Original article

Double negative alpha beta T cells in pediatric hemophagocytic syndromes

Introduction*: Autoimmune lymphoproliferative syndrome (ALPS) and hemophagocytic lymphohistiocytosis (HLH) share clinical and laboratory features including lymphadenopathy, splenomegaly, and pancytopenia. We sought to measure αβ double negative T cells (αβ DNT) in a group of patients with established diagnosis of HLH in relation to disease activity and severity.* ***Methods****: We conducted a follow-up, controlled study that comprised 25 patients with HLH and 25 healthy matched controls. Patients were subjected to clinical evaluation and flowcytometric measurement of αβ DNT Cells at presentation and 9 weeks after start of HLH induction treatment.* ***Results****: In 17 (68%) patients, infection was the trigger of HLH while the cause was malignancy in three (12%), and rheumatological disorders in two patients (8%). At enrollment, 15 patients (60%) had αβ DNT cells levels [median (IQR): 1.71 (1.25-2.12)] that were significantly higher than the control values [median (IQR): 0.7 (0.4-0.8)] (p<0.001). The αβ DNT counts of patients were also higher at enrollment as compared to values at the end of week 9 [median (IQR): 0.76 (0.45-1.17)]; p=0.018. Survivors (n=8) and non-survivors (n=17) had comparable levels of αβ DNT cells at enrollment (p=0.861). αβ DNT cell count correlated positively with ALT (p=0.019) and negatively with CD4/CD8 ratios (p=0.023).* ***Conclusion****: Elevated αβ DNT cell counts might be related to the HLH process and this implies that mild elevation can exist in HLH and are not specific to ALPS. Wider scale studies with longer periods of follow up are needed to validate the results and properly outline the correlation between both medical conditions.*

Keywords: Hemophagocytic lymphohistiocytosis, Double negative T cells, mortality, ALPS.

Elham M. Hossny, Rasha H. El-Owaidy, Hanaa M. Afifi*, Hadeer R. Ahmed**

*Pediatric Allergy and Immunology Unit, Children's Hospital, and *Department of Clinical Pathology, Ain Shams University, **Ministry of Public Health Hospitals, Egypt.*

Correspondence:
Rasha Hassan El-Owaidy, Pediatric Allergy and Immunology Unit, Children's Hospital, Ain Shams University, Cairo, Egypt
Email: rashahasan@med.asu.edu.eg

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH), also known as hemophagocytic syndrome (HPS), is an uncommon, life-threatening, hyperinflammatory immunological disorder that is characterized by prolonged high fever, hyperlipidemia, hepatosplenomegaly and hemophagocytosis of bone marrow cells [1].

TCR alpha beta^{+}CD3^{+}CD4^{-}CD8^{-} "double negative" (αβ DN) T cells comprise a small subset of mature peripheral T cells. While DN T cells constitute a rare and heterogeneous T cell subpopulation in healthy individuals, numbers of TCRαβ^{+} DN T cells are expanded in several inflammatory conditions and may be involved in systemic inflammation and tissue damage as in disorders like SLE, Sjogren's syndrome, and psoriasis.[2] The expansion of these cells in peripheral blood and lymphoid tissues of patients with autoimmune lymphoproliferative syndrome (ALPS) is a consistent feature and is one of the diagnostic criteria of ALPS. [3]

Mild elevations of these cells in several autoimmune diseases suggest that αβ DNTs may perhaps be more common among immune disorders and that these disorders may share a common pathway for immune dysregulation and immune dysfunction**.** [4]

We sought to investigate the expression of αβ DN T cells in a group of pediatric patients with hemophagocytic syndrome. The ultimate objective is to anticipate the possible relation to HLH activity and/or severity.

METHODS

Study design: This is a controlled prospective study that was carried out in the pediatric Allergy and Immunology Unit, Children`s Hospital, Ain Shams University, during the period from April 2015 to June 2016.

Study population:

Patients' group: we enrolled 25 pediatric patients with physician-diagnosed hemophagocytic syndrome, fulfilling the diagnosis of HLH according to the HLH diagnostic criteria put forward by the Pediatric HLH Study Group of the Histiocyte Society.[5] Patients receiving immune-suppressive therapy at time of enrollment were excluded from the study.

Control group: This group comprised 25 age and sex matched healthy children as controls enrolled by one to one approach from the outpatient clinic after exclusion of any chronic illness.

Ethical considerations: Informed consent was obtained from the parents or care givers after explanation of the study before enrollment. The study gained approval of the local Ethics Committee of the Pediatric Department, Ain Shams University.

Study Methods:

1- