Early phenotypic and soluble markers of T cell activity can distinguish sepsis associated HLH from sepsis in children

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Abstract

Sepsis associated Hemophagocytic Lymphohistiocytosis (SHLH) is an underrecognized fatal complication of sepsis which requires early diagnosis and aggressive immunosuppressive treatment. However, overlapping clinical symptoms between sepsis and SHLH makes the early diagnosis challenging. Few recent studies have indicated the utility of immune signatures in distinguishing sepsis from HLH due to various underlying triggers. In this cross-sectional study, we evaluated the proportions of T cell subsets, their activation status (% of HLADR+CD38+ or PD1+ T cells) and cytokine profile within 72 hours of diagnosing the children with sepsis. Among the sepsis children, SHLH were identified if ≥4 HLH-2004 criteria are fulfilled. We report a lower CD4:CD8 ratio, and higher percentages of activated (HLADR+CD38+ or PD1+) CD4 T cells in SHLH children than those with sepsis. We also report an increase in the cytokines/chemokines related to T cell activity (IL-2Ra, IFN-g, MIG/CXCL9 and IL-10) in SHLH. ROC analysis revealed a 100% sensitivity and 78% specificity with CD4:CD8 <1.28 and an 87% sensitivity and 93% specificity with PD1+ CD4 Tcells >23.75% supporting the utility of these immune profiles in differentiating sepsis from SHLH early during the disease. Other markers (HLADR+CD38+CD4+T cells, serum levels of IL2Ra, IL-10 and MIG) also showed a specificity of 81-87%. In conclusion, the PD1+/HLADR+CD38+CD4+T cells, CD4:CD8 ratio, IL-2Ra and IL-10 either individually or together are likely to offer a good diagnostic accuracy in early diagnosis of SHLH from sepsis in children.

Key Points

- Cellular and soluble immune signatures related to T cell activity can distinguish SHLH from sepsis early during the disease.
- Higher % of PD1+/HLADR+CD38+ CD4 T cells, and plasma concentration of IL-2Ra, MIG, and IL-10 offer a good diagnostic accuracy.

Introduction

Sepsis is one of the leading causes of morbidity and mortality in children worldwide and the burden is disproportionately higher in developing countries[1–3]. Timely intervention or treatment can improve the clinical outcomes, however heterogenous host response to infections makes it challenging to decide upon the right treatment regimen[4]. In clinical practice, a small fraction of sepsis children develop Hemophagocytic Lymphohistiocytosis (HLH)-like features characterized by hyperactivated immune response, excessive inflammation and high mortality if not treated appropriately[5]. While immunosuppressive therapy is lifesaving in HLH[6,7], it may be detrimental in sepsis children especially those with poor immune function. However, HLH often remains underdiagnosed in sepsis cases, due to the overlapping clinical phenotypes between the two syndromes[5]. The diagnosis of HLH in children relies on the fulfillment of 5 out of 8 HLH-2004 criteria[7], whereas for the diagnosis of sepsis, the 2005 International Pediatric Sepsis Definitions Consensus Conference is commonly used[8]. In the two mentioned practices, fever and cytopenia can be commonly seen in both the conditions while certain

parameters such as elevated levels of ferritin, triglycerides and hemophagocytosis are also not very specific to HLH and may be seen in critically ill sepsis children[9]. Other challenges in the diagnosis of HLH are the non-availability of certain tests such as NK cell cytotoxicity, plasma sCD25 levels in all clinical settings. In addition, many of these clinical symptoms appear over the course of the disease, thus less useful in early diagnosis of sepsis-associated HLH (SHLH). Therefore, by relying completely on the existing methods to identify HLH, it is likely to miss out on SHLH children. Therefore, in order to improve the treatment response in children with sepsis, it is extremely important to distinguish the two conditions.

Sepsis and HLH are both characterized by dysregulated host immune response to infection or other underlying triggers. A few recent studies have demonstrated some of the unique immune signatures and their diagnostic utility in HLH and sepsis patients. *A* recent study by Chaturvedi et. al., has demonstrated the utility of activated-CD8 T cell > 7% as an good diagnostic marker to distinguish sepsis from HLH (with a sensitivity of 100% and specificity of 89%)[10]. Another large prospective study demonstrated that IFNg > 75 pg/ml and IL-10 > 60 pg/ml were 98.9% sensitive and 93% specific for HLH[11]. Both these studies have assessed immune parameters in HLH children with both active secondary or primary HLH cases pooled together. However, the activation of immune cells may differ between the infection triggered HLH and other HLH forms[12]. Thus, we wanted to investigate whether the similar immune markers will be useful in the diagnosis of infection associated HLH at an early stage in sepsis children. Also, such studies from developing countries where the burden of deaths due to sepsis is much higher, are lacking. Therefore, in this study we aim to identify early markers to distinguish sepsis associated HLH among children with sepsis based on the T cell activation status and cytokine profile.

Methods

Study design and Patients:

This is an observational cross-sectional study in which we recruited a total of 82 children from two IRB approved studies done on HLH in children (IRB Min: 13382 and IRB Min:12765) between the year 2020–2022, after obtaining consent. Children between 1 to 17 years of age who satisfied the definition of severe sepsis according to the 2005 International Pediatric Sepsis Definitions Consensus Conference criteria of severe sepsis/septic shock[8] or those who had fever ≥ 5 days and any two of the three criteria i.e bicytopenia (Hb ≤ 8 gm%; TC ≤ 2000/cumm; Platelets ≤ 1 L/cumm), organomegaly (palpable liver or spleen) or ferritin ≥ 500 mcg/L were included and followed up till their discharge from the hospital. Amongst the recruited children those who fulfilled 4 or more criteria from HLH-2004 guidelines[7] any time during the hospitalization were classified as SHLH (N = 12). We enrolled 5 primary HLH pediatric patients, confirmed with genetic testing and bone marrow biopsy and 14 healthy control group with age ranging from 2–14 years, and without any history of recent /ongoing infection. Children with the presence of underlying disorders which can predispose to HLH; such as malignancy, autoimmune diseases, and primary immunodeficiencies were excluded from the analysis. During the study period, children

diagnosed with Multisystem Inflammatory syndrome secondary to COVID(MIS-C), and those with missing values of certain important clinical variables were also excluded from the analysis.

Sample collection

Blood sample was collected from children within 72 hours of satisfying the inclusion criteria for the study. For a few children we also collected 2–3 follow-up samples once a week. Processing of samples for plasma and peripheral blood mononuclear cells (PBMCs) separation was started immediately upon receiving the blood sample. PBMCs were isolated by layering the diluted blood over a Ficoll-Paque density gradient medium followed by centrifugation at 400g for 30 minutes and brakes off. All PBMCs and serum/plasma samples were stored in -80°C freezer until the assays were performed.

Phenotyping of T cells

Phenotypic evaluation of T cells was performed on stored peripheral blood mononuclear cells (PBMCs) by flowcytometry. PBMCs were thawed, counted and stained for T cell phenotyping only for samples with sufficient cell counts. For a very small number of children (3 in severe sepsis and 1 in SHLH group) if the cell counts were not sufficient after thawing, their follow-up sample (collected a week later) was used for phenotyping. A small subset of samples from each group were initially stained with a limited panel of antibodies as follows, anti CD14-PECy7 (Clone-M5E2), CD56-BV510 (Clone-NCAM16.2), CD4-horizonV450 (Clone L200), CD8-Alexa Fluor700 (Clone RPA-T8), HLADR-FITC (Clone G46-6) and CD38-PerCPCy5.5 (Clone HIT2), fixable viability stain FVS-660 (Cat. no. 564405) for trial run or standardization purpose. Remaining samples were stained with a modified panel and run over four batches along with an internal control sample in each batch Table-S1. The list of antibodies used in the modified panel for T cells surface staining was anti CD3-FITC (Clone UCHT1), CD4-PECy7 (Clone RPA-T4), CD8-APC-R700 (Clone SK1), CD38-PerCPCy5.5 (Clone HIT2), HLADR-PE (Clone G46-6), CD56-BV510 (Clone NCAM16.2), CD137-APC (Clone 4B4-1), PD1-BV421 (Clone EH12.1), SLAMF7-Alexa Fluor 647 (Clone 235614) along with fixable viability stain FVS-780 (Cat.no.565388). All the antibodies for staining were obtained from BD Biosciences. For samples in which Foxp3 staining was performed to assess T regulatory cell frequencies, surface staining was followed by fixation & permeabilization (ebioscience, Foxp3 kit, Cat.no. 00-5523-00) for 20 mins at 4 degrees and intracellular staining with Foxp3-PECF594 (Clone 259D/C7) for 30 mins at 4 degrees in dark. Samples were acquired the same day on FACS ARIA-III (BD, USA). For the analysis of percentages of activated T cells within CD4 and CD8 subsets in severe sepsis and SHLH children, data from all the batches were pooled including the trial run. Detailed gating strategy for the samples run in the four batches is shown in Fig S1.

Soluble CD25

Plasma CD25 or IL2 receptor (sIL2r) concentrations were measured by enzyme-linked immunosorbent assay using ELISA kit (DY223, R&D systems) as per manufacturer’s instruction.

Luminex

Plasma cytokines and chemokines were measured on a subset of samples using a human magnetic 30-plex kit (LHC6003M, Invitrogen) on a Luminex platform (Bioplex-200 system). Assay was performed as per manufacturer's protocol, with 3-fold dilution for standards and 2-fold dilution of the samples. Standards were run in duplicates and standard curve for each analyte was plotted only with the values showing recovery of 70% -130%. For analysis, Bio-Plex Manager software, (v6.0 Bio-Rad) was used. Analytes for which more than 60% of the samples were out of the standard curve range (TNF-alpha, IL-4, IL-5, IL-17& RANTES, G-CSF) were not included in the further analysis across different patient categories. Within the analytes selected as per the above-mentioned QC criteria, the individual samples with values below/above the limits of detection were replaced with the lower limit of quantitation (LLOQ) or the upper limit of quantitation (ULOQ). As another measure of assay quality, we also observed a good correlation between same set of samples evaluated for sCD25/IL-2R with conventional ELISA (R&D systems) and Luminex (R2 = 0.84 and r = 0.9) (Fig S2).

Statistical Analysis

Comparison of median values between any two groups were performed using non-parametric Mann-Whitney U test. P-value ≥ 0.05 was considered as non-significant (ns) in all the analyses. Receiver operating characteristics (ROC) curve analysis was performed to determine the diagnostic accuracy of the T cells and cytokine variables which were significantly different between the sepsis and SHLH children. The data was plotted and analyzed using GraphPad Prism (version 9.4.1). Heatmap generation for the all the cytokines/chemokines analyzed and principal component analysis (PCA) for a limited number of variables were performed using RStudio version 1.4.1717 (www.r-project.org).

Results

Clinical features of children with sepsis and HLH

For the diagnosis of HLH, the most widely used method is HLH-2004 guidelines which require at-least 5 out of 8 criteria to be satisfied[7] (Henter et al). However, majority of the children in this study were assessed for six clinical variables from the HLH-2004 guidelines which are: fever, cytopenia, splenomegaly, hypertriglyceride/hypofibrinogen, ferritin and soluble CD25 (sCD25) levels (Table 1). Also, it is less likely that all the HLH features show up early during the disease (within 48 hrs after the diagnosis of sepsis). Thus, considering the number of clinical parameters available and collection of samples early after the diagnosis of sepsis, we adapted the same guidelines and those who fulfilled 4 or more criteria of the HLH-2004 parameters were categorized to SHLH group. This approach of using a modified HLH-2004 guidelines with ≥ 4 fulfilled HLH criteria has been used previously in another study as well[13] (Debaugnies et al). For sCD25, we used a stringent cut-off > 20,000 pg/ml which is within the range of concentrations observed in the primary HLH children in our study. We observed that the clinical features of HLH were less common in children with sepsis except fever which is seen in 74%, and hyperferritinemia (> 500ug/ml), is observed in 38% of them (Table 1). While the predominant features observed in the SHLH group were fever (in 100%), bicytopenia (in 75%), and hyperferritinemia (in 91%).

Splenomegaly was restricted to SHLH children alone (in ~ 42%), while hepatomegaly was seen in both. Recent studies have shown diagnostic utility of H-Score > 169 in adult secondary HLH patients, our data also showed a higher H-Score (median; IQR = 192.5; 175.3-237.8) in SHLH children than in those without HLH (median; IQR = 130; 91–142) and around 83% of the SHLH and only 6% of the sepsis children without HLH showed a score above 169. Median levels of all the other clinical and laboratory parameters are mentioned in Table 2. Interestingly, the two groups of children in our study also differ by the infection causing pathogens with majority of the SHLH children tested positive for bacterial infections.

Table 1
Basic characteristics and clinical details of children enrolled in the study in each category

	Healthy Controls (HC, n = 14)	Sepsis (S, n = 19)	Sepsis-HLH (SHLH, n = 12)	Primary-HLH (PHLH, n = 5)
AGE: median years (IQR)	4.50 (3.25, 6.0)	6 (2, 10)	7 (3, 11)	1.0 (0.49, 3.5)
SEX	M = 9, F = 5	M = 9, F = 10	M = 8, F = 4	M = 3, F = 2
Fever		15/19 (78.9%)	12/12 (100%)	3/5 (60%)
Ferritin > 500 ng/ml		7/18 (38.8%)	11/12 (91.6%)	5/5 (100%)
Triglyceride > 265 g/dl		3/16 (18.7%)	6/12 (50%)	2/5 (40%)
Fibrinogen < 150 mg/dl		1/17 (5.8%)	6/12 (50%)	1/5 (20%)
Bicytopenia		2/19 (10.5%)	9/12 (75%)	4/5 (80%)
Hb </= 9 g/dL		4/19 (21%)	10/12 (83.3%)	4/5 (80%)
Platelet < 100 × 10^3/mm^3		6/19 (31.5%)	8/12 (66.6%)	5/5 (100%)
Absolute Neutrophil Count < 1 × 10^3/mm^3		0/19	1/12 (8.3%)	2/5 (40%)
sCD25 > 20,000 pg/ml		1/16 (6.2%)	7/12 (58.3%)	5/5 (100%)
H-Score > 169		1/16 (6.2%)	10/12 (83.3%)	
Organomegaly: Splenomegaly (S), hepatomegaly (H), both (S-H)		S = 0/19 H = 9/19 (47.3%) S-H = 0/19	S = 5/12 (41.6%) H = 7/12 (58.3%) S-H = 5/12 (41.6%)	S = 4/5 (80%) H = 5/5 (100%) S-H = 4/5 (80%)
Infections: Bacterial(B), Fungal(F) Viral(V), Unknown(U), both viral and bacterial (VB)		B = 7, F = 3 V = 6, U = 3	B = 8, F = 0 V = 2, U = 2	

	Healthy Controls (HC, n = 14)	Sepsis (S, n = 19)	Sepsis-HLH (SHLH, n = 12)	Primary-HLH (PHLH, n = 5)
Outcome		Died = 1/19 (5.2%) DAMA = 3/19 Total = 21%	Died = 3/12 (25%) DAMA = 1/12 Total = 33.3%	Died = 1/5 (20%) DAMA = 1/5 Total = 40%

Table 2
Median and Interquartile ranges of clinical parameters of children in each group.

Parameters	Healthy Controls (HC, n = 14)	Sepsis (S, n = 19)	Sepsis-HLH (SHLH, n = 12)	Primary-HLH (PHLH, n = 5)
H-Score		130 (91–142)	192.5 (175.3-237.8)	210 (199-243.5)
Ferritin (ng/ml)		551.9 (157.2–3662)	2187 (1373–3777)	1,692 (1,194-5,230)
Soluble CD163 (ng/ml)		0.57 (0.26–1.67)	1.94 (1.09–2.91)	2.16 (1.34–6.82)
Triglyceride (g/dl)		181.5 (101.5-227.5)	264.5 (189.3-328.3)	458.5 (182–1013)
Fibrinogen (mg/dl)		309 (230.5–450.0)	167.5 (72.7-531.5)	230.5 (161.0-269.3)
Soluble CD25 (pg/ml)	1041 (641–1626)	2546 (1835–4491)	22,826 (12,786 – 29,022)	44,000 (30,083–111,709)
Hb (g/dl)		9.9 (9.2–10.6)	7.5 (7.0-8.6)	7.2 (6.25–11.8)
Platelet (counts/mm^3)		146,000 (94000-222,000)	66,000 (27,500 – 112,500)	53,000 (13,500 – 70,000)
Total Leukocyte Counts (cells/ul)		9,900 (9,100 – 14,800)	9,450 (4,725 – 20,100)	2,300 (900 – 11,800)
Absolute Neutrophil Counts (counts/ul)		6,734 (5610–9916)	5,475 (2,510 – 15,059)	1,675 (135-7,127)
SGOT		84.5 (39.5–138.0)	101.5 (49.5-197.3)	67 (26-240.5)

T cell activation in children with sepsis and those with SHLH

In this study, we compared the frequencies of total T cells, it's subsets and the activated T cells between sepsis children and those with SHLH. We found total CD3 + T cells frequencies to be similar between the two conditions (Fig. 1A), while the proportion of its subsets CD4 and CD8 T cells were altered (Fig. 1A). SHLH children had lower CD4 T cells and elevated CD8 T cell frequencies within total T cells, which was also reflected in their ratio (CD4:CD8) with a median (IQR) of 1.07 (0.35–1.17) in comparison to sepsis children who had a median of 1.87 (0.96–2.84) (Fig. 1A). Within the T cell subsets, the frequencies of activated (HLADR + CD38+) cells were elevated in SHLH, though the increase observed in comparison to sepsis was significant only for activated cells in CD4 subset (Fig. 1B and 1C). Similarly, the frequencies of PD1 expressing CD4 + T cells also showed a considerable enhancement in SHLH children than those with sepsis alone (Fig. 1B-C). Since, in the SHLH group of children bacterial infections were more common (~ 80% of children), we analyzed the frequencies of activated CD4 and CD8 T cells among those with confirmed diagnosis of bacterial infection alone, excluding children with other viral, fungal or undetermined infections. In keeping with our unstratified data, activated CD4 + T cells were significantly higher in SHLH than sepsis children even in bacterial infected group (Figure S3). We further assessed the T cell phenotype on a few follow-up samples collected at weekly intervals for upto two to three weeks. One SHLH patient (in blue line) who received HLH directed immune-suppressive treatment showed substantially elevated frequencies of activated T cells initially which gradually declined over the next two weeks, while the CD4:CD8 ratio were low initially and showed recovery over subsequent weeks (Fig. 1D). On the other hand, the four sepsis children showed a delayed activation in CD8 T cells and no activation was seen for CD4 T cells even after a week (Fig. 1D). Though the numbers are very small, these results may also indicate that activated CD4 T cells may be of more advantage in differentiating those with SHLH from sepsis.

Serum cytokine levels associated with T cell activity in children with SHLH

Both sepsis and HLH are often characterized with cytokine storm. However, with the differences seen in the activation profile of immune cells, it is likely to be reflected in their cytokine profile as well. We compared a wide range of cytokines, chemokines and growth factors between sepsis (n = 17) and SHLH (n = 12) children. Both the heatmap (Fig. 2A) and comparison of median concentrations between the two groups (Fig. 2B) showed an increase in the levels of cytokines associated with T cell activation/function (IL2-R and IFNg), chemokines involved in recruitment of activated T cells to the sites of inflammation MIG/CXCL9) and IL-10, while all the remaining cytokines were similar in the two groups of children (Fig. 2B). SHLH children in this study are mostly having bacterial infections, as opposed to sepsis children which were mostly culture negative. For the same set of cytokines, similar analysis was again performed between bacterial sepsis and SHLH only among those with confirmed bacterial infection. The results

showed similar trends for IL-2Ra, IFNg and IL-10 levels as observed previously with the whole data including all infections, although the differences did not reach statistical significance (FigS4).

Diagnostic utility of the T cells and cytokines to distinguish sepsis and SHLH

Since so far, our data indicated activated T cells and its associated cytokines might be more useful in differentiating SHLH from sepsis in children, we further evaluated the diagnostic utility of these cellular and soluble immune parameters using the ROC analysis. We summarized the sensitivity and specificity for the parameters which demonstrated the AUC above 0.7 in Fig. 3A-B. Among the T cell phenotypes described, PD1 expressing CD4 T cells > 23.75% demonstrated a better diagnostic ability with a sensitivity of ~ 86% and specificity of ~ 93% and a highest likelihood ratio of 12 as compared to other T cell parameters. Activated (HLADR + CD38+) CD4 cells with frequency > 2.1% showed a relatively better sensitivity (81.2%) and specificity (75%) than the activated CD8 T cells which showed a sensitivity of 72.3% and specificity of 68.7% at a value > 11.06%. However, both the cell types demonstrated a higher specificity equivalent to PD1 + CD4 T cells (~ 93%) at frequencies above 7.46 and 19%, respectively. On the other hand, the ratio of CD4 to CD8 T cells at a value < 1.28 showed highest sensitivity (100%) and also a good specificity of around 79% (Fig. 3A). Among the cytokines analyzed; IL-2Ra > 1257pg/ml, IL-10 > 54pg/ml, and MIG > 479.9pg/ml demonstrated a good specificity between 81–87%, and a sensitivity of ~ 92%, 83% and 67%, respectively (Fig. 3B). After analyzing the diagnostic accuracy individually for each of the above parameters, we further performed a principal component analysis with the same set of T cell and cytokine parameters. Our analysis demonstrated clear separation of sepsis from SHLH children when both the T cell and cytokine parameters were used together (Fig. 3C). These results indicate that a selective panel of T cell phenotype (HLADR + CD38+, PD1 + CD4+ & CD4:CD8) and cytokines (IL-10, IL-2Ra, and MIG) together are likely to offer enhanced diagnostic accuracy in distinguishing sepsis from SHLH early during the course of disease. Additionally, we also performed ROC analysis for the existing clinical variables for the diagnosis of secondary HLH (such as Ferritin, sCD163, sCD25 and H-Score) and the results are summarized in Fig S5.

