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Original Article

Analysis of cytokine risk factors in the early death of patients with secondary phagocytic lymphocytic histiocytosis

Zengzheng Li[1,2,3*], Jianqiong Liu[1,2,3*], Yan Man[1,2,3,4], Fusheng Liu[1,2,3,4], Lili Gao[1,2,3], Peng Hu[1,2,3], Renbin Zhao[1,2,3], Yajie Wang[1,2,3,4], Tonghua Yang[1,2,3,4]

[1]Department of Hematology, The First People's Hospital of Yunnan Province, Kunming, China; [2]Yunnan Blood Disease Clinical Medical Center, Kunming, China; [3]Yunnan Blood Disease Hospital, Kunming, China; [4]Kunming University of Science and Technology, Kunming, China. [*]Equal contributors.

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Abstract: Secondary hemophagocytic lymphohistiocytosis (sHLH) is an excessive inflammatory response syndrome caused by immune abnormalities. Up to date, the risk factors for cytokines causing early death in sHLH patients have not been elucidated. Our study reviewed the cytokine expression levels in peripheral blood of 50 sHLH patients. Through Cox proportional hazard model analysis, we found that IL-17F ≥2.835 pg/mL (HR = 5.922, 95% CI = 1.793-19.558, P = 0.004) was an independent death risk factor in sHLH patients, and it was also 30 days (Cutoff-value = 2.890 pg/mL, HR = 16.568, 95% CI = 1.917-143.195, P = 0.011), 60 days (Cutoff-value = 2.890 pg/mL, HR = 7.559, 95% CI = 1.449-39.423, P = 0.016), 90 day death risk factor (Cutoff-value = 2.835 pg/mL, HR = 7.649, 95% CI = 1.965-29.778, P = 0.003); IL-10 ≥16.730 pg/mL (HR = 4.821, 95% CI = 1.151-20.116, P = 0.031) is not only a death risk factor within 90 days, but also within 10 days (Cutoff-value = 944.350 pg/mL, HR = 13.321, 95% CI = 1.123-158.03, P = 0.027); and IL-5 ≥2.495 pg/mL (HR = 15.687, 95% CI = 1.377-178.645, P = 0.04) was also a death risk factor within 10 days. Besides, IL-17F, IL-10, IL-5, and the previously reported common risk factors Age, platelets, activated partial thromboplastin time, triglyceride, and lactate dehydrogenase were analyzed together. It was found that the patient age ≥56 years-old is was an important risk factor for death within 30 days, IL-17 ≥2.89 pg/mL and IL-10 ≥16.73 pg/mL are important risk factors for patient death. In summary, our data indicate that age, IL-10 and IL-17F are important risk factors for early death in sHLH patients.

Keywords: Secondary hemophagocytic lymphohistiocytosis, risk factors for early death, cytokine, interleukin 17F, interleukin 10, age

Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a rare life-threatening immune disease. It includes major types caused by genetic defects and immunodeficiency (such as PRF1, Munc13-4, Roquin-1, STX11, STXPB2, ITK, XIAP, LYST, SRGN), and secondary Hemophagocytic lymphohistiocytosis (sHLH) types caused mainly by tumors, infections, and rheumatic diseases [1-3]. In HLH patients, due to inflammatory response targets that cannot be eliminated, the persistence of inflammatory responses leads to uncontrolled activation of macrophages [4]. The primary hemophagocytic syndrome occurs more commonly in children and adolescents. The etiology of sHLH is complicated, and the clinical manifestations and laboratory indicators lack characteristics. Currently, it is believed that the uncontrolled regulation of the immune system may be the pathogenesis of sHLH. The imbalance of Th1/Th2 cells leads to intensive activation of cytotoxic T cells ($CD8^+$ cells). Furthermore, cytokines are also produced in high concentration and stimulate the proliferation and activation of tissue macrophages. This in turn leads to high fever, liver function damage, hyperlipidemia, coagulopathy, phagocytosis and decreased NK cell activity [5, 6]. Macrophage colony-stimulating factor (M-CSF), TNF-α, IL-1, and IL-6 are produced after excessive activation of tissue mac-

rophages, which further aggravates cytokinineemia [5, 7, 8], activated macrophages engulf blood cells, IFN-α, TNF-α, and other factors to inhibit bone marrow hematopoiesis, accompanied by abnormal consumption of coagulation function, thus resulting in pancytopenia [9, 10]. The causes of sHLH include infections, tumors, and autoimmune diseases. Secondary HLH is most commonly characterized by an infection caused by the Epstein-Barr virus (EBV), especially in Asia [11]. In the past few decades, HLH has been considered a genetic disease in children. But accumulating evidence indicates that the disease is susceptible to occur at any age, and about 40% of the patients are adults [12]. The cancer-related mortality rate of adult patients is generally higher, about 20%-60% [13]. HLH represents only 1-2 months median survival time, while there was only a 5% 1-year survival rate in the 1980s [14]. However, the current research shows that HLH-94 or HLH-04 treatment regimens have the potential to increase the disease response rate from less than 10% in the past to about 70% [15, 16]. Unfortunately, the HLH-94 study exhibited only a 54% 5-year survival rate, while it was 62% for the HLH-04 study [17]. For patients with HLH-94 and HLH-04 schemes that are ineffective and refractory to relapse, there is currently no unified treatment plan. In addition to combined chemotherapy, a traditional treatment plan for the allogeneic hematopoietic stem cell transplantation, with the continuous deepening of the pathogenesis of HLH, a series of targeted drugs have been gradually applied in clinical practice. At present, it is relatively certain that Emapalumab (human anti-interferon-γ antibody) is an effective targeted therapy for primary HLH [18, 19]. But most of the applications of these drugs are currently case reports, like JAK1/2 inhibitor Ruxolitinib was used as first-line treatment for sHLH [20, 21], and the cytokine storm and multiorgan failure rapidly reversed, and sHLH patients' condition improved after treatment with Tocilizumab. These targeted drugs can really treat sHLH patients. Despite this, there is no definitive study reporting that these targeted therapies can reduce the early mortality of sHLH. The early deaths rate of sHLH patients within one month still remains about 50% [22].

Previous studies on the relationship between cytokines and HLH risk mostly focused on children, which proved that cytokines had a predictive effect. There are few studies on the relationship between cytokines and sHLH risk (For example, IL-10 is a risk factor for early death in children) [23, 24]. Based on the role of cytokines in predicting the risk of HLH related deaths in children, combined with the analysis of cytokine expression levels may be beneficial to predict the risk of sHLH related deaths. In this study, we tested serum Th1/Th2 cytokines including interferon (IFN)-γ, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-17F, IL-22, tumor necrosis factor (TNF)-α, and tumor necrosis factor (TNF)-β in 50 sHLH patients. Cox proportional hazards model was used for investigating the risk factor of described cytokines for early deaths in the sHLH patients. Then, four different groups of patients were prepared according to their different survival rates: (1) Survival time 1-10 days and >10 days, (2) Survival time 1-30 days and >30 days, (3) Survival time 1-60 days and >60 days, (4) Survival time 1-90 days and >90 days. The risk of deaths in different time zone was analyzed by the Cox proportional hazard model for the analysis of the risk factors associated with the early deaths of sHLH.

Statistical analysis

Interquartile range (IQR) and median were used to describe continuous variables, while categorical variables were shown as percentages and frequencies (%, n). The student's t-test was used to differentiate the survivors and non-survivors. The parameters optimal cutoff values were analyzed with the help of receiver operating characteristic (ROC). The difference between the two sides of the Cutoff-value was determined by the student's t-test. The Kaplan-Meier method and the Log-Rank Test was utilized to analyze the survival curves. Cox proportional hazard model-based analysis (univariate and multivariate) was performed. Statistical software package IBM SPSS 21.0 (SPSS, USA) was used for all statistical calculations. $P<0.05$ means there is statistical difference.

Patients and data collection

In the five years from January 2014 to February 2019, the sHLH patients in the First People's Hospital of Yunnan Province were analyzed retrospectively. Diagnosis for sHLH was conduct-

Table 1. Basic patient cohort information

Parameters	Survivors (n = 12)	Non-survivors (n = 38)
Sex (Male/Female)	4/8	21/17
Nationality	10/2	8/30
Lymphoma	25%	36.80%
Epstein-Barr virus infection	25%	23.70%
Autoimmune disorders	8.30%	5.30%
Unknown triggers	41.70%	34.20%
Age (years) Median (IQR)	28 (16.0-54.0)	42.5 (16.0-76.0)
Temperature (°C) Median (IQR)	39.45 (36.20-42.00)	39.5 (36.50-41.00)
WBC ($\times 10^9$/L) Median (IQR)	2.25 (1.40-10.60)	3.07 (0.50-25.90)
HGB (g/L) Median (IQR)	109.5 (71.00-163.00)	98 (45.00-600.00)
PLT ($\times 10^9$/L) Median (IQR)	76.5 (15.00-354.00)	35 (8.00-177.00)

The proportion of patients with lymphoma, Epstein-Barr virus infection, and autoimmune diseases in the study cohort. And the distribution of patient age, gender, ethnicity, body temperature at the first visit, WBC, HGB, PLT in the study cohort.

ed by using the HLH-2004 protocol of the International Histiocyte Society [16, 25]. Those patients were enrolled in the study that met at least five of the eight given criteria: (1) Fever for more than 1 week, with a peak of more than 38.5°C. (2) Splenomegaly. (3) Pancytopenia. (4) Fibrinogen <1.5 g/L and/or Triglyceride ≥2.0 mmol/L. (5) Hemophagocytosis in lymph nodes, spleen, or bone marrow. (6) Reduced or lacking activity of NK cell. (7) Ferritin ≥500 ug/L and (8) Soluble CD25 ≥2400 U/mL. The patients were excluded who possessed concurrent malignancy, previous history of macrophage activation syndrome, or immunosuppressive therapy. Through reviewing the patient's medical records, laboratory results and clinical features were obtained at the time of diagnosis. Follow-up of the study was accomplished by making phone calls or examining medical records and the results were recorded on the last follow-up day. We measured the overall survival (OS) of patients from the diagnosis of HLH to the last follow-up or death for any cause. The preliminary research content of this project strictly follows the Declaration of Helsinki, the International Code of Ethics for Biomedical Research Involving Humans jointly formulated by the World Health Organization and the Council of International Medical Science Organizations, and the relevant regulations of the Ethics Committee of the First People's Hospital of Yunnan Province (2014YXLH077).

Cytokine determination

Quantitative determination of serum cytokines levels (Th1/Th2) including Interferon (IFN)-γ, Interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-17F, IL-22, Tumor necrosis factor (TNF)-α and TNF-β, was carried out using the Aimplex Cytokine (QuantoBio, Tianjing, China) with the detection range of 1-2500 pg/mL. Data of cytokines were collected for all patients during their diagnosis.

Results

Patient characteristics

Fifty sHLH patients [25 males and 25 females; with 40.5-year median age (range: 16-76 years)] were selected in this study. In this cohort, the OS rate was 24% (12/50), the 30-day OS rate was 56% (28/50), and the 60-day OS rate was 42% (21/50). All patients had a high prolonged fever and ferritin >500 ng/mL. Other commonly occurring clinical manifestations included hyperferritinemia 96% (48/50), and splenomegaly 84% (42/50). Laboratory examination abnormalities included leukopenia 70% (35/50), anemia 88% (44/50), and thrombocytopenia 80% (40/50). In HLH patients, the triggering factors were classified as: malignancies 34% (17/50), followed by Epstein-Barr virus infection 24% (12/50), autoimmune disorders 6% (3/50), and unknown triggers 36% (18/50) (**Table 1**). Laboratory and clinical indicators between survivors and non-survivors were compared and the overall mortality rate for this study was observed as 76% (38/50). Comparisons of clinical and laboratory parameters for survivors and non-survivors are shown in **Tables 1** and **2**. The age of non-survivors (median age 42.5 years) was generally higher than survivors (median age 28 years), which is statistically significant (P = 0.047) (**Table 1**). Similarly, no statistical difference in cytokines level exists between the two groups.

