EBV-HLH children with reductions in CD4+ T cells and excessive activation of CD8+ T cells

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BACKGROUND: In this report, we analyzed the number and activation profiles of T cells in Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis (EBV-HLH) patients and assessed their value for the diagnosis of EBV-HLH.

METHODS: We compared the quantity of CD4+ T cells and CD8+ T cells in peripheral blood among HLH patients, a sepsis group, and healthy controls. We then analyzed variations in the activation marker in CD4+ T and CD8+ T cells.

RESULTS: The percentage of CD4+ T cells decreased in the EBV-HLH patients when compared with healthy controls, which induced significant differences in the ratio of CD4+ T cells/CD8+ T cells. The percentage of CD4+HLA-DR+ T cells, CD8+CD69+ T cells, CD8+HLA-DR+ T cells, and CD8+HLA-DR+ T cells/CD4+CD25+ T cells increased in EBV-HLH patients when compared with that in healthy controls. Compared with EBV-HLH survivors, the median percentage of CD4+CD25+ T cells (7.9 vs. 3.0) were decreased, but the ratios of CD8+HLA-DR+T cells/CD4+CD25+T cells (6.3 vs. 20.3) were increased in the dead patients.

CONCLUSIONS: There was a reduction in CD4+ T cells and abnormal activation of CD8+ T cells in the EBV-HLH patients. The percentage of CD4+CD25+ T cells and the ratios of CD8+HLA-DR+ T cells/CD4+CD25+ T cells in EBV-HLH patients had a relation to the prognosis of the disease.

Hemophagocytic lymphohistiocytosis is a rare hyper-inflammatory syndrome that is fatal (1). There are two forms of HLH: primary and secondary. Primary HLH is an autosomal-recessive disease that exhibits genetic defects. Secondary HLH is associated with infection, and EBV is one of the most frequent triggers of infection-associated HLH (2). HLH is characterized by a combination of signs, including persistent fever, splenomegaly with cytopenia, hypertriglyceridemia, hypofibrinogenemia, and an elevated serum ferritin level (3). An HLH study group of the histiocyte society developed a set of diagnostic criteria for HLH (4), and the diagnosis mainly depends on clinical and laboratory findings exhibited by the affected patients. Although these findings

used to diagnose HLH were nonspecific, they often lead to delayed treatment (5,6). Despite attempts to differentiate HLH from sepsis patients, the symptomatic presentations overlap highly (7). Timely diagnosis is significant for starting therapy before the damage becomes irreversible (8). Therefore, specific diagnostic indices are urgently needed.

Therefore, in this article, we focused on expression of T cells activation markers and evaluated the usefulness of these activation markers on the surface of T cells for differential diagnosis of HLH disease.

METHODS

Participants

This prospective study was conducted from January 2013 to December 2015 at the Children's Hospital of Zhejiang University School of Medicine. Ethics approval was obtained from the Institutional Review Board of Zhejiang University (Approval number: 2013015). All participants provided informed consent. Patients who were recruited had documented EBV infections and met the HLH-2004 protocol (4). All the patients provided detailed medical histories and underwent physical and laboratory examinations. Those participants suffering from other diseases that may have interfered with this study were excluded. Sepsis patients and healthy children who matched in age and gender were also included as control.

Measurement of Activated T-Cell Subsets

Blood was collected directly and resuspended at a concentration of 1×10^6 cells/ml in RPMI 1640 medium (Biomed, Lublin, Poland). Next, the blood was incubated in 24-well flat-bottomed plates at a final volume of 0.2 ml (per well) and was collected after 24 h at 37 °C, 5% CO₂. Flow cytometric immunophenotyping was performed using flow cytometry with double staining (BD Biosciences, San Jose, CA). The main reagents used in this study were as follows: CD3-FITC, CD8-PreCP, CD4-APC, CD25-PE, CD69-PE, HLA-DR-PE, and FACS Lysing Solution. Then, 100 µl of blood was mixed and incubated for 30 min at room temperature with appropriate quantities of antibodies or controls. After a short period of incubation and rinsing, the samples were fixed with 1% paraformaldehyde and analyzed with flow cytometry. The data were collected on a BD FACSCanto II (BD Biosciences, San Jose, CA, USA) and analyzed with Cell Quest software (BD Biosciences, San Jose, CA, USA).

