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Research Article

Primary and secondary hemophagocytic lymphohistiocytosis have different patterns of T-cell activation, differentiation and repertoire

Sandra Ammann[1,2], Kai Lehmberg[3], Udo zur Stadt[4], Gritta Janka[3], Anne Rensing-Ehl[1], Christian Klemann[1,5], Maximilian Heeg[1,5], Sebastian Bode[1,5], Ilka Fuchs[1], Stephan Ehl[1,5] for the HLH study of the GPOH

[1] Center for Chronic Immunodeficiency (CCI), Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Germany
[2] Faculty of Biology, University of Freiburg, Germany
[3] Pediatric Hematology and Oncology, University Medical Center Hamburg Eppendorf, Germany
[4] Center for Diagnostic, University Medical Center Hamburg Eppendorf, Germany
[5] Center for Pediatrics, Department of Pediatric Hematology and Oncology, University Medical Center, University of Freiburg, Faculty of Medicine, University of Freiburg, Germany

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening inflammatory syndrome characterized by hyperactivation of lymphocytes and histiocytes. T cells play a key role in HLH pathogenesis, but their differentiation pattern is not well characterized in patients with active HLH. We compared T-cell activation patterns between patients with familial HLH (1°HLH), 2°HLH without apparent infectious trigger (2°HLH) and 2°HLH induced by a viral infection (2°V-HLH). Polyclonal $CD8^+$ T cells are highly activated in 1°HLH and 2°V-HLH, but less in 2°HLH as assessed by HLA-DR expression and marker combination with CD45RA, CCR7, CD127, PD-1 and CD57. Absence of increased HLA-DR expression on T cells excluded active 1° HLH with high sensitivity and specificity. A high proportion of polyclonal $CD127^-CD4^+$ T cells expressing HLA-DR, CD57, and perforin is a signature of infants with 1°HLH, much less prominent in virus-associated 2°HLH. The similar pattern and extent of $CD8^+$ T-cell activation compared to 2° V-HLH is compatible with a viral trigger of 1°HLH. However, in most 1°HLH patients no triggering infection was documented and the unique activation of cytotoxic $CD4^+$ T cells indicates that the overall T-cell response in 1°HLH is different. This may reflect different pathways of pathogenesis of these two HLH variants.

Keywords: Hemophagocytic lymphohistiocytosis · HLA-DR · Perforin · Primary HLH · Secondary HLH · T-cell activation

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Correspondence: Dr. Stephan Ehl
e-mail: stephan.ehl@uniklinik-freiburg.de

Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a hyperinflammatory syndrome currently defined by a number of clinical and laboratory features that can develop in the context of various diseases [1]. About a third of patients with HLH have a monogenetic predisposition affecting lymphocyte cytotoxicity, including familial hemophagocytic syndromes (FHL2-5) [2], several albinism syndromes with immunodeficiency (GS2, CHS, HPS2) [3] and X-linked lymphoproliferative syndromes (XLP1+2) [4]. These conditions can be summarized as 1°HLH. EBV infection is a key trigger of the HLH syndrome in XLP, but EBV and other viral or bacterial infections can also trigger disease in patients with FHL or albinism syndromes [5]. However, in most FHL patients under the age of one year routine microbiological investigations do not detect an infectious agent in our German cohort. Therefore, it remains unclear if and which trigger is required for disease manifestation in these patients.

In the other two thirds of HLH cases, no clear genetic predisposition can be identified apart from a few patients with inborn errors of metabolism or primary immunodeficiencies (2°HLH) [1, 6]. A possible contribution of monoallelic mutations in FHL associated genes remains debated [7, 8] (Ammann et al., in preparation). About a quarter of 2°HLH patients have underlying diseases associated with immune activation such as autoimmune or autoinflammatory disease or malignancy [9]. In these cases, infections, in particular with viruses, are frequent additional disease triggers. Secondary HLH induced by various infections, most prominently EBV and leishmania [1], without obvious underlying disease, are responsible for an additional 50% of cases with 2° HLH. In the remaining quarter of 2°HLH in our German cohort, neither a clear disease-associated condition nor a triggering infection is reported (Ammann et al., in preparation).

Hyperactivation of lymphocytes and histiocytes are eponymous features of HLH. This is reflected by infiltrations of T cells and macrophages in HLH target organs such as the liver [10], bone marrow, and brain, as well as by the detection of excessive levels of inflammatory cytokines produced by these cells [11]. Studies in perforin knock-out mice have shown that T cells, mainly $CD8^+$ T cells are critical for disease pathogenesis [12]. Both $CD4^+$ and $CD8^+$ T cell populations are significantly more activated in virus-infected mice with genetic defects of cytotoxicity than in virus-infected controls [13]. These studies have defined T-cell hyperactivation as a key feature of 1°HLH providing a rationale for treatment approaches mainly targeting activated T cells such as ATG [14], etoposide [15] or alemtuzumab [16]. It remains far from clear, however, whether activated T cells are as important in 2°HLH. Elevated sCD25 is frequently taken as indirect evidence of T-cell activation in HLH, but CD25 can also be shed from other cells including non-hematopoetic cells [17, 18] and its correlation to cellular markers of T-cell activation remains to be examined.

We hypothesize that the activation and differentiation phenotype of T-cell populations in the different human HLH variants may mirror their involvement in disease pathogenesis. Underlying defects in cytotoxicity may lead to a phenotypic "footprint" in the T-cell compartment. Furthermore, patterns of T-cell activation may differ between virus-triggered and non-infection-associated HLH. Hence, we analyzed markers of T-cell activation and differentiation in patients with 1°HLH in comparison to patients with non-infection associated 2°HLH and patients with virus-triggered 2°HLH (2°V-HLH). We show that despite similarities to 2°V-HLH in $CD8^+$ T cells, the activation profile of $CD4^+$ T cells is a unique signature of 1°HLH and may indicate a different pathogenesis.

Results

Patient population recruited for this study

Between 2012 and 2015, we recruited 93 patients who fulfilled at least 5/8 clinical HLH criteria into this study (Supporting Information Table 1A). 31 patients were classified as 1° HLH. This included 11 patients with FHL-2, 12 patients with FHL-3, 1 patient with FHL-4, 4 patients with FHL-5, two patients with GS2 and 1 patient with CHS. Only three of these patients had proven viral infections, two with EBV and one with CMV. Thirty patients were classified as 2° HLH, all without infections. They included 12 patients with MAS, 1 patient with metabolic disease, 1 patient with osteopetrosis and A91V mutation in perforin and 16 patients who could not be assigned a clear underlying disease. Thirty-two patients were classified as 2° V-HLH, 4 of them had underlying diseases. Among these 32 patients, 19 had evidence of acute EBV infection by serology and/or PCR. Other acute viral infections diagnosed in the context of HLH by PCR included CMV (4), ParvovirusB19 (3), Adenovirus (2), HHV6 (2), Coronavirus (1), Parainfluenza (1), HSV (1), RSV (1). Two patients had positive PCR for two viruses (HHV6+CMV and EBV+CMV) (Supporting Information Table 1a). HLA-DR expression on $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells and sCD25 levels (by chemoluminescence) from the serum, were measured in all 93 patients, since the age-dependent variability of HLA-DR expression and sCD25 level in healthy donors is limited. For all other investigations, we only included patients younger than one year (18 1°HLH, 6 2°HLH, 6 2°V-HLH) because of the extensive variability in T-cell differentiation marker expression beyond that age.

High HLA-DR expression on $CD8^+$ and $CD4^+$ T cells is characteristic for 1° HLH

While naïve human T cells do not express MHC class II molecules such as HLA-DR, TCR mediated activation as well as a number of cytokines, in particular type I and type II interferons, but also IL-2, IL-12 and GM-CSF can upregulate HLA-DR expression [19, 20]. We analyzed HLA-DR expression on $CD4^+$ and $CD8^+$ T cells in patients with active HLH ($n = 93$) in the absence of immunosuppressive therapy. This included patients with 1° ($n = 31$) and 2°HLH with ($n = 32$) or without documented viral infection ($n = 30$) as summarized in Table 1. Irrespective of age, healthy donors show HLA-DR expression on $CD4^+$ or $CD8^+$