Univariate analysis for risk factors

Due to the high early mortality rate of HLH, about 50% HLH patients died within 30 days. To explore whether there are risk factors for cytokines between survivors and non-survivors, we set Cutoff-value. **Table 3** and Supp-

Table 2. Comparison of cytokines between survivors and non-survivors

Parameters (pg/mL)	Survivors (n = 12)		Non-survivors (n = 38)	
	mean-value	Median (IQR)	mean-value	Median (IQR)
IL-1β	1.889	1.78 (0.590-4.920)	23.903	2.365 (0.050-499.870)
IL-2	31.64	2.54 (0.100-345.480)	33.69	2.24 (0.000-697.930)
IL-4	3.388	3.53 (0.140-6.870)	2.648	1.755 (0.330-9.780)
IL-5	1.499	1.35 (0.400-4.100)	1.805	1.76 (0.900-3.100)
IL-6	26.847	7.81 (1.680-93.090)	155.693	66.76 (1.870-1081.870)
IL-8	36.076	14.24 (4.540-133.950)	111.364	41.775 (2.550-996.410)
IL-10	231.775	14.875 (2.300-1434.000)	181.687	31.955 (1.510-1719.440)
IL-12P70	4.96	4.445 (0.5-20.040)	4.273	3.85 (0.230-10.970)
IL-17A	1.792	1.4 (0.100-4.330)	3.09	2.32 (0.980-7.900)
IL-17F	2.611	1.1 (0.50-013.700)	2.835	2.88 (0.400-5.500)
IL-22	6.425	1.4 (0.440-32.550)	2.542	1.7 (0.150-9.600)
IFN-γ	42.141	2.195 (1.210-464.200)	54.297	5.66 (0.430-860.510)
TNF-α	7.307	1.21 (0.630-57.390)	6.753	3.42 (1.120-42.280)
TNF-β	2.52	3.025 (0.790-3.900)	4.153	2.93 (0.720-20.803)

Continuous variables are presented as median with interquartile range (IQR).

Table 3. The Cutoff-value of cytokines between survivors and non-survivors

Parameters (pg/mL)	Cutoff-value	*P*-value	95% Confidence Interval
IL-1β	2.695	0.1005	-
IL-2	3.35	0.0351	6.986-181.200
IL-4	4.835	<0.0001	2.806-5.551
IL-5	1.245	0.0009	0.527-1.758
IL-6	38.68	0.0024	82.410-349.200
IL-8	18.005	0.0608	-
IL-10	16.725	0.0321	28.360-595.500
IL-12P70	3.53	<0.0001	2.833-6.745
IL-17A	1.93	0.0002	1.358-3.751
IL-17F	2.835	0.0009	1.744-5.765
IL-22	1.685	0.0106	2.090-14.090
IFN-γ	2.605	0.2307	-
TNF-α	1.265	0.1045	-
TNF-β	1.82	0.0775	-

The Cutoff-value of cytokines was determined by the ROC curve, and the difference between the two sides was tested by the student's t-test.

lementary Table 1 show the outcomes of univariate analysis of the potential predictors of death in patients with HLH. The IL-2 (P = 0.0351), IL-4 (P<0.0001), IL-5 (P = 0.0009), IL-6 (P = 0.0024), IL-10 (P = 0.0321), IL-12P70 (P≤0.0001), IL-17A (P = 0.0002), IL-17F (P = 0.0009), IL-22 (P = 0.0106), Age (P<0.0001), Temperature (P = 0.0028), WBC (P = 0.0002), HGB (P<0.0001), and PLT (P<0.0001) were significantly different, but no significant differences were found in other cytokines. Besides, in **Figure 1A-E** and Supplementary Figure 1A, the Kaplan-Meier method described the HLH patient's survival curve with different risk factors between survivors and non-survivors. As for cumulative survival probability of patients, IL-1β <2.695 pg/mL was greater than IL-1β ≥2.695 pg/Ml (P = 0.047), IL-17A <1.930 pg/mL was greater than IL-17A ≥1.930 pg/mL (P = 0.015), IL-17F <2.835 pg/mL was greater than IL-17F ≥2.835 pg/mL (P = 0.001), IFN-γ <2.605 pg/mL was greater than IFN-γ ≥2.605 (P = 0.023), and TNF-α <1.263 pg/mL was greater than TNF-α ≥1.263 pg/mL (P = 0.032). Similarly, patients whose age was less than 54.5 years had a survival rate greater than ≥54.5 years-old patients (P = 0.027).

Multivariate analysis

Based on the 9 important cytokines determined by univariate analysis, forward conditional Cox region model analysis was accomplished to investigate the independent predictors of death in HLH patients. We found that only IL-17F (Cutoff-value = 2.835 pg/mL, HR = 5.922, 95% CI = 1.793-19.558, P = 0.004) had