Measurement of natural killer (NK) cell Activity In Vitro

The lytic activities of NK cells were measured with an MTT-based assay. Approximately 5×10^3 K562 cells were plated on round-

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bottom 96-well titer plates. The NK cells isolated from peripheral blood of each EBV-HLH child were added into each well to yield effector cells to target cells (E:T) at ratios of 10:1 in a final volume of 0.2 ml, in triplicate. After incubation for 4 h at 37°C and 5% CO₂, 10 µl MTT (5 mg/ml) was added to each well for an additional 4 h. The plates were then centrifuged at 2,000g for 15 min and 100 µl of the supernatant was replaced with 100 µl 10% sodium dodecyl sulphate. The plates were incubated overnight to completely dissolve the pellet. Optical density (OD) was analyzed at 570 nm and cytotoxicity was assessed by the following formula: cytotoxicity (%) = $[1 - (OD_{E+T} - OD_E) / OD_T] \times 100\%$. Thirty healthy children who matched in age and gender were included as normal control.

Statistical Analysis

All statistical analyses were performed using SPSS Version 18.0 (PASW Statistics for Windows, Version 18.0, SPSS, Chicago, IL). The Mann-Whitney *U*-test was used to compare the differences in continuous variables. The χ^2 test was used to compare the differences in categorical variables. $P < 0.05$ was considered to be significant. We used receiver operating characteristic (ROC) curves to assess the diagnostic value, the optimal diagnostic threshold was determined according to Youden's index, and the relative sensitivity and specificity were calculated.

RESULTS

Characteristics of EBV-HLH Patients

The study participants included 182 EBV-HLH patients (95 boys and 87 girls with a median age of 2.4 years, range: 1.1–14.2 years). The clinical features of these patients are shown in **Figure 1**. The cardinal symptoms and signs were fever (99%). Most of the patients presented characteristic laboratory abnormalities, including elevated ferritin (98%), thrombocytopenia (91%), hemophagocytosis (90%), hepatosplenomegaly (84%), hypofibrinogenemia (81%), and neutropenia (81%). In addition, 80% of cases showed signs of hypertriglyceridemia and anemia. Impaired NK cell cytotoxicity was also a characteristic finding in EBV-HLH patients (84%).

Figure 1. Characteristics of EBV-HLH patients.

T-Cell Subset and its Activation Levels in EBV-HLH Patients

Our data demonstrated a statistically significant decrease in EBV-HLH patients of the median percentage of CD4+ T cells (21.0 vs. 34.6) and the ratio of CD4+ T cells/CD8+ T cells (1.0 vs. 1.5) when compared with the ratios in the control group; however, there was no significant difference in the percentage of CD8+ T cells. To discover variations of the activation markers on the surface of T cells, we measured the markers in EBV-HLH patients in comparison with healthy controls (**Table 1** and **Figures 2** and **3**). There were significant differences in the percentage of CD4+HLA-DR+ T cells, CD8+ CD69+ T cells, and CD8+HLA-DR+ T cells and the ratio of CD8+HLA-DR+ T cells/CD4+CD25+ T cells between the HLH patients and healthy control groups ($P < 0.05$). However, there were no significant differences in the percentage of CD4+CD69+ T cells, CD4+ CD25+ T cells, and CD8+CD25+ T cells between the two groups ($P > 0.05$). The median percentages of CD4+HLA-DR+ T cells, CD8+ CD69+ T cells, and CD8+HLA-DR+ T cells and the ratio of CD8+HLA-DR+ T cells/CD4+CD25+ T cells (12.9 vs. 5.7, 3.4 vs. 1.2, 57.3 vs. 4.2, and 10.5 vs. 0.7, respectively) in HLH patients were significantly elevated when compared with the healthy control group.

