamma\) were virus-dependent, CD8\(^+\) T cell dominated in LCMV infection versus shared between CD4 and CD8 T cells and NK cells in MCMV infection, associated with a different kinetics and extent of individual HLH criteria. Importantly, in contrast to LCMV-induced primary HLH, neither CD8 elimination or IFN-\(\gamma\) blockade could prevent the fatal HLH progression in PKO mice after the MCMV trigger.

Results

Different disease progression in PKO mice after triggering HLH with MCMV versus LCMV infection

Mice with cytotoxicity defects do not spontaneously develop HLH. However, after LCMV infection, PKO mice fulfil all criteria used for the diagnosis of human HLH, with the exception that fever in patients is mirrored by a drop of ear temperature in mice [10,34]. To establish a second model of infection-driven primary HLH, we infected PKO mice with different doses of tissue culture propagated MCMV\(_{\text{Smih}}\) intraperitoneally versus intravenously and determined HLH disease parameters (not shown). Intravenous infection with 2 \(\times\) 10\(^5\) pfu MCMV\(_{\text{Smih}}\) yielded consistent results and was used in all further experiments.

To directly compare disease severity in LCMV versus MCMV-infected PKO mice, we investigated diagnostic HLH criteria [35] in both models. A drop in ear temperature (Fig. 1A) and weight loss (Fig. 5F) occurred earlier after MCMV (day 4 and day 3) than after LCMV infection (day 5 and day 7). Correspondingly, peak disease severity requiring mouse elimination was reached at day 7 after MCMV and at day 12 after LCMV infection. These time points were chosen for evaluation of HLH criteria. Criteria used for HLH diagnosis in patients were fulfilled after both infections (Fig. 1A–I and data not shown for impaired cytotoxicity). However, in addition to the different kinetics of disease evolution, some further differences in disease parameters were observed. Leukopenia and anemia were less pronounced after MCMV infection (Fig. 1C and D), but fulfilled the diagnostic criteria for bi-lineage cytopenia in at least two-thirds of animals. Moreover, the levels of the inflammatory parameters ferritin, triglycerides, and sCD25 were significantly higher (Fig. 1G–I). Thus, the specific manifestation of primary HLH in PKO mice is influenced by the infectious trigger.
Figure 1. Intravenous infection with tissue-propagated MCMVSmith induces most clinical features of primary HLH in PKO mice. (A–J) Perforin-deficient (PKO) and C57BL/6 (WT) mice were infected with 200 pfu LCMV-WE or with $2 \times 10^5$ pfu MCMVSmith, i.v. (A) Peripheral body temperature was monitored daily until mice were sacrificed on day 12 or 7 post infection, respectively, for analysis of disease parameters. Data are shown as mean ± SD. (B–J) (B) Spleen weight and (C–E) peripheral blood counts measured using a Sysmex KX-21 hematology analyzer. The shaded area represents normal values obtained in uninfected WT and PKO mice (F) Representative histological picture of hemophagocytosis observed in liver sections of all 10 investigated MCMV-infected and LCMV-infected PKO mice. Serum levels of ferritin (G), triglycerides (H), sCD25 (I), and IFN-γ (J). The graphs show pooled results from five independent experiments with three to five mice per group. The horizontal lines represent mean values. **p < 0.01, ****p < 0.001 (Student’s unpaired t-test).