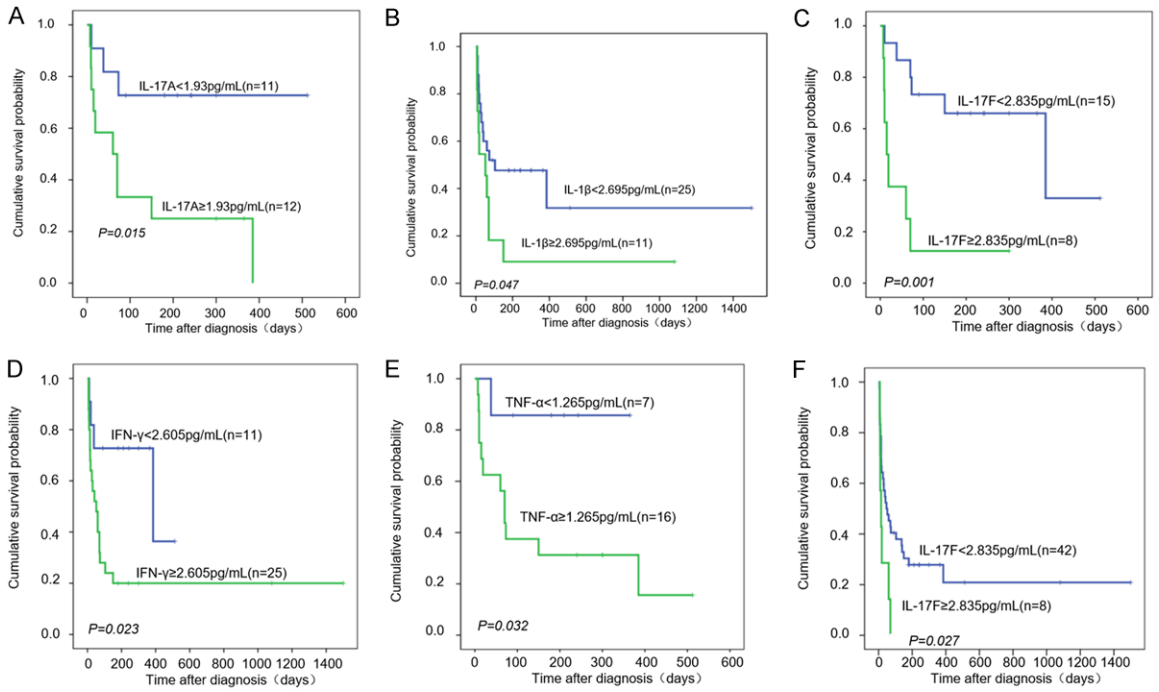

Figure 1. Kaplan-Meier survival curve of HLH patients with Cytokine's different risk between survivors and non-survivors. Kaplan-Meier curve of patients Overall survival (OS) with (A) IL-17A ≥1.93 pg/mL or IL-17A <1.93 pg/mL, (B) IL-1β <2.695 pg/mL or IL-1β ≥2.695 pg/mL, (C) IL-17F <2.835 pg/mL or IL-17F ≥2.835 pg/mL, (D) INF-γ <2.605 pg/mL or INF-γ ≥2.605 pg/mL, (E) TNF-α ≥1.265 pg/mL or TNF-α <1.265 pg/mL, (F) IL-17F <46.35 pg/mL or IL-17F ≥46.35 pg/mL.

Table 4. Multiple factor analysis of cytokines for Risk factors of different long-term death in sHLH

Duration of survival	Parameters (pg/mL)	Adverse factor	*P*-value	Hazard ratio
Survivors and non-survivors	IL-17F	≥2.835	0.004	5.922
1-10 days and >10 days	IL-5	≥2.495	0.04	13.321
	IL-10	≥944.35	0.027	15.687
1-30 days and >30 days	IL-17F	≥2.890	0.011	16.568
1-60 days and >60 days	IL-17F	≥2.890	0.016	7.559
1-90 days and >90 days	IL-10	≥16.73	0.031	4.812
	IL-17F	≥2.835	0.003	7.649

Through multivariate analysis of risk factors, significantly different level of IL-17F was observed in survivors and non-survivors with a hazard ratio of 5.922. Serum levels of IL-10 and IL-5 are also significantly different among patients with survival time of 1 to 10 days and >10 days, and the hazard ratios were 15.687 and 13.321 respectively. Furthermore, IL-17F also significantly different among patients with survival time of 1-30 days and >30 days, with a Hazard ratio of 16.568. Patients with survival time of 1-60 days and >60 days had significant differences in IL-17F, the hazard ratios were 7.559. IL-10 and IL-17F exhibited significant differences between 1-90 days and >90 days, with hazard ratios of 4.812 and 7.649 respectively.

significant difference (**Table 4**). Besides, **Figure 1F** describes the different IL-17F levels containing the HLH patent's survival curve using the Kaplan-Meier method.

In our study, we found that IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17A, IL-17F, IL-22 are different between survivors and non-survivors. To further analyze the difference of these cytokines and their effect on early death in HLH patients, according to the survival time, we divided HLH patients into four groups: (1) Survival time 1-10 days and >10 days, (2) Survival time 1-30 days and >30 days, (3) Survival time 1-60 days and >60 days, (4) Survival time 1-90 days and >90 days. Through multivariate analysis of cytokines by forward conditional Cox regression model, we found that: (1) IL-10 (Cutoff-value = 944.350 pg/mL, HR = 13.321, 95% CI = 1.123-158.033, P = 0.027) and IL-5 (Cutoff-value = 2.495 pg/mL, HR = 15.687, 95% CI = 1.377-178.645, P = 0.04) were risk factors for death within 10 days (**Table 4**, Supplementary Table 2). (2)

IL-17F was risk factors for death within 30 days (Cutoff-value = 2.890 pg/mL, HR = 16.568, 95% CI = 1.917-143.195, P = 0.011), and also the risk factors for death within 60 days, the Cutoff-values are (HR = 7.559, 95% CI = 1.449-39.423, P = 0.016) (**Table 4**, Supplementary Tables 3, 4). (3) IL-17F (Cutoff-value = 2.835 pg/mL, HR = 7.649, 95% CI = 1.965-29.778, P = 0.003) and IL-10 (Cutoff-value = 16.730 pg/mL, HR = 4.821, 95% CI = 1.151-20.116, P = 0.031) were risk factors for death within 90 days (**Table 4**, Supplementary Table 5).

Next, we performed Kaplan-Meier survival curve and forward conditional Cox region model analysis on IL-17F, IL-10, IL-5, and the previously reported common risk factors age, platelets (PLT), activated partial thromboplastin time (APTT), triglyceride, and lactate dehydrogenase (LDH) [4, 5, 26, 27]. The results show that APTT showed a significant difference in the Kaplan-Meier survival curve of patients on both sides of 33.7 s (P = 0.038) (Supplementary Figure 2G). The Kaplan-Meier survival curve of patients with LDH ≥1996 U/L and LDH <1996 U/L tends to be significantly different (P = 0.095) (Supplementary Figure 2F), regardless of death within 30 days or death within 60 days. Although ATPP and LDH did not show superiority in our forward conditional Cox region model analysis (Supplementary Table 6), there are significant differences or tend to be significant differences in the Kaplan-Meier survival curve of patients on both sides of the Cutt-off value. Kaplan-Meier survival curve for patients aged ≥56 years-old and patients <56 years-old who died within 30 days tended to be significantly different (P = 0.061) (Supplementary Figure 2A). Kaplan-Meier survival curve for patients ≥49.5 years-old and <49.5 years-old who died within 60 days There was a significant difference (P = 0.064) (Supplementary Figure 2B). Also, forward conditional Cox region model analysis shows that age ≥56 years-old is an important risk factor for death in sHLH patients within 30 days (Supplementary Table 6), which is similar to previous studies reporting the risk index of age greater than 54 years-old [8]. The results of IL-17F analysis are similar to those that did not include Age, APTT, LDH, PLT, and TRIG (**Table 4**, Supplementary Table 6; **Figure 1C**, Supplementary Figure 2H), and IL-10 ≥16.73 pg/mL is the risk factor for death in the patients (HR = 4.762, P = 0.05) (**Table 4**, Supplementary Table 6).

Generally speaking, our research has similar results with other people's research work, but there are also different contents. This may be attributed to the following reasons: 1. The area where patients are collected is different, 2. The characteristics of patients is different (including age range, primary disease proportion, degree of infection, regulation of the autogenous immune system, etc.), 3. The included detection indicators are inconsistent.

Discussion

The early mortality rate of sHLH remains the main challenge for clinicians. In our cohort, 44% (22/50) of patients died within 30 days. The most common cause was malignant tumors (34%, 17/50) and EBV infection (24%, 12/50). It was consistent with the results of most retrospective sHLH studies. Our results are similar to those previously reported that age ≥56 years-old is a risk factor for sHLH (Their results show age ≥54 years-old as a risk factor for early deaths [5]) (Supplementary Table 6).

It has been found in a previous study that the TNF and IFN-γ enhanced in children with familial HLH [28]. Subsequently, accumulating evidence indicates that multiple cytokines including IL-1β, IL-2, IL-6, IL-10, IL-12, IL-16, IL-18, TNF-α, and IFN-γ are elevated in patients with HLH [29, 30]. The increased level of IL-10 indicates that the patient does retain the mechanism that inhibits the activation of monocytes, macrophages, and T lymphocytes (although we are not yet clear) [24, 31]. Among the cytokines with a sharp increase in HLH, IFN-γ is the noteworthy one. Increased IFN-γ level results in the activation of macrophage which subsequently increases the other pro-inflammatory cytokines production. In the analysis of hematopoietic kinetics in the sHLH mouse model, it was found that the positive regulatory factors encoding myelopoiesis (granulocyte colony stimulating factor (G-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF)) were increased [32]. In clinical analysis, it was also found that GM-CSF expression was increased in sHLH patients [33]. While $CD8^+$ T cells and IL-33/ST2 axis are still important mediators of HLH, IFN-γ regulated $CD8^+$ T cell expression of GM-CSF and neutrophil survival [34, 35]. In a

mouse model with perforin deficiency developed by inducing HLH via lymphocytic choriomeningitis virus infection, the HLH phenotype uniquely required a combination of $CD8^+$ T cells and IFN-γ, indicating that the inhibition of IFN-γ can be considered as a potential target for HLH therapy [36, 37], such as Emapalumab [18, 19]. Although IFN-γ is not required for the fulminant HLH development, using therapies that focus to target the $CD8^+$ T cells activators (upstream), i.e. IL-33/ST2 signaling, may be an effective treatment with more universal applicability [38]. In our research, the cumulative survival probability of IFN-γ ≥2.605 pg/mL is significantly lower than the cumulative survival probability of IFN-γ <2.605 pg/mL (**Figure 1D**). Elevated early cytokine i.e. IFN-γ, IL-6, and IL-10 and are the models of childhood cases, while an independent early death prognostic factor in HLH children is an increased level of IL-10 [24, 39]. The important anti-inflammatory factor (IL-10) that protects the host from overreactions of pathogens and microbiota, is also significantly involved in wound healing, autoimmune response, cancer, and homeostasis maintenance [40]. Th2 cells are the very first cellular source of IL-10 [41]. However, lymphoid and myeloid cells, and some non-hematopoietic cells (including tumor cells) also secrete IL-10, in response to different stimulation [42, 43]. In our study cohort, the death risk factor is IL-10 ≥944.35 pg/mL within 10 days (HR = 15.687) while IL-10 ≥16.73 pg/mL within 90 days (HR = 4.812) (**Table 4**). Even if other risk indicators are included, IL-10 ≥16.73 pg/mL is still a risk factor for death in sHLH patients (HR = 4.762) (Supplementary Table 6; Supplementary Figure 2E). This may be related to most patients with lymphoma in our cohort. So, IL-10 is not only an independent risk factor for early childhood death, but also a death risk factor for sHLH.

IL-5 is the only cytokine involved in differentiation, and plays a substantial role in eosinophils induction and proliferation. IL-5 regulates various inflammatory responses, thus promotes the frequent clearance of pathogens. Meanwhile, it contributes to the pathology of chronic inflammation, overproduction of IL-5 will lead to the rise of eosinophils [44, 45]. Anti-IL5 therapy is considered an effective drug intervention for the treatment of eosinophilia. At present, it is mainly used to treat asthma. An important humanized monoclonal antibody, mepolizumab, can rapidly cause the neutralization of IL-5 which leads to eosinophil reduction both in tissues and circulation [46-48]. Besides, Anti-IL-5 therapy with mepolizumab can effectively inhibit the middle ear recruitment of eosinophil in patients having eosinophilic otitis media (EOM) [49]. IL-5 has been widely reported in many immune diseases, but its relationship with HLH is rarely reported. In our study, IL-5 ≥2.495 pg/mL was a risk factor for sHLH patient's death within 10 days (HR = 13.321) (**Table 4**). Although IL-5 does not show an advantage after the inclusion of other risk indicators (Supplementary Table 6), it is still necessary to pay attention to the abnormal expression of IL-5 in sHLH patients.