Immune markers associated with survival in sepsis or SHLH children

Risk of mortality and adverse outcomes have been reported to be high in both sepsis and HLH patients. Our data also demonstrated an overall high incidence of poor prognosis in both sepsis and HLH conditions; with higher mortality seen in SHLH children (25%) than in sepsis children (5%) (Table 1). Thus, we further investigated if the immune markers are different in those with poor disease outcome and if it can be useful in early prediction of adverse outcome. We analyzed the immune markers at presentation in children who died during their hospitalization (non-survivors, n = 4; one sepsis, 3 SHLH) and those who

were discharged (survivors, n = 24). Among the survivors, those who were discharged against medical advice (DAMA) were excluded from the analysis. Those who died in this study had higher total leukocyte count & higher absolute neutrophil counts regardless of their underlying condition; sepsis or SHLH. (Fig-S6A). In line with these cellular components, the soluble markers of macrophage/neutrophil activation (Ferritin, IL-6, IL-8) were also significantly elevated in the plasma of children who died during their hospitalization period (Fig-S6B). Interestingly, the non-survivors, showed reduced or no activation of T cell subsets as compared to the survivor's group (Fig-S6D). However, cytokine related to T cell activity (sCD25, IFNg, MIG) were similar in both the groups, with IL-10 levels are slightly higher among the non-survivors as compared to the survivors (Fig-S6C). Thus, certain markers such as TLC, ANC, IL-6, IL-8 and activated T cell frequencies can be of significance in determining the risk of poor prognosis in sepsis patients with or without co-occurrence of HLH which needs to be validated further on a larger cohort size.

Discussion

Both sepsis and HLH often present with overlapping clinical symptoms and hyperinflammation. Hence, early distinction between the two syndromes and timely intervention remains a challenge, more so when the underlying cause of HLH is infection. Since, both the conditions show an obvious dysregulation of the immune system, in this study we compared the immune profiles; i.e., soluble and cellular markers of activation of immune system and cytokines/chemokines reflecting the state of inflammation in children with sepsis and those which develop sepsis-associated HLH. We report differences in the T cell subsets, their activation states and the cytokines associated with T cell activity in sepsis children with and without HLH.

In this study, SHLH children presented with similar T cell frequencies but lower CD4/CD8 ratios in comparison to the sepsis children without HLH. Similar findings were also reported in another study in children with EBV-associated HLH when compared with sepsis children[14]. Increased apoptosis of T cells, preferentially in activated CD4 is a common phenomenon noticed in sepsis patients[15–18]. Whether a marked reduction in the CD4/CD8 ratio in SHLH patients is either governed by preferential apoptosis of CD4 T cells or excessive proliferation of activated CD8 T cells which is often reported in HLH patients; or both, needs further investigation. A number of studies have clearly demonstrated expansion of activated T cell population as a characteristic feature in HLH patients; more evidently in CD8 T cells[10,12,14] while in sepsis, suppressive T cell phenotypes or function was noticed[17,19–21]. More recently, the diagnostic utility of CD38hi HLADR + activated T cells has also been reported in distinguishing active HLH from early sepsis in children[10] (Chaturvedi et al). In keeping with these studies, we also report higher frequencies of activated (CD38 + HLADR+) CD4 T cells in children with SHLH compared to those with sepsis without HLH. In contrast to these studies[12,14], however, the levels of activated CD8 T cells (though elevated in SHLH), did not reach statistical significance in our study. Besides HLADR and CD38, PD1 expression also upregulates upon activation of T cells, and at the same time its expression is often linked with exhaustion in T cells[22,23]. Our data showed that similar to the activated T cells, the PD1 expressing CD4 T cells were also remarkably elevated in SHLH children than those with sepsis alone which is in line with previous

studies[24]; however, we find that these differences were not statistically significant for CD8 PD1 + cells. Whether these cells exhibit suppressive immune function may not be concluded from our study, however a recent study have shown partial impairment of cytotoxic function in PD-1 + T cells in HLH patients[24]. Also, the lower CD4:CD8 ratio observed in SHLH may indicate reduced CD4 help to CD8 cells, which in turn can result in impaired CD8 T cell proliferation and effector functions[25–27]. Although previous studies on HLH patients have emphasized more on activated CD8 T cells, our data in contrast suggest CD38 + HLADR + or PD1 expressing CD4 T cells to be more useful in differentiating SHLH from sepsis cases at an early stage or a may be few weeks later as well; as seen in a couple of follow up samples in this report. These differences in results may be explained by the differences in our study design or patient characteristics, differences in sample size & statistical power, heterogeneity in etiological factors, differences in genetic composition, as well as technical variability in laboratory methodologies across study cohorts. Also, activation of CD8 T cells is common in viral infections and other intracellular pathogens which can further amplifies with the severity of disease[28,29]. As a significant proportion of sepsis children in this study were severe with septic shock/multiorgan dysfunction, it is likely that the background activation of CD8 T cells is already very high in some patients and hence making it less suitable for early diagnosis of SHLH.

Multiple studies have shown cytokines/chemokines related to both T cell (IFNg, CXCL9, CXCL10, IL-10 etc.) and monocyte/macrophage activation (IL-18, IL-6, IL-1b, IL-1RA, TNFa etc) to be elevated in HLH in comparison to those with sepsis or other inflammatory conditions[11,30–33]. However, in this study where we compared the cytokine profile between sepsis and sepsis-associated HLH, only a set of cytokines which are largely involved in T cell function/regulation were found to be different. Thus, in corroboration with our T cell data, we also report higher levels of IFNg (secreted by effector Th1 cells), IFNg induced chemokines MIG/CXCL9 (monokine induced by interferon-gamma, a chemoattractant for CXCR3 expressing effector T cells), IL-2Ra (which is considered as a surrogate for T cell activation) and IL-10 (an anti-inflammatory cytokine produced by regulatory T cells, Th-2 cells or activated macrophages; regulating T cell response to viral infections) (*ref Ejrnaes et al*). These findings are also supported by a recent study[31] where the both gene expression and plasma cytokine profile indicated upregulation of IFNg inducible genes in pediatric HLH patients in comparison to those with severe sepsis/SIRS. Though IFN-g levels were not seen to be elevated in all the sepsis-HLH samples in this study, yet higher MIG levels reflects an active and increased IFNg signaling predominantly in SHLH children. Thus, as reported previously, a shorter half-life may explain poor detection of IFNg in some of the SHLH patients[34]. When the same set of cytokines were evaluated for their diagnostic potential to distinguish between the two syndromes, IL-10, MIG and IL2Ra also demonstrated a good diagnostic capacity with > 80% specificity. A few reports have also suggested altered IL6/IL-10 or IL-6/CXCL9 ratio in HLH patients with lower IL-6 and higher IL-10 or CXCL9 in differential diagnosis of HLH from sepsis. We observed a similar trend in primary HLH children, however between SHLH and sepsis IL-6 levels were similar. In keeping with this a recent study also indicated higher levels of IL-6 in infection associated HLH (other than EBV) when compared to other forms of HLH[11].