Interferon-γ-reporter mice as a tool to study excessive IFN-γ production

One hallmark of LCMV-induced HLH in PKO mice is the dramatic elevation of IFN-γ [10]. Notably, IFN-γ levels were in a similar range at the peak of MCMV-induced HLH (Fig. 1J). Since CD8+ T cells, CD4+ T cells and NK cells all produce IFN-γ, but play different roles in the two infection models, we studied the cellular origin of IFN-γ. Using intracellular staining for IFN-γ, we only detected a low signal in CD8+ T cells from LCMV-infected PKO mice ex vivo [10, 30] and restimulation with the immunodominant gp33 peptide did not improve detection of increased IFN-γ production by PKO T cells (Fig. 2A). We therefore crossed PKO mice with IFN-γ reporter mice [33], in which transcription of IFN-γ and Thy1.1 is linked. Since production and turnover of Thy1.1 and IFN-γ proteins were shown to differ [36], we validated this experimental system for our setting. LCMV infection of WT IFN-γ reporter mice induced intermediate Thy1.1 expression in 84% and high expression in 1% of CD8+ T cells at day 12 (Fig. 2B), which corresponds to estimates of T cells with LCMV specificity at that time point [22,23]. However, while the fraction of responding cells was similar, the expression level was more impressive in PKO-IFN-γ reporter mice enabling the distinction between high and intermediate expression. Overall 53% of PKO T cells were Thy1.1 high whereas WT cells remained largely Thy1.1 intermediate (Fig. 2B). Intracellular co-staining for IFN-γ revealed that only in Thy1.1 high expressors IFN-γ could be detected ex vivo and upon peptide restimulation. Thy1.1+ CD8+ T cells thus contained all cells reacting to the main MHC-I-restricted viral epitope (Fig. 2C). To validate that Thy1.1 expression at d12 was still associated with high IFN-γ expression and not just caused by slow turnover of Thy1.1 expression, we sorted Thy1.1 negative, intermediate, and high CD8+...
**Figure 2.** IFN-reporter mice as a tool to study excessive IFN-γ production in murine HLH. (A) PKO and WT mice (4 mice/group) were infected with 200 pfu LCMV-WE i.v. At day 12 after infection, splenocytes were stained for surface CD8 and intracellular IFN-γ expression ex vivo (left panel) or after 4 h restimulation with LCMV-peptide gp33 in the presence of brefeldin (right panel). (B–D) PKO and WT IFN-γ reporter mice (PKO/IFN, WT/IFN; 4 mice/group) were infected with 200 pfu LCMV-WE. (B) Blood samples were taken from tail vein on days 0 and 12 and stained for CD8 and Thy1.1. The gate used to define Thy1.1 negative T cells was set with CD8+ spleen cells from uninfected mice (A), the gate to define Thy1.1int cells was set above the main CD8+ T cell population in WT/IFN mice at day 12 after infection. CD8+ T cells with Thy1.1 expression above this threshold were defined as Thy1.1high (excessive IFN-γ producers). (C) Splenic CD8+ T cells were analyzed for expression of surface Thy1.1 and intracellular IFN-γ. Representative flow cytometry plots are shown for (A–C) and the respective frequencies are shown within each gate. (D) Thy1.1low, Thy1.1int and Thy1.1high CD8+ splenocytes of WT and PKO mice were flow cytometry sorted and subsequently analyzed by quantitative RT PCR for IFN-γ mRNA expression. Box plots show the mean IFN-γ expression and SD for the cell populations sorted from WT and PKO mice. Data shown in this figure are a representative illustration of data acquired in three independent experiments.

T cells from spleens of WT-IFN-γ and PKO-IFN-γ reporter mice. We found that high Thy1.1 expression corresponded well with high amounts of IFN-γ mRNA in the respective cells (Fig. 2D).