Diagnostic Accuracy of the T-Cell Subset and its Activation Markers in EBV-HLH

To distinguish sepsis from EBV-HLH, a T-cell subset and its activation markers were measured in both EBV-HLH patients and a sepsis group. The percentage of CD4+ T cells, CD4+ CD25+ T cells, and CD8+HLA-DR+ T cells, and the ratios of CD4+ T cells/CD8+ T cells and CD8+HLA-DR+ T cells/CD4+ CD25+ T cells were significantly different between the EBV-HLH patients and the sepsis group. When compared with EBV-HLH patients, the median percentages of CD4+ T cells (33.2 vs. 21.0), CD4+ T cells/CD8+ T cells (1.9 vs. 1.0), and CD4+CD25+ T cells (8.3 vs. 5.5) were increased in the sepsis group, but the median percentage of CD8+HLA-DR+ T cells (25 vs. 57.3) and the ratios CD8+HLA-DR+ T cells/CD4+ CD25+ T cells (2.9 vs. 10.5) were decreased in the sepsis group when compared with the EBV-HLH patients. As analyzed above, we used ROC curves to assess their diagnostic value for distinguishing sepsis and EBV-HLH. The percentage of CD8+HLA-DR+ T cells and the ratio of CD8+HLA-DR+ T cells/CD4+CD25+ T cells were found to be the most efficient indicators (**Figure 4**). When the percentage of CD8+HLA-DR+ T cells exceeded 76.44%, the sensitivity was 28.21% and the specificity was 97.44%. When the ratio of CD8+HLA-DR+ T cells/CD4+CD25+ T cells exceeded 9.125, its sensitivity was 56.41% and the specificity was 97.44%.

Predictive Value of T-cell subset and its Activation Levels for the Outcome of Disease

In order to assess the predictive value of T-cell subset and its activation levels for the outcome of EBV-HLH children, their levels were compared between the EBV-HLH survivors and EBV-HLH dead patients. The result shows that the percentage

Table 1. T-cell quantity and state of T-cell activation in HLH patients

Parameters	Normal control (n = 156)	HLH (n = 182)	Sepsis (n = 178)
CD3+ (%)	64.6 (57.9–69.5)	61.4 (5.8–564.2)	59.4 (22.9–83.6)
CD3+CD4+ (%)	34.6 (29.3–46.4)	21.0 (1.1–53.1)	33.2 (8.9–51.6)
CD3+CD8+ (%)	22.6 (16.7–29.8)	21.3 (1.3–68.0)	17.3 (7.3–61.2)
CD3+CD4+/CD3+CD8+	1.5 (1.1–2.8)	1.0 (0.1–5.2)	1.9 (0.3–5.0)
CD3+CD4+CD25+ (%)	6.3 (3.9–8.4)	5.5 (0.2–18.3)	8.3 (1.5–25.8)
CD3+CD4+CD69+ (%)	0.8 (0.2–1.7)	1.2 (0.1–14.9)	0.5 (0.03–25.0)
CD3+CD4+HLA-DR+ (%)	5.7 (3.4–8.0)	12.9 (0.2–70.3)	7.7 (0.04–73.1)
CD3+CD8+CD25+ (%)	1.2 (0.3–2.3)	0.5 (0.03–24.5)	0.5 (0.03–8.4)
CD3+CD8+CD69+ (%)	1.2 (0.3–1.9)	3.4 (0.2–21.4)	1.5 (0.03–28.0)
CD3+CD8+HLA-DR+ (%)	4.2 (2.9–8.1)	57.3 (0.3–94.2)	25.0 (1.1–80.2)
CD3+CD8+HLA-DR+/CD3+CD4+CD25+	0.7 (0.6–1.0)	10.5 (0.03–325.0)	2.9 (0.2–24.2)

Values are the median (range).