Previous studies have reported a positive association of IL10 levels with the poor outcomes in both pediatric and adult HLH patients[33–36]. We also observed, higher levels of IL10 in SHLH children who died than those who survived. Interestingly, none of the IFN-g regulated cytokines were found to be further elevated in children who died. Instead, those who died showed a higher concentration of IL6, IL8, ferritin, IL10 in comparison to those who survived. These immune signatures were previously shown to be associated with poor survival and high severity/SOFA score in sepsis children[37]. Additionally, higher absolute total leukocyte and neutrophil counts indicate that the hyperinflammation in those who died, likely to be contributed by enhanced monocyte/macrophage and neutrophils activation. Whereas, a poor percentage of activated T cells among the non-survivors may be a result of either enhanced apoptosis of activated T cells or their enhanced recruitment to the sites of infection or inflammation[38]. Similar trend of lower proportion of activated T cells in a small number of poor-outcome HLH patients was also noticed in another report by Chaturvedi et al, hence this needs further validation and investigation on a larger sample size.

Though the study's limitation is its smaller sample size as it was exploratory, yet the findings on increased T cell activation along with elevated IFN-g and it's inducible proteins are similar to the other two similar studies published recently[10,31]. In addition, this study is unique, as the pediatric HLH patients in our study are a clean infection associated HLH children and those with primary HLH were analyzed separately. Another, important factor is the study design where we assessed the immune profile at an early timepoint within a couple of days of diagnosing sepsis, thus the results indicate early markers to differentiate SHLH from sepsis. Thus, together our data show, reduced CD4 to CD8 ratio, increased T cell activity with significantly higher proportions of activated CD4 T cells and IFNg, MIG and IL-10 levels in SHLH than those with sepsis. Out of these, CD4 to CD8 ratios, percentage of HLADR + CD38+/PD1 + CD4 cells and IL-10 plasma concentrations; together may provide an advantage in early diagnosis of SHLH from those with sepsis without HLH.

Declarations

Data sharing: For original data, please contact drdeepthiboddu@gmail.com

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Authorship and conflict-of-interest statements

D.B and S.B.P contributed in study conception and design. A.C, A.K, P.S performed phenotyping, A.C and C.S participated in Luminex assay and B.V and Vinod performed soluble CD25 ELISA. A.C analysed the data and drafted the manuscript. A.C, D.B, S.B.P edited and finalized the manuscript. D.B, J.P.C and others provided HLH and sepsis patient samples. All authors reviewed and approved the final report. None of the authors have declared any competing interest.

Footnotes

For original data, please contact email id of the corresponding author.

Supplementary data is available in the online version of this article.

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Figures

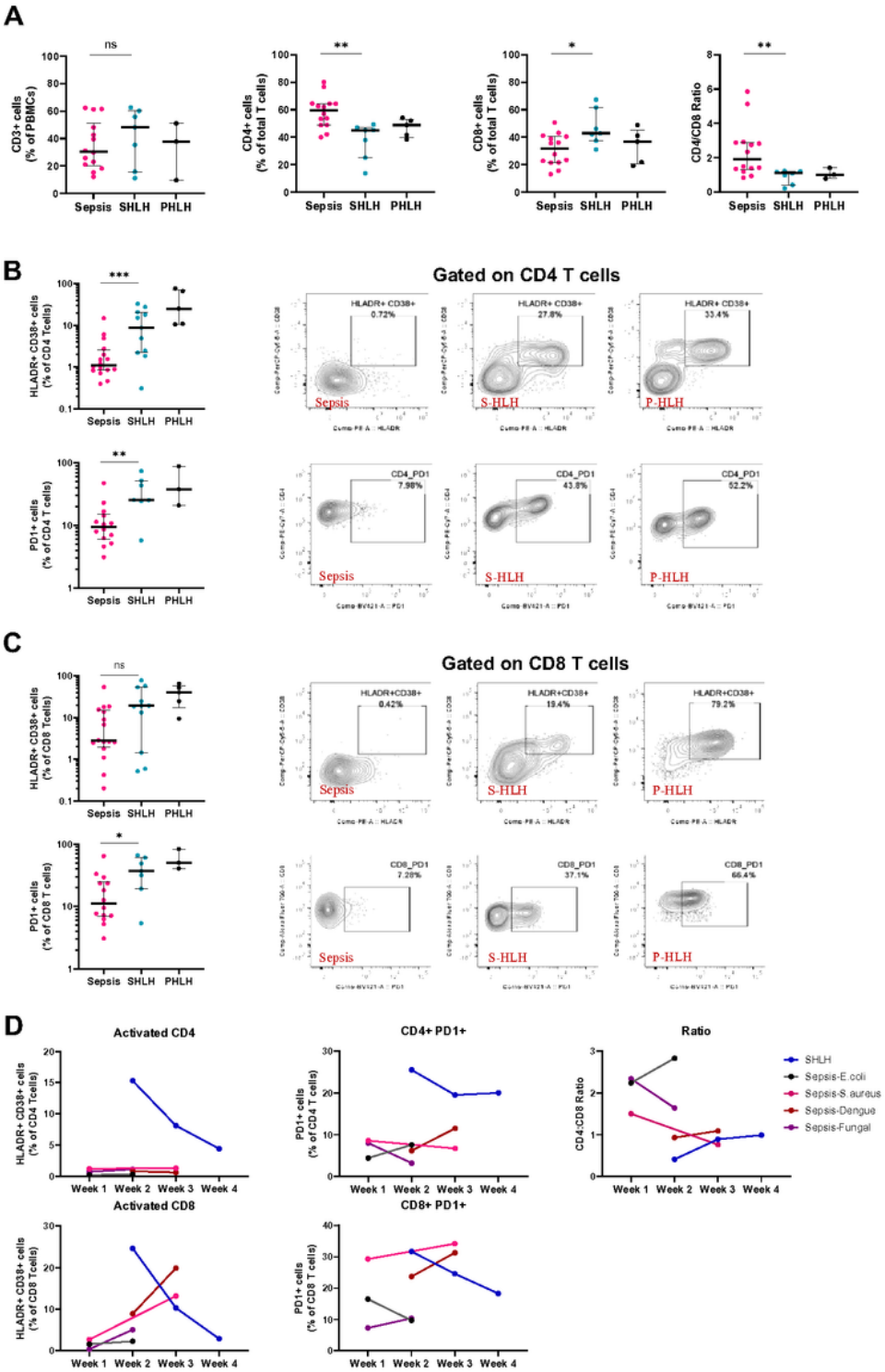


Figure 1

T cell profile in sepsis and SHLH children. Comparison of median frequencies of CD3, CD4, CD8 T cells and median ratio of CD4 to CD8 cell subsets **(A)**, median percentage of activated (HALDR+CD38+) CD4 and PD1+ CD4 T cells and CD8 T cells **(B & C,** respectively**)** between sepsis and SHLH children. The representative gating graphs for the activated and PD1+ CD4 and CD8 T cells in sepsis, SHLH and PHLH are shown on the right. **(D)** Trajectories of CD4:CD8 ratio, activated and PD1+ CD4 and CD8 T cells in

weekly follow-up samples for one SHLH (blue) and 4 sepsis children. Mann-Whitney test was performed to compare medians between sepsis and SHLH groups and only comparisons with p-values <0.05 are displayed on each graph as *, **, *** & **** for values <0.05, <0.01, <0.001 & <0.0001, respectively.

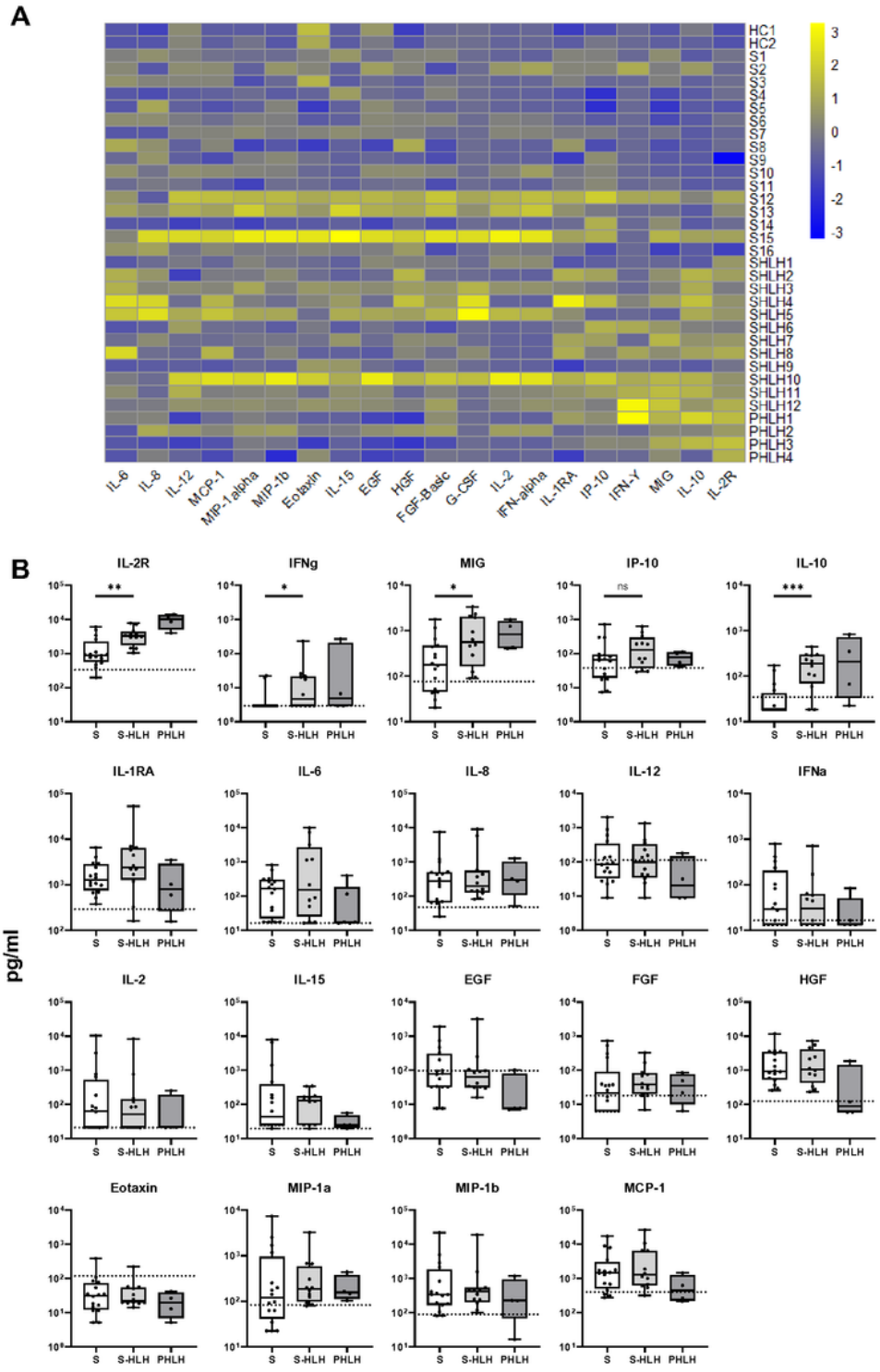
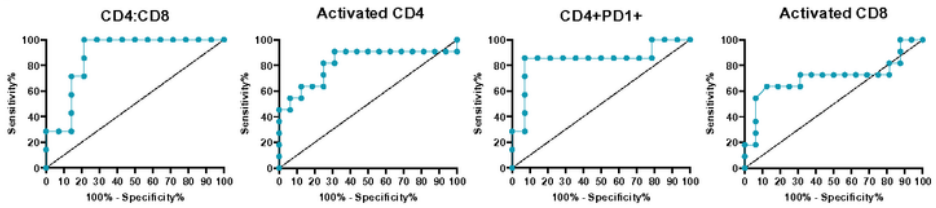