**The initial infectious trigger determines the cellular sources of excessive IFN-γ**

To understand the different HLH course triggered in PKO mice by infection with LCMV versus MCMV, we evaluated the contribution of different lymphocyte subsets to excessive IFN-γ production by determining Thy1.1 expression in IFN-γ reporter mice (representative gating shown in Supporting Information Fig. S1). We analyzed blood CD8+, CD4+, and NK cells at days 0, 5, 8, and 12 after LCMV (Fig. 3A and B) and at days 0, 3, 5, and 7 after MCMV infection (Fig. 3C). Following LCMV infection, the cell fraction producing excessive IFN-γ was highest in the CD8+ compartment, rising rapidly after day 5 to a maximum on day 8 and only slightly leveling off by day 12. CD4+ T cells followed a similar kinetic, but at a much lower level. The response was lowest in NK cells, where the difference between PKO and WT mice was only transiently significant at day 8 (Fig. 3B). Interestingly, the pattern was different after MCMV infection. While the fraction of CD8+ T cells producing excessive IFN-γ at days 5 and 7 was similar to the corresponding time points after LCMV infection, the CD4+ T cell contribution was higher. More
Figure 3. Cellular sources of IFN-γ differ in LCMV versus MCMV induced HLH. PKO/IFN and WT/IFN mice were either infected with 200 pfu LCMV-WE or $2 \times 10^5$ pfu MCMV-Smith i.v. Blood samples were taken from the tail vein on days 0, 5, 8, and 12 or days 0, 3, 5, and 7, respectively, and stained for CD4, CD8, NK1.1, and Thy1.1 and analyzed by flow cytometry. (A) Representative flow cytometry plots of Thy1.1 expression on CD8$^+$ T cells of LCMV-infected animals. Numbers indicate the frequencies of Thy1.1low, Thy1.1int, and Thy1.1high fractions in the CD8$^+$ populations defined as described in Fig. 2. (B) Frequencies of Thy1.1high expressing CD8$^+$ T cells, CD4$^+$ T cells, and NK cells (upper panel) and the mean fluorescence intensity (MFI) of the Thy1.1 expression level of the three lymphocyte populations (lower panel) over time after LCMV-infection or (C) after MCMV-infection. Data were pooled from three independent experiments with three to five mice per group. (B and C) Data are shown as mean ± SD. Statistical analysis was performed using Student’s unpaired t-test. $^{****}p < 0.001$. 
impressively, NK cells produced excessive IFN-γ in PKO and WT mice, but while production leveled off in WT, it remained high in PKO mice (Fig. 3C). Thus, in primary HLH the kinetics, extent and cellular sources of IFN-γ vary across the two triggering infections.

Excessively IFN-γ producing cells accumulate in the BM

The IFN-γ reporter mice allowed us to address the question of tissue-specific accumulation of different IFN-γ-producing lymphocyte subsets. While on day 12 after LCMV infection around 40% of CD8+ T cells in liver and spleen expressed excessive IFN-γ, this fraction was higher in BM (Fig. 4A and B). A similar albeit less pronounced pattern was observed for CD4+ T cells (Fig. 4B). The numbers of CD8+ T cells producing excessive IFN-γ appeared tenfold higher in spleen than in BM (Fig. 4A). However, only a single femur was flushed for the determination of BM cell counts, leading to underestimation [37]. Notably, the percentage of different lymphocyte subsets producing excessive IFN-γ and their organ distribution showed some differences at day 7 after MCMV infection (Fig. 4B, lower panel). While BM remained the site with the highest accumulation of excessive IFN-γ producing T cells, there was a higher relative contribution by CD4+ and NK cells in all organs.

The infectious trigger determines outcome in HLH-prone mice with an attenuated cytotoxicity defect