Along with IL-10 and IL-5 mentioned above, IL-17F is the most important cytokine in our research. The IL-17A-F 6 cytokines are the family members of IL-17 which can be used as new targets for drug intervention in certain inflammatory and immune diseases [50]. To treat colitis, IL-17F was reported as an effective target as it can inhibit the development of colitis [51]. Moreover, IL-17F overexpression can induce airway inflammation and can be used as a diagnostic indicator for patients with allergic asthma [52]. The hallmark pro-inflammatory cytokines of $CD4^+$ cells included IL-17A and IL-17F while IL-17A production or signal transduction disorder often leads to an autoimmune response and tissue destruction, however, its mechanism is not clear [53]. IL-17A and IL-17F possess 50% sequence homology and overlapping biological functions, they are up-regulated in various inflammatory tissues and synergistically enhance the inflammatory response along with the rest of the pro-inflammatory cytokines i.e. TNF [54-56]. In our study cohort, the cumulative survival probability of IL-17F ≥2.835 pg/mL was significantly lower than that of IL-17F <2.835 pg/mL, and IL-17A ≥1.93 pg/mL was considerably lower as compared to IL-17A <1.93 pg/mL, the cumulative survival probability of TNF-α ≥1.265 pg/mL was significantly lower than that of TNF-α <1.265 pg/mL (**Figure 1A**, **1C**, **1E**). IL-1 is considered an important component to differentiate Th17 cells and is capable of stimulating a variety of responses associated with T cells, including $CD8^+$ T cells [57]. Whether or not T cell receptor (TCR) binds IL-1α and IL-1β, IL-23 can synergistically support the production of the IL-17A by T cells in both mice and humans [58]. Obtained results revealed a

significant difference in the cumulative survival probability between the two sides of the IL-1β threshold (**Figure 1B**). This may be associated with the synergistic impact of cytokines IL-17A, IL-17F, and TNF-α, but the mechanism is still not known. IL-17F is included in various immune diseases, however, the relationship between IL-17F and sHLH is rarely reported. IL-17 is a highly versatile pro-inflammatory cytokine in itself and is important for host defense, tissue repair, the pathogenesis of inflammatory diseases, and cancer progression [52]. The main predisposing factors for sHLH are infection, tumors, and autoimmune diseases. TH17 cells are the main instigator of autoimmune pathology [50, 59]. There are many conditions for the production of IL-17 by TH-17 cells, but IL-23 and/or IL-1 are usually implicated, in conjunction with signals from pathogen derived PAMPs (pathogen-associated molecular patterns) or the inflammatory environment [60]. In sHLH patients with immune disorder and organ injury, a variety of immune cells and cytokines are not controlled and interacted, and the immune network among them has not been clarified. In our study, IL-17F was found a very important risk factor. IL-17F ≥2.835 pg/mL is an independent risk factor for deaths in sHLH patients (HR = 5.922) and is also a death risk factor of sHLH patients within 90 days (HR = 7.649). In addition, IL-17F ≥2.890 pg/mL is an independent risk factor for sHLH patients who died within 30 days (HR = 16.568) and 60 days (HR = 7.559) (**Table 4**). We were surprised to find that no matter what period the patient died, the Cutoff-value of IL-17F was approximately 2.890 pg/mL. We were surprised to find that regardless of the length of time the patient died and whether other risk indicators were included, the cut-off value of IL-17F was about 2.890 pg/mL (Supplementary Table 6, **Table 4**). Therefore, IL-17F is an important death risk factor of sHLH patients. But We did not test the patient's immune cells, so our data do not support information about which immune cells are represented by abnormal cytokine expression.

Conclusion

In this retrospective study, we found that the cytokine risk factors for the early death of sHLH patients are IL-17F and IL-10. These results may help clinicians make better treatment decisions for the sHLH patients having abnormally elevated cytokines to reduce their early mortality. At the same time, they may help the development of clinical drugs for abnormal sHLH cytokines. However, it is not clear how these cytokines are involved in the process of sHLH disease, thus further research is needed for its understanding on a molecular level.

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Disclosure of conflict of interest

None.

Address correspondence to: Tonghua Yang, Renbin Zhao and Yajie Wang, Department of Hematology, The First People's Hospital of Yunnan Province, 157 Jinbi Road, Kunming 650032, China. Tel: +86-0871-63639027; Fax: +86-0871-63639027; E-mail: ynanblood@aliyun.com (THY); ZhaoRenbin@126.com (RBZ); kbb165wyj@sina.com (YJW)

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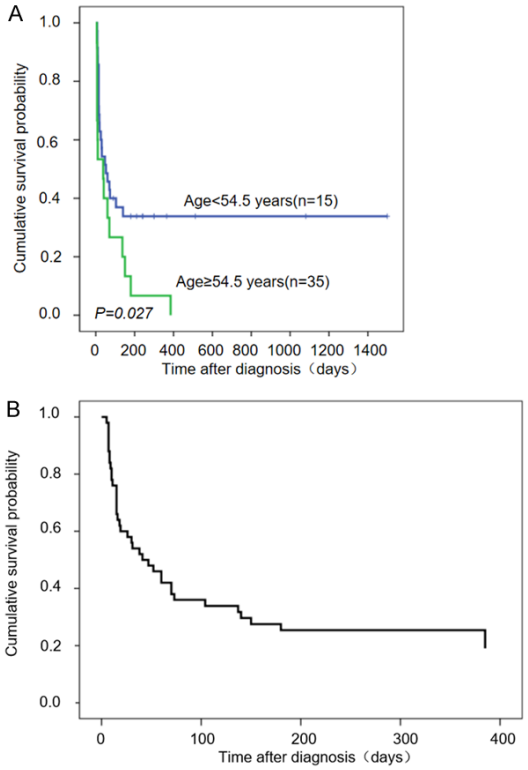
Supplementary Table 1. The Cutoff-value and the difference between two sides of Age, Temperature, WBC, HGB and PLT between survivors and non-survivors

Parameters	Cutoff-value	*P*-value	95% Confidence Interval
Age (years)	54.5	<0.0001	12.23-30.900
Temperature (°C)	39.15	0.0028	1.268-2.138
WBC ($\times 10^9$/L)	2.515	0.0002	2.293-6.891
HG (g/L)	115	<0.0001	97.660-156.500
PLT ($\times 10^9$/L)	1.505	<0.0001	1.486-3.695

Abbreviations: WBC: White blood cell; PLT: Platelet; HGB: Hemoglobin.