Figure 2. The percentage of T-cell subgroups. The median percentage of CD3+ cells (a), CD4+ T cells (b), and CD8+ T cells (c), and the ratio of CD4+ T cells/CD8+ T cells (d) in HLH patients, the sepsis group, and healthy controls. The circle and star on behalf of outliers.

of CD4+CD25+ T cells and the ratios of CD8+HLA-DR+ T cells/CD4+CD25+ T cells were significantly different between them ($P < 0.01$). However, there were no significant differences in the percentage of CD8+HLA-DR+ T cells between the two groups ($P > 0.05$). When compared with EBV-HLH survivors, the median percentage of CD4+CD25+ T cells (7.9 vs. 3.0) was decreased, but the ratios of CD8+HLA-DR+ T cells/CD4+CD25+ T cells (6.3 vs. 20.3) were increased in the dead patients (Figure 5).

DISCUSSION

EBV-HLH is a form of acquired, infection-related HLH that typically represents a fulminant presentation of an acute EBV infection with a 30–50% mortality rate (9). It has even been suggested that treatment should be started based on a strong

clinical suspicion of HLH before overwhelming disease activity occurs, which usually leads to irreversible damage in some patients (10). Currently, a diagnosis of HLH disease mainly depends on clinical symptoms and laboratory tests. We investigated 182 EBV-HLH patients and found that 81% of patients had hypofibrinogenemia, 91% had thrombocytopenia, 84% had hepatosplenomegaly, 80% of cases showed hypertriglyceridemia, and 81% revealed neutropenia. It can be seen that the patient's symptoms cannot meet all of the diagnostic criteria and did not appear at the same time. In addition, these symptoms were nonspecific, which led to an indefinite diagnosis (11). Thus, reaching a diagnosis of EBV-HLH is challenging.

HLH is a disorder of the regulatory pathways of immune/inflammatory responses (11,12). It is currently believed that

Figure 3. The expressions of CD69, CD25, and HLA-DR on the surface of CD4+ T cells and CD8+ T cells. The percentage of CD4+CD69+ T cells (a), CD4+HLA-DR+ T cells (b), CD4+ CD25+ T cells (c), CD8+ CD69+ T cells (d), CD8+HLA-DR+ T cells (e), and CD8+CD25+ T cells (f), and the ratio of CD8+HLA-DR+ T cells/CD4+ CD25+ T cells (g) in HLH patients, the sepsis group, and healthy controls. The circle and star on behalf of outliers.

Figure 4. ROC curve for the differential diagnosis values of the percentage of CD8+HLA-DR+ T cells and the ratio of CD8+HLA-DR+ T cells/CD4+CD25+ T cells in patients with EBV-HLH or sepsis. ROC of CD8+HLA-DR+ T cells (a), with an area under the curve (AUC) of 0.78 and ROC of CD8+HLA-DR+ T cells/CD4+CD25+ T cells (b), with an AUC of 0.84. ROC, receiver operating characteristic.

Figure 5. The percentages of CD4+CD25+ T cells and CD8+HLA-DR+ T cells, and the ratios of CD8+HLA-DR+ T cells/CD4+CD25+ T cells in patients with different prognosis. When compared with EBV-HLH survivors, the percentage of CD4+CD25+ T cells (a) was decreased, but the ratios of CD8+HLA-DR+ T cells/CD4+CD25+ T cells (c) were increased in the dead patients. The percentage of CD8+HLA-DR+ T cells (b) was comparable. The circle and star on behalf of outliers.