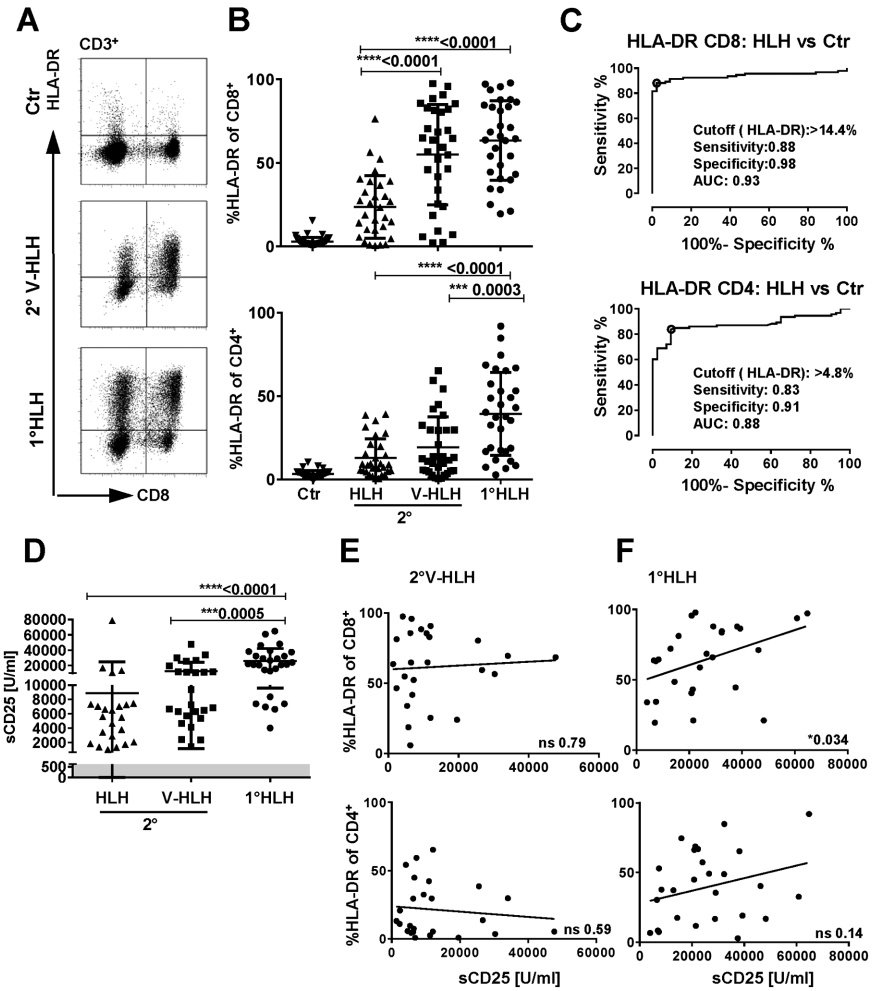


Figure 1. Activation state of CD4+ and CD8+ T cells by HLA-DR expression and soluble CD25 levels. (A-C) PBMCs from healthy controls (Ctr), patients with 2°HLH with viral infection (2°V-HLH) or without viral infection (2°HLH) and patients with primary HLH (1°HLH) were analyzed by flow cytometry. PBMCs of all groups were analyzed once for each individual, no replicates were performed. Data were pooled and grouped from Ctr: $n = 43$, 2°HLH: $n = 30$, 2°V-HLH: $n = 32$, 1°HLH: $n = 31$ donor samples. (A) Representative dot-plots showing HLA-DR expression by flow cytometry on $CD8^+$ and $CD8^-$ T cells from Ctr, 2°V-HLH and patients with 1°HLH. Plots were gated on $CD3^+$ lymphocytes. (B) Percentage of HLA-DR^+ $CD8^+$ T cells among total $CD3^+CD8^+$ T cells (upper panel) and HLA-DR^+ $CD4^+$ T cells among total $CD3^+CD4^+$ T cells (lower panel) in the indicated groups of patients. Differences between the three groups were analyzed by Mann–Whitney U test. Only significant differences are shown (defined as ***for $p \leq 0.0005$ and ****for $p < 0.0001$). (C) ROC curves showing the cut-off for optimal sensitivity and specificity for % HLA-DR^+ $CD8^+$ (upper panel) and $CD4^+$ T cells (lower panel) for patients with any form of HLH and controls. (D) Serum level of soluble CD25 (sCD25) were measured by Chemiluminescence Immunoassay (CLIA) patients with 2°HLH ($n = 23$), 2°V-HLH ($n = 25$) and 1°HLH ($n = 27$) given in U/mL. Differences between the three groups were analyzed by Mann–Whitney U test mean +/− SD is shown. Only significant differences are shown (defined as ***for $p \leq 0.0005$ and ****for $p < 0.0001$). (E) Correlation of sCD25 levels and percentage of HLA-DR expressing $CD8^+$ T cells (upper panel) or $CD4^+$ T cells (lower panel) in patients with 2°V-HLH. (F) Correlation of sCD25 and percentage of HLA-DR expressing T cells in patients with 1°HLH. Linear regression analysis was performed for all data pairs and the level of significance is indicated.

T cells below 12% [21, 22] (Fig. 1A). The proportion of T cells with increased HLA-DR expression was significantly higher in patients with HLH. Among $CD8^+$ T cells, this was moderate (mean 21%) in patients with 2°HLH in the absence of an apparent infection, but much higher (median 61.5%) in patients with 2°HLH triggered by a virus infection (2°V-HLH) as well as in patients with 1°HLH (mean 64.4%) (Fig. 1B, upper panel). T-cell activation was not significantly different between the 19 patients with EBV-associated HLH and the 13 patients with HLH in the context of other viral infections (data not shown). Interestingly, the pattern of HLA-DR expression was different among $CD4^+$ T cells, which showed a moderate increase in the fraction of HLA-DR expressing cells in both groups of 2° HLH patients (mean 8.7 and 12%), while it was much higher (mean 37.8%) in patients with 1°HLH (Fig. 1B, lower panel). We performed ROC analysis to determine the cut-off for optimal sensitivity and specificity of HLA-DR in discriminating between healthy donors and patients with any form of active HLH. A percentage of HLA-DR expression below 14% among $CD8^+$ T cells and 5% among $CD4^+$ T cells achieved sensitivities and specificities well above 80%. However, HLA-DR expression could not differentiate between 1° and 2°V-HLH (Fig. 1C).

Poor correlation of sCD25 serum levels with T-cell activation as assessed by HLA-DR expression

An increased serum level of soluble IL-2 receptor α chain (sCD25) is a characteristic marker of immune activation in HLH. sCD25 is shed from activated T cells, but is also expressed by NK cells, B cells, macrophages, dendritic cells and non-immune cells such as fibroblasts and pulmonary endothelial cells [23]. It therefore remains unclear to which extent sCD25 levels reflect T-cell activation in different HLH conditions. sCD25 serum levels were highly elevated in all three groups of patients. However, 1°HLH patients had significantly higher serum levels of sCD25 (all above 6000 U/mL) compared to both 2°HLH groups (Fig. 1D). Unexpectedly, there was a poor correlation between HLA-DR expression on $CD4^+$ or $CD8^+$ cells and sCD25 levels in all patient groups (Fig. 1 E,F). The only significant correlation was observed between the fraction of HLA-DR^+CD8^+ T cells and sCD25 levels in patients with 1° HLH (Fig. 1F). This poor correlation could either be explained by the fact that we only looked at the percentage of circulating T cells and not at their absolute numbers, including activated T cells in lymphoid tissues, or point to other sources of sCD25 than T cells.

$CD4^+$ T cells show more advanced differentiation in 1°HLH than in virus induced 2°HLH

We then addressed the question whether the differences in HLA-DR expression were reflected in the T-cell differentiation pattern in the different subtypes of HLH. According to Appay [24], naïve T cells were defined as $CD45RA^+CCR7^+$, central memory cells (TCM) as $CD45RA^-CCR7^+$, effector-memory (TEM) cells as $CD45RA^-CCR7^-$ and terminally differentiated or effector T (TE) cells as CD45RA+CCR7- (Fig. 2A). Since the variability of the percentage of naïve and memory cells increases with age [25], we restricted our analysis to patients and controls younger than one year of age (Supporting Information Table 1B). Patients with 2°HLH without apparent infection ($n = 6$) had about 90% naïve $CD4^+$ and $CD8^+$ T cells, similar to healthy controls and to asymptomatic patients with 1°HLH. In contrast, the majority of $CD8^+$ T cells had an effector-memory phenotype in about 60% of patients with 1°HLH and 50% of patients with 2°V-HLH ($n = 6$). Advanced T-cell differentiation was less pronounced among $CD4^+$ T cells, where a predominant effector-memory phenotype was observed in 14.5% in patients with 2°V-HLH. Notably, this was more pronounced in patients with 1°HLH (35%) (Fig. 2B). Overall, the pattern of T-cell activation was variable between different individuals (Supporting Information. Fig. 1), probably favored by the fact that samples were taken at different time points in the course of the disease.