To further compare HLH pathophysiology after different triggers, we infected Lyst-mutant souris, stx11 deficient, and munc13-4 mutant jnxx mice, in which LCMV-induced primary HLH is attenuated [34] due to less severe cytotoxicity defects [12]. MCMV control in all organs was similar in all strains and only slightly higher than in B6 mice (Supporting Information Fig. S2A). Nevertheless, while all other strains survived, souris mice died after MCMV infection, (Supporting Information Fig. S2B), revealing that impaired virus control is not the only factor determining survival or death after MCMV infection. We chose souris mice for further comparative analysis. Inflammatory disease parameters such as sCD25, ferritin, and GPT were less pronounced in souris than in PKO mice upon LCMV and even more so upon MCMV infection (Fig. 5A–C). In line with this finding, the attenuation of IFN-γ production in souris compared to PKO mice was more pronounced after MCMV infection. This was reflected both by serum levels (Fig. 5E) and decreased IFN-γ reporter activity in CD8+ T cells in different organs (Fig. 5D). Reduced IFN-γ levels corresponded to better control of virus replication in souris versus PKO mice after MCMV, but not after LCMV infection (Fig. 5G). Surprisingly, however, despite attenuation of HLH disease parameters and improved virus control similar to WT mice, MCMV-infected souris mice showed weight loss similar to MCMV infected PKO mice and had to be eliminated at day 7. In contrast, LCMV-infected souris mice regained weight after day 12 and survived (Fig. 5F). Both, MCMV-infected PKO and souris mice demonstrated a higher percentage of NK cells in the liver (Supporting Information Fig. S3A) than LCMV-infected animals. In contrast, serum levels of TNF and IL-6 were not correlated with disease severity (Supporting Information Fig. S3B and C). Thus, in a primary HLH model with a less severe cytotoxicity defect, the infectious trigger determines disease outcome.

The infectious trigger impacts the success of targeted immunotherapy

Depletion of CD8+ T cells and IFN-γ blockade can ameliorate LCMV-induced primary HLH [10,17]. We confirmed that application of anti-CD8 or anti-IFN-γ antibodies during active disease prolonged the survival of LCMV-infected PKO mice (Fig. 6A). In contrast, both therapeutic approaches were not effective in MCMV-infected PKO mice (Fig. 6A). This was not due to a further increase in virus titers at the time of analysis (Fig. 6B). To confirm that the rapid death of MCMV-infected PKO mice was immune-mediated and not a mere consequence of MCMV-induced cytopathology, we infected RAG2−/− mice depleted of NK cells. These lymphocyte-deficient mice did not show any signs of disease including weight loss at day 7, when MCMV-infected PKO mice had to be eliminated because of severe disease. The mice eventually died after a mean of 18 days after infection. Thus, early lethality in PKO mice is immune-mediated and can be separated from MCMV lethality induced by viral cytopathogenicity that occurs significantly later in lymphocyte-deficient mice.

When analyzing HLH criteria in LCMV-infected PKO mice, anti-CD8 treatment improved five of six and anti-IFN-γ improved three of six evaluated disease parameters, while in MCMV-infected mice, only three of six and none of six criteria were improved (Fig. 7A; Supporting Information Fig. S4). Notably, leukopenia reverted to leukocytosis in anti-IFN-γ treated MCMV infected mice (Fig. 7B). Ferritin (Fig. 7C) and liver disease as reflected by GPT and LDH (Fig. 7D and E) were not significantly attenuated. Analysis of TNF-α (Fig. 7F) and IL-6 (Fig. 7G) revealed that IFN-γ blockade led to higher levels of these proinflammatory cytokines in MCMV than in LCMV infection, indicating that this intervention shifted, but did not control overall inflammation. These data show that depending on the inducing virus, the clinical syndrome of primary HLH can reflect different pathologies, leading to different responses to therapeutic interventions.

