

Observational study on dual marker of T cell activation for early diagnosis of HLH on peripheral blood

ABSTRACT

Introduction-

HLH is a fatal disorder, characterized by a cytokine storm due to cytotoxic T cell activation, the causes of which are various. Its detection is first on clinical awareness and proved by several tests. However, there is not one specific test to diagnose HLH. We present an observational study to assess T cell activation dual marker (CD 38 high/ HLA -DR) as one of the important diagnostic parameters for HLH for an early rapid diagnosis for this condition.

Methodology

This is a retrospective observational study conducted in 22 patients. Demographic information, clinical characteristics, diagnostic impressions, pathological features, and laboratory values were collected for each patient. Flow cytometric immunophenotyping for dual positive markers was performed. The panel included antibodies specific for CD45, HLA-DR, CD3, CD4, CD8 and CD 38 T-cell receptor (TCR).

Results

Routine markers such as soluble CD 25 , CRP, LDH, triglycerides, LDH and ferritin were unable to differentiate between HLH and other similar conditions. T-cell activation panel performed in these patients showed that 63.64% patients were positive and 36.36% negative for dual markers ((CD 38 high/ HLA -DR))

Conclusion

T cell activation marker CD 38 high/ HLA -DR was found to be a rapid marker for identification of HLH. This will aide clinicians with a rapid specific diagnosis although the benefit of an early diagnosis did not yield a better outcome indicating that a larger study is needed.

Key words: HLH, Flowcytometry

INTRODUCTION:

HLH is a rare, aggressive life threatening inflammatory disorder resulting in a cytokine storm and immune mediated multi organ failure.¹ The systemic inflammation is the result of inappropriate and dysregulated hyperactivation of cytotoxic T lymphocytes, macrophages and natural killer cells.² Patients with primary disease present early in childhood, affecting approximately 1.2 cases per million children per year.³ Familial HLH (FHLH) is associated with a group of inherited immunodeficiencies including autosomal recessive inherited Chediak – Higashi syndrome

and griselli syndrome as well as the X – linked proliferative syndrome. Secondary HLH occurs in adults with an associated acute illness, most commonly malignancies, autoimmune diseases and infections.⁴

According to the HLH – 2004 diagnostic guidelines, acquired HLH is defined as the presence of at least 5 out of 8 of the diagnostic criteria which includes fever, splenomegaly, cytopenia, hypertriglyceridemia and/or hypofibrinogenemia, hemophagocytosis on tissue examination (bone marrow, spleen or lymph node tissue), hyperferritinemia, high serum levels of soluble CD25, Low or absent natural killer cells activity.⁵

The presentations may mimic sepsis, acute liver failure and disseminated intravascular coagulation and flu-like illness such as corona virus disease 2019 (COVID-19), hence differentiation of HLH from these is important. The underlying pathology of HLH is a malignant over response of the patient's own immune system resulting in hyperactive macrophages, lymphocytes with release of cytokines, resulting in hemophagocytosis followed by a life-threatening multiorgan failure.⁶

In murine models of primary HLH, significant and prolonged CD8⁺Tcell activation was observed, which is the result of continuous stimulation by activated antigen- presenting cells leading to the excess release of proinflammatory cytokines, such as interferon – gamma, tumor necrosis factor – alpha, interleukin – 2, and IL-6 collectively, these cytokines mediate much of the mortality and morbidity associated with HLH.⁷ Activated T- cells in patients with HLH were characterized as CD 38^{high}/ HLA -DR⁺effector cells with activation of CD8⁺T cells being most pronounced.⁸ Circulating activated T-cells appear to be broadly characteristic of HLH, as they were seen in children with and without genetic lesions or identifiable infections and resolved with conventional treatment of HLH.⁹ Hence, this study was conducted to determine whether T-cell activation can be used as a diagnostic parameter for HLH.