Two further prominent phenotypic changes after T-cell activation are downregulation of the IL-7 receptor alpha chain (CD127) [24, 25] and upregulation of the inhibitory receptor PD-1. Consistent with the results obtained above, patients with 2°HLH without a viral trigger had low percentages of PD-1^+ expressing $CD4^+$ or

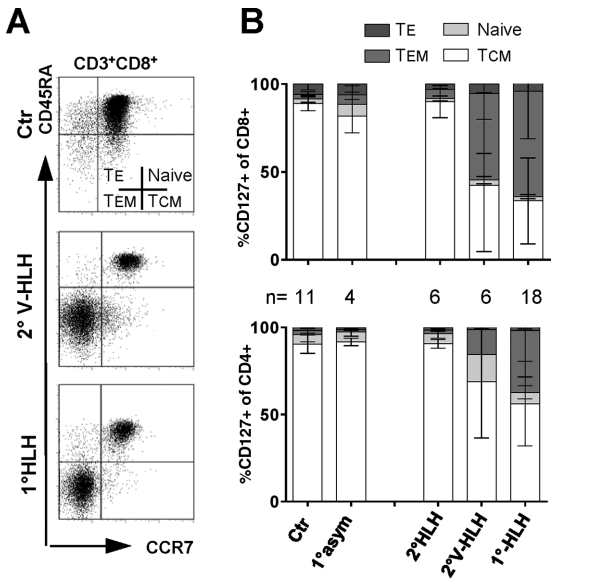
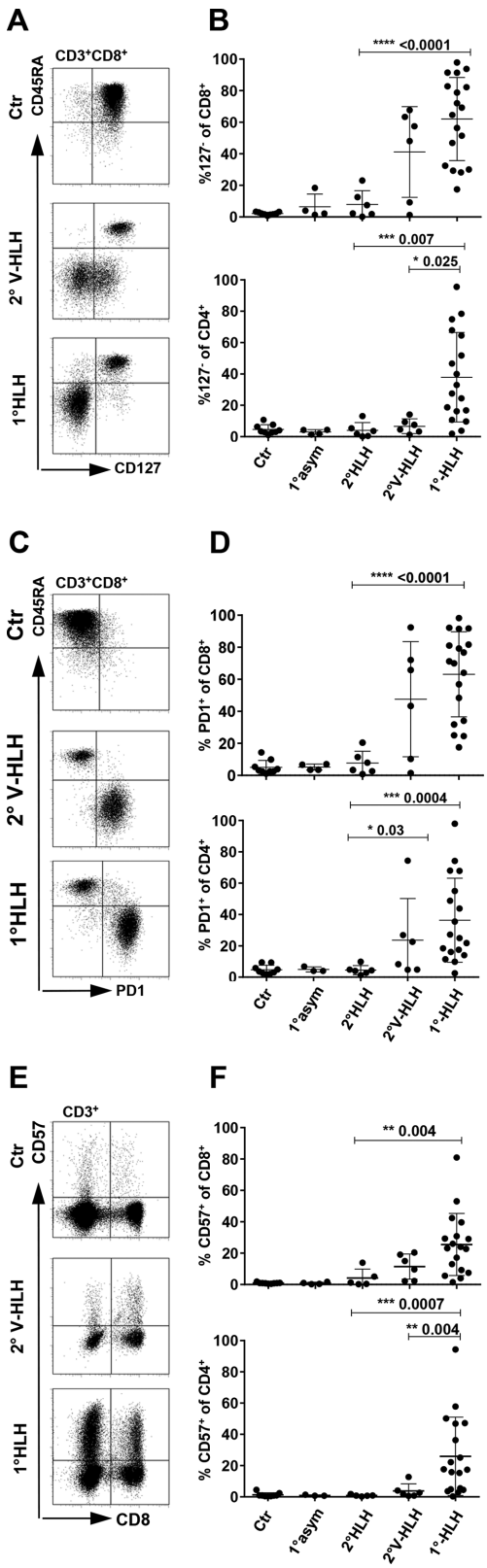


Figure 2. $CD8^+$ T-cell differentiation is similar in 1° and 2° V-HLH. PBMCs from healthy controls (Ctr), asymptomatic patients with a genetic defect (1°asym), patients with 2°HLH without viral infection (2°HLH) or with viral infection (V-2°HLH) and patients with primary HLH (1°HLH) were analyzed by flow cytometry. PBMCs of all groups were analyzed once for each individual, no replicates are performed. Data were pooled and grouped from Ctr ($n = 9$), 1° asym ($n = 4$), 2°HLH ($n = 6$), 2°V-HLH ($n = 6$) and 1°HLH ($n = 18$) donor samples. (A) Representative dot-plots of naïve ($CD45RA^+$,$CCR7^+$), central memory TCM ($CD45RA^-$, $CCR7^+$), effector-memory TEM ($CD45RA^-$,$CCR7^-$) and effector TE ($CD45RA^+$,$CCR7^-$) $CD8^+$ T cells of a healthy control (Ctr), a patient with 2°V-HLH and a patient with 1°HLH. Plots are gated on $CD3^+CD8^+$ T cells. (B) Summary of differentiation state of $CD3^+$ $CD8^+$ (upper panel) and $CD3^+$ $CD4^+$ (lower panel). The mean fraction (+/−SD) of each of the 4 cell populations in a given patient group is shown.

$CD8^+$ T cells, similar to healthy donors or asymptomatic patients with 1° HLH (Fig. 3C, D). In contrast, in patients with 1°HLH or patients with 2°V-HLH, the percentage of $PD1^+$, as well as $CD127^-$ cells was highly increased (Fig. 3 A–D). Again, changes in the $CD4^+$ T-cell population were more pronounced in 1° HLH, although the difference to 2°V-HLH only reached statistical significance for CD127 expression (Fig. 3B).