Supplementary Figure 1. Overall survival function and age Kaplan-Meier survival curve. Kaplan-Meier curve of patients Overall survival (OS) with (A) Age <54.5 years or ≥54.5 years (n = 15). (B) The cumulative survival probability function of HLH patients.

Supplementary Table 2. The Cutoff-value and the difference between two sides of cytokines in the survival time of HLH patients between 1-10 days and >10 days

Parameters (pg/mL)	Cutoff-value	*P*-value	95% Confidence Interval
IL-1β	2.835	0.0592	-
IL-2	3.35	0.0351	6.986-181.200
IL-4	4.035	<0.0001	2.680-4.767
IL-5	2.495	<0.0001	1.266-2.336
IL-6	58.39	0.0009	103.500-366.800
IL-8	17.335	0.0608	-
IL-10	944.35	<0.0001	1225.000-1626.000
IL-12P70	6.855	<0.0001	5.259-9.709
IL-17A	1.93	0.0002	1.358-3.751
IL-17F	2.845	0.0009	1.744-5.765
IL-22	0.87	0.2337	-
IL-γ	3.895	0.1066	-
TNF-α	4.43	0.0005	10.910-33.150
TNF-β	2.895	0.0427	0.1186-6.483

Supplementary Table 3. The Cutoff-value and the difference between two sides of cytokines in the survival time of HLH patients between 1-30 days and >30 days

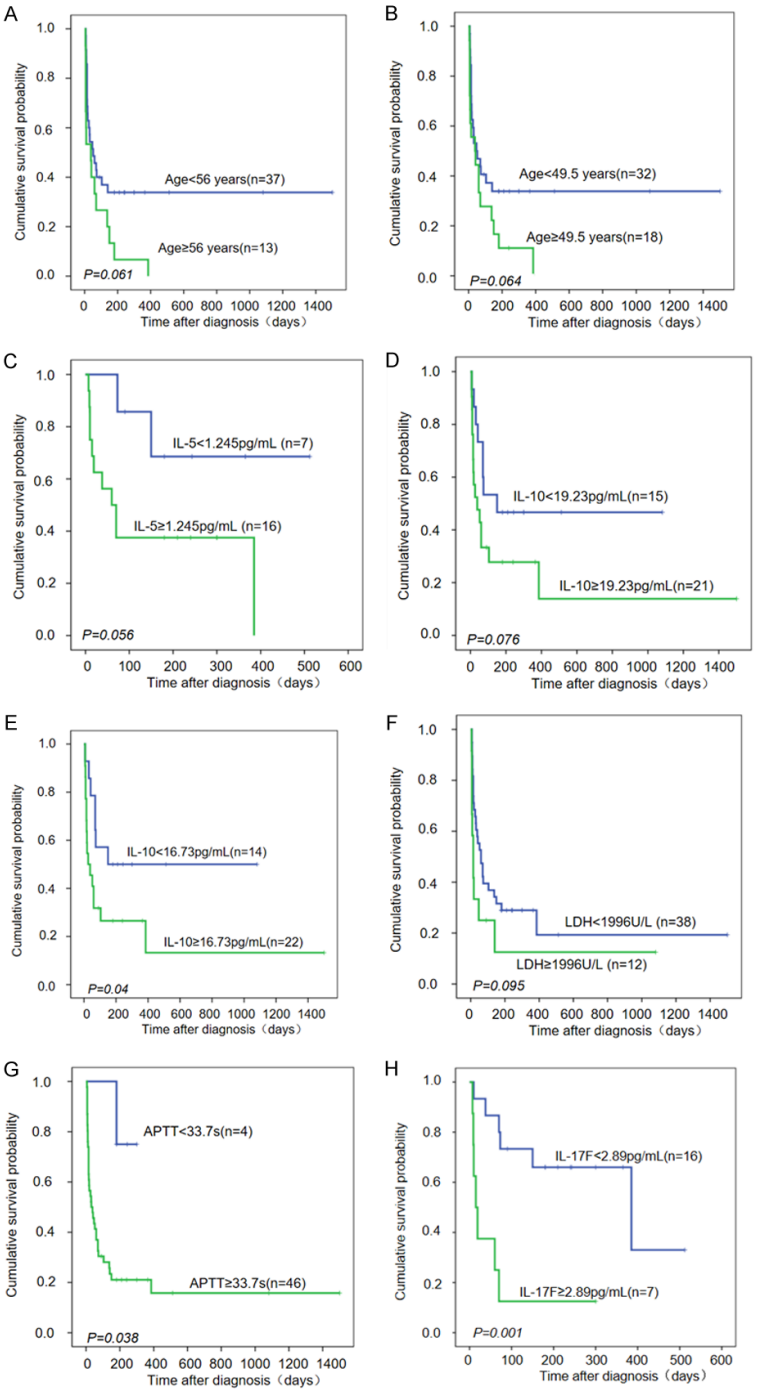
Parameters (pg/mL)	Cutoff-value	*P*-value	95% Confidence Interval
IL-1β	2.05	0.2944	-
IL-2	2.38	0.1166	-
IL-4	3.315	<0.0001	2.665-4.416
IL-5	1.245	0.0009	0.527-1.758
IL-6	58.39	0.0009	103.500-366.800
IL-8	18.005	0.0608	-
IL-10	16.73	0.0321	28.360-595.500
IL-12P70	5.685	<0.0001	4.477-8.601
IL-17A	1.93	0.0002	1.358-3.751
IL-17F	2.89	0.0005	1.954-6.032
IL-22	1.685	0.0106	2.090-14.090
IL-γ	1.655	0.4672	-
IL-α	1.265	0.1796	-
TNF-β	4.04	<0.0001	5.613-14.240

Supplementary Table 4. The Cutoff-value and the difference between two sides of cytokines in the survival time of HLH patients between 1-60 days and >60 days

Parameters (pg/mL)	Cutoff-value	*P*-value	95% Confidence Interval
IL-1β	2.05	0.2944	-
IL-2	2.375	0.1166	-
IL-4	2.675	<0.0001	2.593-4.342
IL-5	1.245	0.0009	0.5273-1.758
IL-6	58.39	0.0009	103.5-366.800
IL-8	58.39	0.0008	90.090-311.700
IL-10	16.725	0.0321	28.36-595.500
IL-12P70	5.685	<0.0001	4.477-8.601
IL-17A	1.93	0.0002	1.358-3.751
IL-17F	2.89	0.0005	1.954-6.032
IL-22	1.34	0.0889	-
IL-γ	2.895	0.2017	-
TNF-α	3.08	0.0302	1.307-23.500
TNF-β	4.04	0.0071	1.598-9.037

Supplementary Table 5. The Cutoff-value and the difference between two sides of cytokines in the survival time of HLH patients between 1-90 days and >90 days

Parameters (pg/mL)	Cutoff-value	*P*-value	95% Confidence Interval
IL-1β	2.835	0.0592	-
IL-2	3.35	0.0351	6.986-181.200
IL-4	3.315	<0.0001	2.665-4.416
IL-5	1.105	0.0017	0.485-1.812
IL-6	38.68	0.0024	82.410-349.200
IL-8	18.005	0.0608	-
IL-10	16.73	0.0321	28.360-595.500
IL-12P70	6.855	<0.0001	5.259-9.709
IL-17A	2.45	<0.0001	2.009-4.123
IL-17F	2.835	0.0009	1.744-5.765
IL-22	1.685	0.0106	2.090-14.090
IL-γ	2.895	0.2017	-
TNF-α	3.08	0.0302	1.307-23.500
TNF-β	4.04	0.0071	1.598-9.037



Supplementary Figure 2. Age, APTT, LDH, IL-5 and IL-10 Kaplan-Meier survival curve. A. Kaplan-Meier survival curve of patients with Death within 30 days and 60 days of age ≥56 years-old or age <56 years-old. B. Kaplan-Meier survival curve of patients with Death within 30 days and 60 days of age ≥49.5 years-old or age <49.5 years-old. C. Kaplan-Meier survival curve of patients with Death within 10 days, 30 days, 60 days, 90 days and non-survivors of IL-5 ≥1.245 pg/mL or IL-5 <1.245 pg/mL on both sides. D. Kaplan-Meier survival curve of patients with Death within 10 days of IL-10 ≥19.23 pg/mL or IL-10 <19.23 pg/mL. E. Kaplan-Meier survival curve of patients with Death within 30 days, 60 days and non-survivors of IL-10 ≥16.73 pg/mL or IL-10 <16.73 pg/mL. F. Kaplan-Meier survival curve of patients with Death within 60 days and 90 days of LDH ≥1996 U/L or LDH <1996 U/L. G. Kaplan-Meier survival curve of patients with Death within non-survivors of APTT ≥33.7 s or APTT <33.7 s. H. Kaplan-Meier survival curve of patients with Death within 10 days, 30 days, 60 days, 90 days non-survivors of IL-17F ≥2.89 pg/mL or IL-17F <2.89 pg/mL.

Supplementary Table 6. Age, PLT, APTT, TRIG, LDH, IL-5, IL-10, IL-17F multi-factor analysis

Duration of survival	Parameters	Adverse factor	*P*-value	Hazard ratio
Survivors and non-survivors	Age (years)	54.5	0.055	4.286
	IL-10 (pg/mL)	16.73	0.05	4.762
	IL-17F (pg/mL)	2.89	0.025	9.044
	PLT ($\times 10^9$/L)	115	0.873	1.129
	LDH (U/L)	-	-	-
	IL-5 (pg/mL)	1.245	0.669	0.636
	APTT (s)	33.7	0.983	>100
1-10 days and >10 days	Age (years)	56	0.016	8.452
	IL-10 (pg/mL)	19.23	0.582	1.557
	IL-17F (pg/mL)	2.89	0.021	8.002
	PLT ($\times 10^9$/L)	-	-	-
	LDH (U/L)	1996	0.484	2.429
	IL-5 (pg/mL)	1.245	0.589	1.557
	APTT (s)	41.75	0.315	2.117
1-30 days and >30 days	Age (years)	56	0.021	8.098
	IL-10 (pg/mL)	16.73	0.640	1.458
	IL-17F (pg/mL)	2.89	0.009	12.298
	PLT ($\times 10^9$/L)	16	1.000	1.000
	LDH (U/L)	360	0.750	0.604
	IL-5 (pg/mL)	1.245	0.837	0.782
	APTT (s)	-	-	-
1-60 days and >60 days	Age (years)	49.5	0.248	2.817
	IL-10 (pg/mL)	16.73	0.771	1.277
	IL-17F (pg/mL)	2.89	0.033	5.056
	PLT ($\times 10^9$/L)	115	0.766	1.255
	LDH (U/L)	1996	0.172	4.876
	IL-5 (pg/mL)	1.245	0.561	1.968
	APTT (s)	34.55	0.997	1.006
1-90 days and >90 days	Age (years)	-	-	-
	IL-10 (pg/mL)	7.27	0.102	3.108
	IL-17F (pg/mL)	2.835	0.048	4.946
	PLT ($\times 10^9$/L)	115	0.536	1.476
	LDH (U/L)	1996	0.149	3.857
	IL-5 (pg/mL)	1.105	0.784	1.309
	APTT (s)	34.55	0.855	4.946

Through the multivariate analysis of risk factors, IL-17F in the Cox proportional hazards model analysis is that each stage of survival is a risk factor for patient death. HR are 9.044 (P = 0.025), 8.002 (P = 0.021), 12.298 (P = 0.009), 5.056 (P = 0.033), 4.946 (P = 0.048). Age ≥56 years-old are risk factors in survival times of 1-10 days and >10 days, 1-30 days and >30 days (HR = 8.452, P = 0.016; HR = 8.098, P = 0.021). IL-10 ≥16.73 pg/mL was a risk factor for Survivors and non-Survivors group (HR = 4.762, P = 0.05).