Discussion

In this study, we analyzed to which extent an infectious trigger dictates the pathology of the clinical syndrome of primary HLH and whether this has therapeutic implications. We established a model of MCMV infection in perforin-deficient mice fulfilling the diagnostic criteria for human primary HLH and compared this to the PKO-LCMV model. We chose MCMV, because similar to LCMV the virus is controlled by perforin [38–40], but immune cells mediating virus control and infection-associated tissue damage differ.
Figure 4. Excessively IFN-γ producing cells accumulate in the BM. PKO/IFN and WT/IFN reporter mice were either infected with 200 pfu LCMV-WE or with $2 \times 10^5$ pfu MCMV-Smith i.v. Mice were sacrificed on day 12 or 7 post infection, respectively, and blood, spleen, liver, and BM (obtained from a single femur) lymphocytes were analyzed for Thy1.1 surface expression by flow cytometry. (A) Representative flow cytometry plots of Thy1.1 expression on tissue CD8$^+$ T cells upon LCMV infection (left panel) with respective frequencies shown in each gate. Absolute numbers of Thy1.1$^{\text{high}}$ expressing CD8$^+$ T cells (right panel). (B) Absolute numbers of Thy1.1$^{\text{high}}$ expressing CD8$^+$ T cells per indicated organ. (C) Percentage of Thy1.1$^{\text{high}}$ cells among the indicated lymphocyte populations obtained from the indicated tissues on day 12 after LCMV (upper panel) or on day 7 after MCMV infection (lower panel). Data were pooled from two independent experiments with four mice per group (LCMV) and two and three mice per group (MCMV). (B and C) Data are shown as mean ± SD. Statistical analysis was performed using Student’s unpaired t-test. *$p > 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$.

CD8$^+$ T cells are essential for LCMV control [41], while MCMV control is organized more redundantly with prominent roles for NK, CD4$^+$, and CD8$^+$ T cells [29,40,42]. IFN-γ contributes to MCMV control [38,40], but plays a minor role in LCMV control [43]. In the absence of perforin, both infections are lethal [11,39]. After LCMV infection, lethality is driven by CD8$^+$ T cell- and IFN-γ-mediated immunopathology [10,11]. Immunopathology in MCMV infected PKO mice [30–32] is not improved by elimination of CD8$^+$ T cells or IFN-γ [30], but in part by TNF [30] or IL-18-neutralization [31]. However, these interventions are not sufficient to reduce early immune-mediated lethality [31]. Since cytopathic MCMV can cause more tissue damage than non-cytopathic LCMV [44,45], this could reflect additional direct viral cell damage. Infection of NK cell depleted RAG$^{-/-}$ mice showed that this lymphocyte-independent damage is not responsible for the early severe immunopathological disease observed in PKO mice. Nevertheless, it likely causes the later lethality observed in various immunodeficient mouse strains with impaired MCMV control [38,46–50]. Moreover, this viral cytopathogenicity is also expected to increase and shift the overall inflammatory response.
Building on these previous findings, we used several additional tools to relate the pathology induced by the two viral infections in PKO mice to human primary HLH. Several studies have noted clinical features compatible with HLH in MCMV infected PKO mice such as cytopenia, hemophagocytosis, or splenomegaly [30, 31, 38-40], but no study has formally evaluated all eight human diagnostic criteria [35]. Since no previous study has documented at least five criteria, required for HLH diagnosis in patients, a recent review concluded that MCMV does not induce bona fide primary HLH [25]. On the other hand, it was recently reported that MCMV induces five of eight HLH criteria in WT BALB/c mice, consistent with “secondary” HLH [26]. All these studies have used intraperitoneal infection and mostly crude salivary gland propagated virus, in which tissue-derived components induce variable virus-independent inflammation, particularly in the liver [51]. In our study, intravenous infection of PKO mice with tissue-cultured MCMV Smith [32] induced at least seven of eight HLH criteria. Since we did not specifically measure neutropenia, we could only document bi-lineage cytopenia as the eighth criteria in two-thirds of the animals. This allowed a quantitative comparison of HLH disease parameters, lymphocyte activation, and response to immunomodulatory treatment of LCMV- and MCMV-induced primary HLH.