CD57 is an epitope on human T cells that strongly correlates with their proliferative history [26]. $CD57^+$ T cells produce cytokines and are cytotoxic [27], but poorly proliferate in vitro [26]. As expected, almost no $CD57^+$ T cells were detected in healthy infants or asymptomatic 1°HLH patients and this was similar in patients with 2°HLH (Fig. 3 E+F). In contrast, $CD57^+$ T cells were increased in patients with virus-induced 2°V-HLH and particularly in 1°HLH (Fig. 3F). Again, among $CD4^+$ T cells, CD57 expressing cells were significantly higher in 1°HLH as compared to 2°V-HLH.

Of note, all infants classified as 2°HLH without apparent microbial trigger had little evidence of T-cell activation (HLA-DR and differentiation markers), despite the fact that they fulfilled current clinical diagnostic HLH including sCD25 levels between 5,000-12,000 U/mL.



Perforin expressing CD4+ T cells are a signature of 1°HLH

Perforin expression is mainly induced after activation of $CD8^+$ T cells but can also be detected in terminally differentiated human $CD4^+$ T cells during chronic infections [28, 29]. We analyzed perforin expression in our patient cohort. Besides all infants with degranulation defects, we also included 2 patients with biallelic perforin mutations, who had reduced, but sufficient residual protein expression to separate perforin-positive from perforin-negative T cells. While the proportion of perforin expressing $CD8^+$ T cells was low in controls, it was variably elevated in patients with 2°HLH, irrespective of a viral trigger (Fig. 4 A, B). The highest proportion of perforin-expressing CTL was observed in patients with 1°HLH. This difference was much more pronounced in the CD4 compartment (Fig. 4B). Perforin-positive T cells, in particular $CD4^+$ T cells, are usually characterized by downregulation of CD27 and CD28 [28]. Interestingly, among the 1°HLH infants, in 6/10 $CCR7^-CD45RA^-$ T cells (representing the $perforin^+$ population as shown in 4/4 patients) had a $CD27^+CD28^+$ and 4/10 a mixed $CD27^+CD28^+$ and $CD27^-CD28^+$ phenotype (Fig. 4C+D), indicating that these cells represent early/intermediate differentiated acute effector $CD4^+$ T cells [24, 28].

Moderate perturbation of the T-cell repertoire in 1° and 2°V-HLH

Strong antigen-specific activation of T cells can result in repertoire alterations due to preferential expansion of antigen-specific T-cell populations [29]. We analyzed the T-cell receptor

Figure 3. A high percentage of $CD127^-PD1^+CD57^+$ $CD4^+$ T cells are characteristic for 1°HLH. PBMCs from healthy controls (Ctr), asymptomatic patients with a genetic defect (1°asym), patients with 2°HLH without viral infection (2°HLH) or with viral infection (V-2°HLH) and patients with primary HLH (1°HLH) were analyzed by flow cytometry. PBMCs of all groups were analyzed once for each individual, no replicates were performed. Data were pooled and grouped from Ctr ($n = 9$), 1° asym ($n = 4$), 2°HLH ($n = 6$), 2°V-HLH ($n = 6$) and 1°HLH ($n = 18$) donor samples. (A) Representative dot-plots of CD127 and CD45RA expression gated on $CD3^+CD8^+$ T cells in a healthy control (Ctr), a patient with 2°V-HLH and a patient with 1°HLH. (B) Percentage of $CD127^-$ cells among $CD8^+$ (upper panel) and $CD4^+$ (lower panel) T cells in the indicated patient groups. (C) Representative dot-plots of PD1 and CD45RA expression gated on $CD3^+CD8^+$ T cells in a healthy control (Ctr), a patient with 2°V-HLH and a patient with 1°HLH. (D) Percentage of $PD1^+$ cells among $CD3^+CD8^+$ (upper panel) and $CD3^+CD4^+$ (lower panel) T cells in the indicated control and patient groups. (E) Representative dot-plots of CD57 expression gated on $CD3^+CD8^+$ T cells in a healthy control (Ctr), a patient with 2°V-HLH and a patient with 1°HLH. Plots are gated on $CD3^+$ lymphocytes (F) Percentage of $CD57^+$ cells among $CD8^+$ (upper panel) and $CD4^+$ (lower panel) T cells in the indicated control and patient groups. Significance was defined by Mann–Whitney U test as *for $p \leq 0.05$, **for $p \leq 0.005$, ***for $p \leq 0.0005$ and ****for $p < 0.0001$. Data are shown as mean +SD and are from a single experiment.