high levels of activating cytokines generated by uncontrolled activation of immune cells lead to the death of EBV-HLH patients (13–16). Our previous study found that the levels of interferon- γ , interleukin (IL)-6, and IL-10 were significantly increased in EBV-HLH patients. These cytokines were down-

stream effector molecules and were indirect evidence of activation of T lymphocytes (6,17). CD69+ antigen markers are involved in activating signal transduction and are an early activation marker that leads to the synthesis of various cytokines. CD25+ antigen, which is a medium-term activation

indicator, occurs mainly in activated T cells. HLA/DR+ antigen is expressed in T cells, and it occurs during the later stages of T lymphocyte and NK cell activation (6,18). To determine T-cell activation levels, we measured these three activation markers in CD4+ T cells and CD8+ T cells, respectively. The results showed that there was activation of CD4+ T cells (high expression of HLA-DR) and CD8+ T cells (high expression of HLA-DR and CD69). In addition, the numbers of CD8+ T cells had not changed, and the percentage of CD4+ T cells decreased in the EBV-HLH patients when compared with the healthy controls, which induced significant differences in the ratio of CD4+ T cells/CD8+ T cells.

Research showed that CD8+ T cells were abnormally activated, having a prominent role during the process of HLH disease (19). Thus, the results showed that the disease was caused not only by reducing CD4+ T cells but also through an imbalance between CD8+ T-cell activation and inhibition.

It has been shown that CD4+CD25+ T cells suppress CD8+ T-cell proliferation and interferon- γ production induced by polyclonal or Ag-specific stimuli (20). Prominent but phenotypically variable CD8+ T cells were often present and were efficiently activated, particularly in EBV-associated HLH, and increased the number of CD8+ T cells that were reported before (21); however, we did not find obvious changes in this study. A strong expression of HLA-DR was seen in most cases of EBV-associated HLH in our results. The expression of HLA-DR on granulocytes has been correlated with high levels of cytokines, including IL-2 and interferon- γ . Thus, it may serve as a marker of cytokine release (22). Cytokine storm was known as one of the important causes of death of the EBV-HLH patients. The research found that the decreased percentage of CD4+CD25+ T cells and the increased ratios of CD8+HLA-DR+ T cells/CD4+CD25+ T cells in EBV-HLH patients had a relation with death. Thus, it is concluded that the excessive release of cytokines, such as interferon- γ , resulting from the imbalance of activation and inhibition of T cells is one of the most important causes of death in EBV-HLH patients.

HLH in the context of infection is best described as part of a spectrum of EBV-associated illness that results in clonal proliferation of T lymphocytes (9). This syndrome may be difficult to distinguish from other infectious illnesses, including sepsis, and recognizing this condition is very important. We found that there were significant differences in CD8+HLA-DR+ T cells and CD8+HLA-DR+ T cells/CD4+CD25+ T cells between EBV-HLH patients and the sepsis group. HLH patients exhibited a higher percentage of CD8+HLA-DR+ T cells, and the results suggested that increased T-cell HLA-DR expression was associated with HLH patients, which indicated that antigen expression on the surface of T cells may be a useful tool for predicting and identifying HLH from sepsis. We evaluated the usefulness of the above indicators for the diagnosis of HLH patients. The results showed that the ratio of CD8+HLA-DR+ T cells/CD4+CD25+ T cells was a preferable diagnosis marker of EBV-HLH.

Finally, our novel finding of the association between excessive CD8+ T-cell activation and the decreased number of CD4+ T cells suggests that both the quantity and the activation conditions of T cells may have value for the management of patients with HLH. The ratio of CD8+HLA-DR+ T cells/CD4+CD25+ T cells may be an indicator for the diagnosis of EBV-HLH. The percentage of CD4+CD25+ T cells and the ratios of CD8+HLA-DR+ T cells/CD4+CD25+ T cells in EBV-HLH patients had a relation with the prognosis of the disease. Currently, flow cytometry is widely used for clinical diagnosis, and these specimens were easily extracted from peripheral blood cells. Therefore, this discovery may present a novel application of flow cytometry in EBV-HLH. These findings, along with clinical and laboratory information, may be used for diagnosis and differential diagnosis of HLH patients.

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