We assessed the contribution of different lymphocyte populations to the hyperinflammatory response by their IFN-γ production, a key cytokine in primary HLH. The use of IFN-γ reporter mice allowed quantification of IFN-γ transcription in individual cells without restimulation [33]. Due to its slow turnover, membrane Thy1.1 expression may mark some cells that have only produced IFN-γ in the past [36]. Nevertheless, the fraction of Thy1.1 expressing cells was in the expected range [22, 23] and Thy1.1 expression still correlated with very high IFN-γ mRNA levels at day 12 after infection. The pattern of IFN-γ production differed between LCMV- and MCMV-infected PKO...
mice. Upon LCMV-infection, the IFN-γ-response was dominated by CD8+ T cells with minor contribution of CD4+ T and NK cells. CD8+ T cells also produced excessive IFN-γ after MCMV infection, but there was also significant contribution by CD4+ T and particularly by NK cells, confirming previous observations [30]. Strikingly, IFN-γ production by T cells differed between tissues. The most prominent accumulation of IFN-γ producing cells as well as the highest level of IFNγ expression per cell was observed in BM. Since immune cells recirculate, we cannot differentiate, whether this was due to excessive stimulation in BM or due to preferential BM homing of activated immune cells primed in other tissues. Preferential presence of IFN-γ high producing cells in the BM was also observed after MCMV infection, suggesting that it is a common feature of virus-induced primary HLH, likely associated to the characteristic cytopenia [11].

Interestingly, despite the different infection biology and different immune cell activation by LCMV versus MCMV, the HLH disease pattern as defined by the diagnostic criteria in PKO mice was similar after infection with the two viruses, albeit with different kinetics and extent of disease parameters. Differences in the extent of cytopenias may be explained by a higher fraction of CTL producing excessive amounts of IFN-γ in the BM after LCMV infection. Another difference was the more extensive systemic inflammation after MCMV infection illustrated by ferritin, triglycerides, and sCD25, possibly caused by different and higher levels of inflammatory mediators, including IFN-γ. Despite these differences, the overall clinical course was remarkably similar. Both viruses induced lethal disease fulfilling clinical HLH criteria in PKO mice, while not causing overt disease in WT mice.

Unexpectedly, despite these overlapping features, the response to three different interventions differed between the two infections. The attenuated cytotoxicity defect in souris mice improved virus control and HLH disease parameters including IFN-γ levels after MCMV even to a higher extent than after LCMV infection. Notably, this included serum levels of liver enzymes indicating attenuation of liver damage in mice with reduced MCMV replication. Nevertheless, while all LCMV infected souris mice survived, they succumbed to MCMV with similar kinetics as PKO mice. This was associated with increased NK cell infiltrations of the liver. Furthermore, elimination of CD8+ T cells or IFN-γ blockade after disease induction improved disease and survival in LCMV-infected, but had only minor effects on disease and did not improve lethality in MCMV-infected PKO mice. These observations indicate that MCMV-induced pathology in PKO mice, while associated with similar T cell activation and even higher IFN-γ production, is not exclusively dependent on these immune activation pathways. We did not fully resolve the cause of death in MCMV-infected PKO mice. The lack of disease signs in lymphocyte-deficient NK cell depleted RAG-deficient mice at a time point when PKO mice died with full-blown HLH clearly indicates that this early death (day 7) is largely immune-mediated. Nevertheless, the later death (days 15–20) observed in these and several other immunodeficient mice with impaired MCMV control illustrates an eventually severe additional direct virus-induced effect [38,46–50]. Leukocytosis and
Figure 7. T cell and IFN-γ targeted therapy improves inflammatory disease in LCMV-, but not in MCMV-induced HLH. PKO mice were either infected with 200 pfu LCMV-WE or with $2 \times 10^5$ pfu MCMV-Smith i.v. and treated with 200 μg α-CD8, 500 μg α-IFN-γ, or PBS i.v. as indicated in Fig. 6. On day 12 after LCMV- and day 7 after MCMV-infection mice were sacrificed and assessed for HLH disease parameters apart from cytotoxicity and hemophagocytosis. (A) The table shows the changes in these parameters relative to untreated mice by upward or downward arrows. The number of arrows reflects the level of significance of the parameter alteration. Grey fields indicate no alteration, white fields improvement, and black fields deterioration of a given parameter. (B) Peripheral white blood cell count measured using a Sysmex KX-21 hematology analyzer, (C) serum ferritin levels measured by immunoassay, (D and E) serum levels of GPT and LDH analyzed by photometry, (F and G) serum levels of inflammatory cytokines TNF-α and IL-6 measured by Legendplex FACS assay (Biolegend). (A–G) Data were pooled from three independent experiments with three to five mice per group. The variable number of data points per group is explained by death of some mice before final analysis. Data are shown as mean. Statistical analysis was performed using Student’s unpaired t-test. *p > 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.