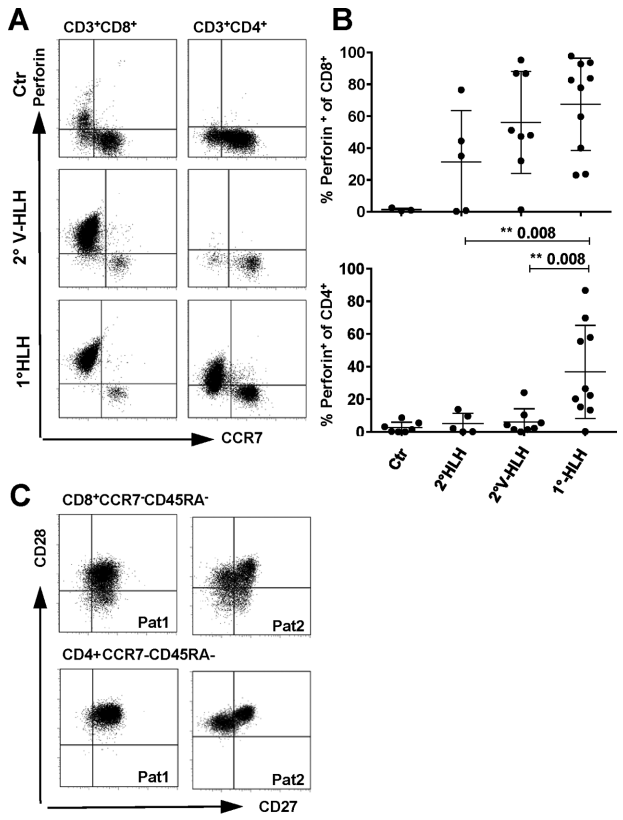


Figure 4. A high percentage of perforin-expressing CD4$^+$ T cells is characteristic for 1° HLH. PBMCs from healthy controls (Ctr), patients with 2°HLH without viral infection (2°HLH) or with viral infection (V-2°HLH) and patients with primary HLH (1°HLH) were analyzed by flow cytometry. PBMCs of all groups were analyzed once for each individual, no replicates were performed. Data were pooled and grouped from Ctr (CD8$^+$ $n = 3$ and CD4$^+$ $n = 7$ (4 beyond 1year), 2°HLH ($n = 5$), 2°V-HLH ($n = 8$ (2 beyond 1year) and 1°HLH ($n = 10$) donor samples. (A) Representative dot-plots of intracellular perforin versus CCR7 expression in CD3$^+$CD8$^+$ (left panels) and CD3$^+$CD4$^+$ (right panels) T cells in a healthy control (Ctr), a patient with 2°V-HLH and a patient with 1°HLH. (B) Percentage of perforin$^+$ cells among CD3$^+$CD8$^+$ (upper panel) and CD3$^+$CD4$^+$ (lower panel) T cells in the indicated control and patient groups (for 1° HLH, the analysis was restricted to MUNC13-4 and MUNC18-2 deficient patients and perforin deficient patients with residual protein expression). Data are shown as mean +SD and are from a single experiments. Differences between the three HLH groups were analyzed by Mann–Whitney U test U test as *for $p \leq 0.05$, **for $p \leq 0.005$, ***for $p \leq 0.0005$ and ****for $p < 0.0001$. Mean +/− SD is shown. (C) Representative dot-plot of CD27 and CD28 expression gated on CCR7$^-$ CD8$^+$ (upper panel) and CCR7$^-$ CD4$^+$ (lower panel) T cells in two patients representing different patterns of expression: one patient with MUNC13-4 deficiency (P1; same patient as in A) and one patient with perforin deficiency (with residual protein expression (P2).

repertoire in HLH patients using antibodies to the 24 most common TCR V-beta chains. Patients classified as 2°HLH without apparent infectious trigger showed a V beta repertoire among CD4$^+$ and CD8$^+$ T cells that was similar to that of control infants (Fig. 5). Compared to these controls, patients with 2°V-HLH had more V beta chain populations that were represented more than 2 standard deviations above the mean of normal donors (mean + 2 SD), indicating a moderate perturbation of the normal repertoire. This picture was similar in 6 patients with 1°HLH. In a single patient, we found a strong dominance of a V beta 3 expressing CD8$^+$ T-cell population (Fig. 5). The TCR repertoire analysis did not reveal obvious general differences between 1° and 2°V-HLH.

Discussion

To characterize patterns of immune activation in patients with different forms of HLH, we performed a phenotypic comparison of their T-cell compartments. Our observations (summarized in Table 1) support three main conclusions: First, T-cell activation is a hallmark of many defined forms of HLH, most prominent in 1°HLH and 2°V-HLH, but also in other forms of 2°HLH including MAS. However, disease states meeting all current diagnostic criteria for the syndrome of HLH can occur without obvious evidence of T-cell activation. Second, the pattern of CD8$^+$ T-cell activation in 1°HLH, in most cases in the apparent absence of a microbial trigger, is very similar to that in 2°HLH triggered by a viral infection. Third, strong activation of cytotoxic CD4$^+$ T cells is a signature of 1°HLH that is much less pronounced in virus-triggered 2°HLH. These findings are relevant for understanding the pathogenesis of different HLH variants and have implications for their differential diagnosis.

Previous studies have reported increased HLA-DR expression on T cells [30–32] and a mild decrease in the percentage of CD45RA$^+$ CD4$^+$ T cells [31, 33] in children with active HLH. However, this has not been analyzed in larger cohorts with clearly defined disease subtypes and the current possibilities to confirm genetic disease. Here, we observed a strong activation of the CD8$^+$ T-cell compartment in patients with 2°V-HLH and 1°HLH, while this was less pronounced in patients with 2° HLH in the absence of infections, including patients with MAS. In some infants, disease states fulfilling all current clinical criteria of 2° HLH even occurred in the absence of any measurable T-cell activation. In patients with 1°HLH and 2° V-HLH, activation was polyclonal with a mild perturbation of the normal repertoire. A large fraction of CD8$^+$ T cells had a CCR7$^-$CD45RA$^-$ effector memory phenotype that was associated with downregulation of CD127, and upregulation of HLA-DR, PD-1, CD57, and perforin. This differentiation pattern is similar to that of virus-specific CD8$^+$ T cells in patients undergoing an acute virus infection in the absence of HLH. In CMV infection, the percentage of CD127$^-$ cells correlates with the level of viral replication [34] and incomplete virus control increases the fraction of CD57$^+$ CD8$^+$ T cells [26]. CD57 defines T cells with a high lytic granule content and cytotoxic activity [27]. Thus, the CD8$^+$ T cell phenotype that was highly represented in 1°HLH and 2° V-HLH (CCR7$^-$ CD45RA$^-$ CD127$^-$ HLA-DR$^+$ PD-1$^+$ CD57$^+$ and perforin$^+$) is a phenotype that is also observed in patients with highly active, uncontrolled viral infections in the absence of features of HLH [35–37].