Methodology

This is a retrospective observational study conducted in 22 patients. Demographic information, clinical characteristics, diagnostic impressions, pathological features, and laboratory values were collected for each patient. Laboratory tests included a complete blood count (CBC) using the Automatic Hematology Analyzer (Sysmex XN-1000). Biochemical parameters (Ferritin, LDH, TG)were measured by using COBAS - 6000 or COBAS - 8000. Bone marrow was done from the posterior superior iliac spine. Flow cytometric immunophenotyping for dual positive markers was performed by BD FACS Canto II flow cytometry system on peripheral blood - EDTA or heparin sample by red cell lysis method. Cell suspensions were incubated with combinations of 4 monoclonal antibodies that were used at concentrations titrated for optimal staining. The panel included antibodies specific for CD45, HLA-DR, CD3, CD4, CD8 and CD 38 T-cell receptor (TCR). Selected antibody combinations were conjugated to fluorescein isothiocyanate, phycoerythrin, peridinin-chlorophyll protein, and allophycocyanin fluorochromes. List-mode data files were acquired and analyzed for each specimen using programs. All flow cytometric data for patients with HLH were reviewed by pathologists with following Interpretation.

1. Increased frequency of CD38^{high} / HLA-DR + CD 8+ T cells is an optimal diagnostics marker for identifying patients with active HLH.

2. A threshold of $>7\%$ CD 38^{high} / HLA-DR + cells among CD8+ T cells has strong positive and negative predictive value for distinguishing HLH from early sepsis or healthy controls. Cytokine storm of HLH is marked by distinctive T-Cell activation whereas early sepsis is not, and that these 2 syndromes can be readily distinguish by T-cell phenotypes.

Statistical analysis was done using: Stata software.

RESULTS:

We studied 22 patients with a mean age of 52.41 ± 20.18 . 45.45% were males and 54.55% were females. The patients were primarily diagnosed with different clinical conditions such as post COVID (59.09%), Dengue (18.8%), CKD with sepsis (4.55%), EBV (4.55%), Metastatic breast carcinoma (4.55%), post operative sepsis (4.55%), septic shock (4.55%) (Table 1). All the patients studied were hospitalized and suspected to have HLH based on clinical grounds

According to the diagnostic guidelines for HLH, only 1 (4.55%) patient had hepatosplenomegaly (Table 1). Other diagnostic identifiers of HLH observed were : In 63.64% of patients hemophagocytosis was evident on pathological examination of bone marrow, 95.45% of the patients had hyperferritinemia, 90.91% of the patients had hypertriglyceridemia, 27.27 % of the patients had leukopenia, neutropenia, lymphocytopenia. All (100.00%) the patients had thrombocytopenia . Importantly, soluble CD25 levels were below the normal range for all patients (100.00%) (Table 2).

T-cell activation panel performed in these patients showed that 63.64% patients were positive and 36.36% negative for dual markers ((CD 38^{high}/ HLA -DR))(Table 1). Other laboratory parameter test results showed that 54.55% of the patients had normal Hemoglobin levels, Mean levels of C-reactive protein (CRP) was observed to be 153.00, 54.55% of the patients had above normal levels of LDH. Mean duration of hospital stay for the HLH patients was 22.05 ± 17.62 days. 63.64 %of the patients were treated with Steroids + IVIG, 27.27% by steroids alone and 4.55% with steroids + IVIG + Anakinra (IL 1 receptor antagonist) (Table 1). 17 patients recovered uneventfully while 5 patients died. At the time of discharge, 77.27% of the patients had improved levels of CBC, 72.73% of the patients had hypertriglyceridemia,86.36% of the patients had hyperferritinemia, 54.55% of the patients had thrombocytopenia (Table 1).

In microbial culture test, no organisms were detected in 15 patients , however 3 patients were found to be positive for *E.coli*, *Klebsiella Pneumoniae* and *Sphingomonas Paucimobilus*.