increased TNF-α and IL-6 production in anti-IFN-γ treated MCMV-PKO mice as well as increased liver NK cells in MCMV infected versus LCMV infected souris mice also implicate additional layers of disease immunopathogenesis that are uncovered by neutralization of IFN-γ. Thus, in MCMV-PKO mice, overall disease includes, but is not limited to IFN-γ driven HLH features. Therefore, control of this “HLH component” of the pathology is not sufficient to control early MCMV-induced disease and lethality under conditions of perforin deficiency. It is possible that the direct viral cytopathogenicity of MCMV adds to the mainly immune-mediated pathology that fulfills the clinical criteria of HLH [27]. This interpretation is supported by the fact that some features associated with MCMV infection even in the absence of HLH, such as cytopenia and hepatitis, are also part of the HLH criteria. These features are “hidden” among the overall manifestations of immune-mediated HLH in PKO mice, but could also contribute to lethality even if the IFN-γ-driven HLH is largely attenuated.

What can these mouse models teach us for HLH patients? Although the current view on HLH pathogenesis has profited immensely from the LCMV-PKO model, its limitations are apparent. All patients with perforin null mutations become symptomatic in the first year of life, while PKO mice do not develop the disease spontaneously and even when infected with various viruses or bacteria—with exception of LCMV and MCMV, both of which cause persistent infections (S. Ehl, unpublished data). In at least two-thirds of patients with early onset primary HLH no persistent infection is detected [20]. Furthermore, human primary HLH can arise in utero or as a CNS-only disease, again usually in absence of a documented infection [20]. Almost a third of patients die despite immunosuppressive therapy [52]. This could imply that immune overactivation is not as uniform as suggested by the LCMV-PKO model and may not be the only factor contributing to disease in all primary HLH patients. The MCMV-PKO model does not fully resolve these issues and has additional limitations related to viral cytopathogenicity. Nevertheless, the comparative analysis of the MCMV-PKO model in this study illustrates that the relatively stereotypic inflammatory syndrome of primary HLH, can occur in association with an underlying triggering disease that makes its own contribution to the overall pathology. Targeted immunotherapeutic control of the inflammatory HLH component of the pathology may uncover this initially “hidden” part of the overall disease, with consequences that are not easy to predict.
Materials and methods

Mice and viruses

C57BL/6J mice were purchased from Janvier (Le Genest St-Isle, France). Perforin-deficient C57BL/6-Prf1tm1Sdz (PKO) mice were obtained from Dr. H. Hengartner (Zurich) [14]. LYST-mutated C57BL/6J-Lystbg-Btlr/Mmucd (souris) mice were obtained by Dr. B. Beutler [34]. Thy1.1 IFN-γ reporter mice (IFNγtm1(Thy1)Weav) were generated by Dr. C. Weaver (Birmingham, Alabama) [33]. Mice were kept under specific pathogen-free conditions. All mouse experiments were approved by the Regierungspäsidium Freiburg (G-14/117, G-17/169).

LCMV-WE was obtained from Dr. F. Lehmann-Grube (Hamburg) and grown on L929 cells. Mice were infected with 200 plaque-forming units ( pfu) LCMV-WE intravenously (i.v.). LCMV was quantified in organ homogenates using a standard focus-forming assay [53]. MCMV-Smith (ATCC VR-194) was propagated on SV40-immortalised mouse embryonic fibroblasts (SV40-MEFs). Mice were infected with 2×10^5 pfu i.v. MCMV titers were determined by plaque-forming assay on murine embryonic fibroblasts (MEFs) [54]. In brief, MEFs were seeded on 48-well plates to reach 70–80% confluency and then organ homogenate was added followed by centrifugation (30 min/800 rpm/RT). After 2 h incubation (37°C/5% CO₂), the organ homogenate was aspirated and replaced with medium. After 5 days incubation (37°C/5% CO₂) plaques were counted microscopically.

Clinical laboratory tests and evaluation of serum parameters

Body temperatures were obtained using an infrared ear thermometer (BRAUN, ThermoScan type 6022). Blood counts were determined by a Sysmex KX-21 hematology analyzer. GPT (glutamate pyruvate transaminase), triglyceride, and ferritin serum levels were analyzed using the Roche Modular Analytics Evo.

Parameter values were evaluated using Student’s unpaired t-test and considered significant at a p-value below *p < 0.05, **p < 0.005, ***p < 0.001, and ****p < 0.0001.

Antibodies and intracellular staining

Antibodies were purchased from eBioscience, BD Biosciences, BioLegend, or Bio X Cell. For intracellular cytokine staining, 10^6 lymphocytes were stimulated with 10^{-7} M LCMV GP33-peptide (KAENVFTAM) for 4 h in the presence of Brefeldin A (5 mg/mL), then surface-stained with anti-CD8a, anti-CD4, anti-NK1.1, or anti-Thy1.1 (Ox-7) antibody followed by fixation and permeabilization, using Cytotox/Cytoperm Kit (BD Biosciences) and anti-IFN-γ antibody staining. Sample analyses were performed using a FACSFortessa flow cytometer (BD Biosciences) and FlowJo software (Tristar). Data are displayed following the guidelines for the use of flow cytometry and cell sorting in immunological studies [55]. For CD8 T cell depletion, YTS169 antibody (200 μg) was administered i.v. 3 and 4 days after LCMV and MCMV infection. Depletion efficacy was confirmed by FACS analysis. NK cell depletion was performed by i.p. injection of 300 μg anti-mouse NK1.1 (Clone PK136; BioXcell) on d-1/d+1/d+8. IFN-γ was neutralized by injecting 500 μg of anti-IFN-γ (XMG-1) i.p. on days 6, 9, and 12 after LCMV or day 3, 6, and 9 after MCMV infection.

Quantitative RT-PCR

After cDNA synthesis with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), qRT-PCR was performed using KAPA™ SYBR® FAST qPCR Kit (peqlab) with an IFN-γ-specific primer pair (forward: 5'-GAGGTCAACAACCCACAGGT-3', reverse: 5'-GGGACAATCTCTTCCCCACCCAGGT-3'). Samples were run on an ABI Prism® 7900 sequence detector and analyzed with SDS2.4 software and RQ manager 1.2.1 (all Applied Biosystems).

Statistical analysis

Data were analyzed using Graphpad Prism 7 software. Differences were evaluated using Student’s unpaired t-test and considered significant at a p-value below *p < 0.05, **p < 0.005, ***p < 0.001, and ****p < 0.0001.

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Author contributions: RG has performed all major experiments and has contributed to drafting the manuscript. P.A., T.K., and J.R. provided experimental advice and reagents. R.Z., H.H., H.P., and C.W. provided mice, viruses, and reagents. A.S.G. performed immunohistological analysis. S.E. designed the study and written the manuscript. All authors have contributed to the manuscript and have approved the final version.

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Abbreviations: ATG: anti-thymocyte globulin · FHL: familial (primary) hemophagocytic lymphohistiocytosis · HLH: hemophagocytic lymphohistiocytosis · LCMV: lymphocytic choriomeningitis virus · MCMV: murine CMV · PKO: perforin knock-out

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