Interestingly, however, in our study, only 3 of 31 infants with 1°HLH had a proven concomitant viral infection and apart from 1 patient with periungual S. aureus infection, no bacterial or opportunistic infections were detected. This was less than expected from a previously reported cohort of 122 patients with HLH, in which 25 patients with a positive family history were reported to have

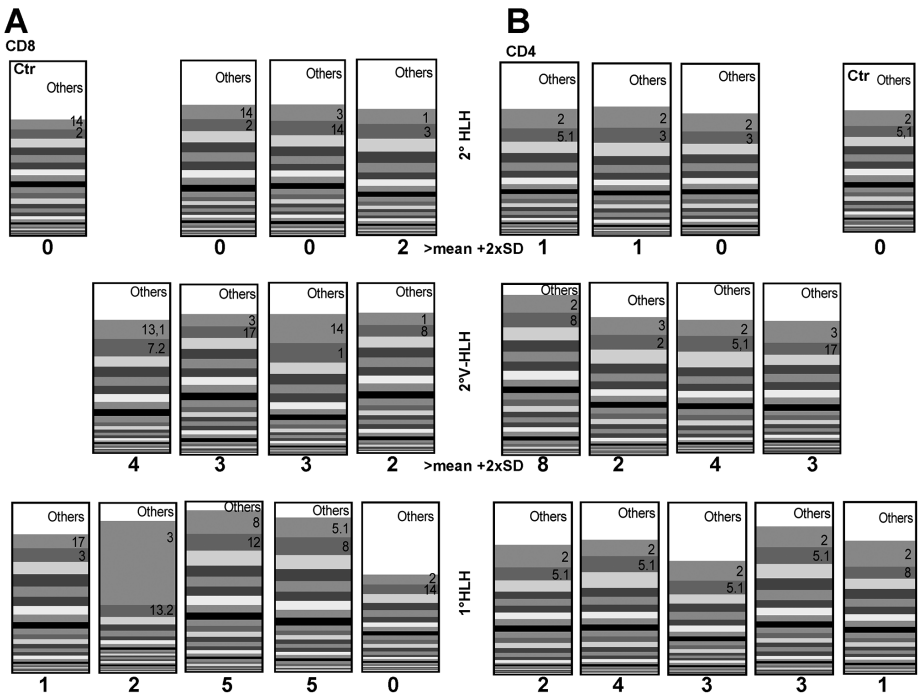


Figure 5. Moderate perturbation of the V beta repertoire analyses among CD8$^+$ and CD4$^+$ T cells in 1° and 2°V-HLH (A, B) Relative expression of the 24 most common TCR V beta chains on CD8$^+$ (A) and CD4$^+$ (B) T cells. T cells were analyzed by flow cytometry. In the individual charts, each vertical bar represents a fraction of T cells expressing one of the 24 chains, "others" indicates the remaining T-cell population not covered by the 24 antibodies. The bars are ordered in increasing size and not according to the V beta chain they represent. The chain numbers of the 2 most abundant V beta expressing populations are indicated in the individual charts. Below each chart, the number of V beta populations extending to more than 2SD above the mean value of controls is indicated. The chart labeled Ctr represents the mean value of controls, the other charts in the upper lane represent three infants with 2°HLH, the charts in the middle lane represent 4 infants with 2°V-HLH and the charts in the lower lane six infants with 1°HLH.

Table 1. Summary of T cell phenotype in different HLH variants

Group		HLA-DR	% EM	CD127-	CD57	Perforin
1°	CD8	+++	+++	+++	++	+++
2°V-HLH	CD8	+++	+++	++	-	+++
2°HLH	CD8	+	-	-	-	+
1°	CD4	++	++	++	++	+++
2°V-HLH	CD4	+	+	-	-	-
2°HLH	CD4	+	-	-	-	-

an infectious trigger [5]. Obviously, although in most patients an extensive search for systemic viral infections including EBV, CMV, adenovirus, HHV-6, and HHV-8 and Parvovirus has been performed, these investigations do not rule out all infections, particularly with respiratory or gastrointestinal viruses. It is unclear, however, whether a local infection without systemic spread is sufficient to trigger and maintain the very strong systemic T-cell activation observed in 1°HLH. At least in perforin-deficient PKO mice, only systemic persistent infection with LCMV or MCMV can induce HLH [12, 38], while local infections with respiratory viruses (RSV, pneumonia virus of mice, influenza) do not cause HLH in PKO mice [39]. Overall, it therefore remains a relevant possibility that the highly activated T-cell phenotype observed in human 1°HLH is in most cases not driven by a persisting viral (or other microbial) infection. This would imply that in humans, perforin-mediated control of T-cell stimulation is required for maintenance of T-cell homeostasis even in the absence of continued viral stimulation.

Such pathophysiological differences between 1°HLH and 2°V-HLH are also suggested by a different pattern of CD4$^+$ T cell activation in the two conditions. In 1°HLH, the percentage of CD4$^+$ T cells with a highly activated HLA-DR$^+$CD127$^-$PD1$^+$CD57$^+$perforin$^+$ phenotype was higher than in 2°V-HLH. Although perforin-expressing CD4$^+$ T cells with cytotoxic activity have been described in persistent virus infections such as HIV, EBV and CMV infections [28, 40] in none of these conditions these cells reach the levels we observed in primary HLH. The stronger activation of CD4$^+$ T cells in 1°HLH than in 2°V-HLH suggests qualitative differences in the activation of T-cell populations. Thus, the cytotoxicity defect and the associated impaired elimination of stimulating APC [41] could favor additional recruitment of CD4$^+$ T cells into the inflammatory response through prolonged presentation of antigen via MHC class II. What is the function of the cytotoxic CD4$^+$ T cells? It has been speculated that they play a role in containing viral infections tropic for MHC class II positive cells such as EBV in B cells [40]. They could also take over a prominent role in antiviral responses to pathogens that have evolved mechanisms to evade antigen presentation on MHC class I molecules [42]. However, in view of the above considerations, it is also conceivable that these cytotoxic CD4$^+$ T cells have a role in human T-cell homeostasis that is independent of infection control, e.g. by controlling APC [41].