Discussion

The diagnosis of HLH may present as a challenge because of it's similarity in clinical manifestations and immunopathology with other diseases. The incidence of Primary HLH ranges from 1 to 225 per 300 000 live births

according to epidemiological studies.¹⁰ Infections, malignancy, drug hypersensitivity, rheumatologic disease, autoimmune/immunodeficiency conditions can trigger HLH, either due to activation of immune responses or by immune suppression.¹¹ Yao S et al (2021) reported Epstein-Barr virus to be the most common cause which accounted for 44.01% of the 1445 cases in 31 regions in their study.¹² In our study, patients were primarily diagnosed with different clinical conditions, with post COVID (59.09%) being the majority. Huang et al. (2020) also reported findings which indicate that severe COVID-19 leads to an increased inflammatory response resembling HLH.¹³ Circulating excessive CD8(+) T-cell activated cells appears to be predominant characteristic feature of HLH resulting in cytokine production by these cells. Results of Humblet-Baron S et al (2016) study showed activation of these cells rewires the IL-2 homeostatic network away from Treg cell maintenance and provide a potential pathway for the persistent inflammation in patients with HLH.¹⁴ Identification of T cell activation panel can be used as surrogate diagnostic marker for identifying patients with active HLH. De Matteis A et al (2022) found that CD4dimCD8+T cells levels were increased in patients with secondary HLH and their frequency correlated with a clinical severity score.¹⁵ Chaturvedi V et al (2021) also observed a threshold of >7% CD38^{high}/HLA-DR⁺ cells among CD8⁺ T cells which held high prediction value in distinguishing HLH positive patients from early sepsis or healthy controls.¹⁶ 63.64% HLH patients in this study were positive for dual markers (CD38^{high}/HLA-DR⁺) suggesting its utility along with classical markers in identification of HLH cases.

The standard diagnostic criteria for HLH includes confirmation of hemophagocytosis in bone marrow examination or liver biopsy, presence of hyperferritinemia to >500 ng/mL, fever, splenomegaly, presence of hypertriglyceridemia to >260mg/ml and cytoopenias affecting two or more cell lines. Basu S et al (2018) found that in HLH group, median ferritin level was significantly higher [6556 (2402–11,734) ng/ml] as compared to non-HLH group [median 1175 (943–2000) ng/ml] ($p < 0.0001$).¹⁷ 95.45% of HLH patients had high ferritin levels in this study in which majority them were positive for T cell panel activation. Chandra H et al. (2014) reported that early differentiation between HLH and non HLH cases can be done by distinguishing presence of pancytopenia and higher grade of hemophagocytosis in bone marrow examination in HLH cases.¹⁸ In our study 100% and 64.28% of HLH patients who were positive for T cell panel activation showed pancytopenia and hemophagocytosis, respectively. Okamoto M et al (2009) reported that hypertriglyceridemia was seen on diagnosis or during the disease period with the treatment-related improvement of its levels.¹⁹ 85.71 % HLH patients with hypertriglyceridemia in our study were positive for T cell activation panel.

In this observational study, the CBC, CRP, ferritin and triglyceride levels were unable to confirm an HLH. On the other hand, Dual T cell marker as a single entity was able to detect HLH in two thirds of the cases suggesting that it could be a more robust solitary marker for HLH. High ferritin, high LDH, high CRP, low platelets, low leucocytes and neutrophils were seen similarly between Dual marker T cell positive or negative patients suggesting that they could be non-specific. Since, soluble CD 25, the conventional marker was negative in all our patients with HLH., T cell activation as a solitary marker then may be a good indicator for onset of HLH.

Treatment of HLH also raises several challenges and is directed towards targeting the underlying disorder and controlling the immune dysregulation. The Histiocyte Society Study Group for HLH gave the HLH-94 and HLH-2004 treatment protocols, which combined chemotherapy and immunotherapy including etoposide, corticosteroids, and cyclosporine upfront and, in selected patients, intrathecal therapy methotrexate.^{20,21} Similar to results of our study, Georgiadou S et al (2019) suggested that early diagnosis and combination treatment with IVIG and

corticosteroids seemed to be an efficient treatment option for this condition.²² Among 5 patients who died, 4 patients had T cell dual marker positive.

The small numbers in our study may be a limitation but the concept could be further assessed in a larger study as it can make a significant impact in the real world to detect and treat HLH early

Conclusion

This is a small observational study on the patients with clinical features suggestive of HLH, where the routine markers such as soluble CD 25, CRP, LDH, triglycerides, LDH and ferritin were unable to differentiate between HLH and other similar conditions. However, T cell activation marker CD 38 high/ HLA -DR+ was found to be a rapid marker for identification of HLH and may allow earlier intervention in such patients. We need a larger study to see if early intervention makes a difference to the outcome of such patients.

Table 1: Summary Statistics of Demographics

PARAMETER	Overall (N=22)
Age (years)	52.41± 20.18
Gender, n (%)	
Male	10 (45.45 %)
Female	12 (54.55 %)
Indication, n (%)	
CKD with Sepsis	1 (4.55 %)
Dengue	4 (18.18 %)
EBV	1 (4.55 %)
Metastatic Breast Carcinoma	1 (4.55 %)
Post Covid	13 (59.09 %)
Post Operative Sepsis	1 (4.55 %)
Septic Shock	1 (4.55 %)
Organomegaly, n (%)	
No	21 (95.45 %)
Hepato Splenomegaly	1 (4.55 %)
Treatment Given, n (%)	

PARAMETER	Overall (N=22)
Steroids	6 (27.27 %)
Steroids + IVIG	14 (63.64 %)
<u>T cell activation panel Positive patients</u>	8 (57.14 %)
<i>CBC Improved</i>	6 (75.00 %)
<i>Died</i>	2 (25.00 %)
<i>Discharge</i>	6 (75.00 %)
<u>T cell activation panel Negative patients</u>	6 (42.86 %)
<i>CBC Improved</i>	5 (83.33 %)
<i>Died</i>	1 (16.67 %)
<i>Discharge</i>	5 (83.33 %)
Steroids + IVIG + Anakinra	1 (4.55 %)
Data Not Available	1 (4.55 %)
T Cell Activation Panel, n (%)	
Positive	14 (63.64 %)
Negative	8 (36.36 %)
CBC Improvement, n (%)	
Improved	17 (77.27 %)
Not Improved	1 (4.55 %)
Data Not Available	4 (18.18 %)
Outcome, n (%)	
Died	5 (22.73 %)
Discharged	17 (77.23 %)
Culture Report, n (%)	
E. Coli	3 (13.64 %)
Gram Negative Bacilli, Sphingomonas	1 (4.55 %)

PARAMETER	Overall (N=22)
Paucimobilus	
Klebsiella Pneumoniae	3 (13.64 %)
No Aerobes Or Anaerobes	15 (68.82 %)
<u>Post Covid</u>	
E. Coli	3 (13.64 %)
Gram Negative Bacilli, SphingomonasPaucimobilus	1 (4.55 %)
Klebsiella Pneumoniae	1 (4.55 %)
No Aerobes Or Anaerobes	8 (36.36 %)
<u>Other than Post Covid</u>	
Klebsiella Pneumoniae	2 (9.09 %)
No Aerobes Or Anaerobes	7 (31.82 %)

Table 2: Summary Statistics of Patients Characteristic based on T cell Activation Panel

Parameter	T cell activation panel positive (n=14)	T cell activation panel negative (n=8)	Inter Group P- value (Chi Square)
Cytopenia (N=22)	14 (100.00 %)	8 (100.00 %)	NA
Hemophagocytosis (N=14)	9 (64.28 %)	5 (62.50 %)	0.9332
Hypertriglyceridemia (N=20)	12 (85.71 %)	8 (100.00 %)	0.2622
Hyperferritinemia (N=21)	13 (95.86 %)	8 (100.00 %)	0.4391

Parameter	T cell activation panel positive (n=14)	T cell activation panel negative (n=8)	Inter Group P-value (Chi Square)
Soluble CD 25 (Below normal range) (N=22)	14 (100%)	8(100%)	NA
Post COVID Patient(n=13)	8 (57.14 %)	5 (62.50 %)	0.8058
Death (n=5)	4 (28.57 %)	1 (12.50 %)	0.3869
Steroids + IVIG (n=14)	8 (57.14 %)	6 (75.00 %)	0.4023

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