Figure 2

Cytokines related to T cell activity are elevated SHLH in comparison to the sepsis children. A) Heatmap showing the analytes evaluated across all samples from 2 pediatric healthy control (HC1-2), 16 sepsis (S1-16), 12 Sepsis-HLH (SHLH1-12) and 4 Primary HLH (PHLH1-4) children. **B)** Comparison of median concentration of the plasma analytes between sepsis and SHLH children. Horizontal line in the median plots is the average concentration of each analyte from pediatric healthy controls. Mann-Whitney test was performed to compare medians between sepsis and SHLH groups and only comparisons with p-values <0.05 are displayed on each graph as *, **, *** & **** for values <0.05, <0.01, <0.001 & <0.0001, respectively.

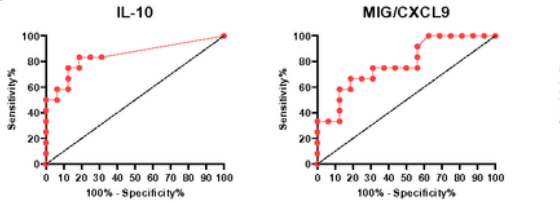
Figure-3

A



Parameters	*Cut-off*	*Sensitivity %*	*Specificity %*	*Area Under Curve (AUC)*	*Likelihood Ratio (LR)*	*P-value*
CD4/CD8 Ratio	< 1.28	100	78.57	0.87	4.6	0.006
% of Activated CD4 cells	> 2.1	81.82	75.0	0.82	3.27	0.006
	> 7.46	54.55	93.75		8.72	
% of PD1+ CD4+ cells	> 23.75	85.71	92.86	0.85	12.0	0.011
% of Activated CD8 cells	> 11.06	72.23	68.75	0.71	2.32	0.07

B



Parameters	*Cut-off*	*Sensitivity %*	*Specificity %*	*Area Under Curve (AUC)*	*Likelihood Ratio (LR)*	*P-value*
IL-10 pg/ml	> 53.95	83.3	81.25	0.85	4.4	0.002
	> 85.70	75.0	87.50		6.0	
	> 151.5	58.3	93.75		9.3	
MIG/CXCL9 pg/ml	> 453.3	66.67	81.25	0.78	3.5	0.011
	> 479.9	58.33	87.50		4.6	
IL-2Ra pg/ml	>1257	91.67	81.25	0.87	4.9	0.0008
	>2760	75.0	87.50		6.0	

C

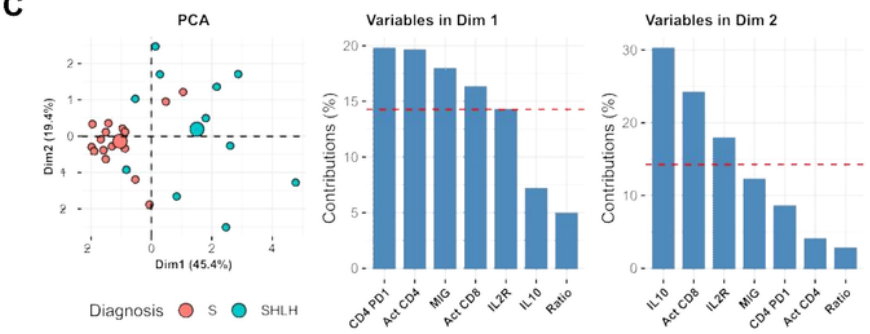


Figure 3

Diagnostic potential of phenotypic and soluble parameters linked to T cell activity/function to differentiate between sepsis and SHLH children. (A and B) ROC curves along with table displaying cut-off values and demonstrating diagnostic potential for CD4:CD8 ratio, HLADR+CD38+ and PD1+ CD4 T cells, HLADR+CD38+ CD8 T cells and for soluble plasma analytes (IL10, MIG and IL-2Ra)**. C)** Principal component analysis with the same set of variables (CD4:CD8 ratio, HLADR+CD38+ and PD1+ CD4 T cells

and HLADR+CD38+ CD8 T cells, IL-10, MIG and IL-2Ra) displaying separation between sepsis and SHLH children when all the parameters are considered together.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.docx
- Supplementaryfile.pdf