A relevant number of infants without cytotoxicity defects fulfilling all current diagnostic criteria for HLH in the absence of an infection had mostly naive CD4$^+$ and CD8$^+$ T cells with very few cells expressing markers of activation or advanced differentiation. They all had sCD25 levels in the range of 5000–12 000 U/mL, but it is well known that immune cells other than T cells as well as non-immune cells [23] can significantly contribute to sCD25 production. In fact, we have recently documented similar sCD25 levels in immunodeficient patients fulfilling clinical HLH criteria that completely lacked T and NK cells [6]. In the current study, we have formally labeled these patients as 2°HLH, but as discussed previously, it is questionable whether the hemophagocytic

inflammatory syndrome in these patients should really be called HLH. This study further illustrates, that the current clinical criteria for HLH cannot differentiate between T cell driven disease ("bona fide" HLH) and hemophagocytic syndromes without T-cell activation [6]. Because of obvious implications for therapy, in particular if targeting T cells, a revision and prospective validation of the diagnostic criteria is needed. Our data suggest that inclusion of markers of T-cell activation should be considered. Their use may help avoid too aggressive treatment of patients that according to current criteria are inappropriately classified as suffering from "lymphocytic" histiocytosis.

Materials and methods

Patient recruitment

Patients were recruited to this project through the HLH study of the German Society of Pediatric Hematology and Oncology, details of which have been described elsewhere (Ammann et al., in preparation). The study was carried out after obtaining institutional review board approval (University of Freiburg ethics committee's protocol numbers 143/12 and 40/08). Control samples from healthy donors under 1 year of age were obtained from a clinic of HIV exposed (but not infected) infants (ethics number 282/11).

Patient characterization, immunological, microbiological, and genetic investigations

Patient inclusion required active HLH at the time of blood sampling, i.e. at least 5 of the 8 criteria defined by the Histiocyte Society. Patients who had received steroids for > 3 days or patients who had received cyclosporine or etoposide were excluded. All patients had microbiological investigations. Recommendations included blood cultures and screening for EBV, CMV, Adenovirus, HHV-6, and HHV-8 and Parvovirus by serology and PCR, but the extent of these investigations was not controlled in this study. As described (Ammann et al., in preparation), all patients had degranulation assays with "fresh" (uncultured) and "activated" (incubated with 100 U/mL IL-2 for 48–72 h) NK cells [43]. In parallel, stains for perforin, SAP and XIAP expression were performed. DNA from patients with abnormal protein expression was sequenced for mutations in *PRF1*, *SH2D1A* or *BIRC4A*. In patients with abnormal degranulation we analyzed *UNC13D*, including selected intronic regions [44, 45], *STXBP2* and *STX11* [46]. In patients with albinism we sequenced *RAB27A*, *LYST,* and *AP3B1*. Exome sequencing was performed in unresolved cases with defective fresh NK degranulation as described [47].

Final patient classification

Patients with reduced perforin expression or defective NK cell degranulation and biallelic mutations in a gene associated with FHL2-5, CHS, or GS2 were classified as 1°HLH. Patients with XLP or malignant disease were excluded. Patients who had negative genetic results including patients with monoallelic variants but no immunological abnormalities or patients in whom no genetic analysis was performed, were classified as 2° HLH, if they did not have a disease relapse for at least 6 months after the first HLH episode (median follow-up 2.3 (0.5 – 4.5) years). Among these, patients who developed HLH in the context of a documented viral infection, were classified as 2° virus-induced HLH (2°V-HLH). The other patients who developed HLH with or without underlying disease without evidence for an infection were classified as 2°HLH. Patients who could not be clearly assigned to one of these groups were excluded.

Siblings identified at birth with biallelic mutations in HLH-associated genes, but without clinical symptoms were included to control for possible alterations in the T cell phenotype due to the genetic defect in the absence of an acute HLH episode (asymptomatic 1° HLH). A group of HIV exposed (but not infected) infants under 1 year of age was included as healthy controls.

Flow cytometry

HLA-DR expression on $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells and sCD25 levels Chemiluminescence Immunoassay (CLIA) were measured in all patients referred for suspected HLH. Since the age-independent variability of HLA-DR expression and sCD25 level in healthy donors is limited, patients of all ages fulfilling the inclusion criteria were considered for analysis (n = 93). For all other investigations, we only included patients younger than one year because of the extensive variability in T-cell differentiation marker expression beyond that age. In that group, ficoll isolated PBMCs were stained with three different stainings using the antibodies indicated in the Supporting Information material.

Statistical analysis

Data were analyzed by Mann–Whitney U test using GraphPad Prism version 6.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad. Significance was defined as * for $p \leq 0.05$, ** for $p \leq 0.005$, *** for $p \leq 0.0005$, and **** for $p < 0.0001$.

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Abbreviations: **ATG**: anti-thymocyte globulin · **CD**: cluster of differentation · **CHS**: Chediak-Higashi Syndrome · **CMV**: Cytomegalovirus · **CTL**: cytotoxic T cell · **EBV**: Epstein-Bar Virus · **FHL**: familial HLH type · **GM-CSF**: Granulocyte-macrophage colony-stimulating factor · **GS**: Griscelli Syndrome Type 2 · **HHV**: Human herpesvirus · **HLA**: Human Leukocyte Antigen · **HLH**: Hemophagocytic Lymphohistiocytosis · **HPS**: Hermansky-Pudlak Syndrome · **MAS**: Macropahge activation syndrome · **MHC**: Major histocompatibilty complex · **PCR**: polymerase chain reaction · **PD-1**: Programmed cell death protein 1 · **SAP**: Slam-associated protein · **sCD25**: soluble IL-2 receptor · **TCM**: central memory T cells · **TCR**: T-cell receptor · **TE**: terminally differentiated or effector · **TEM**: effector memory T cells · **V-HLH**: viral induced HLH · **XIAP**: X-linked inhibitor of apoptosis · **XLP**: linked lymphoproliferative disease

Full correspondence: Dr. Stephan Ehl
e-mail: stephan.ehl@uniklinik-freiburg.de

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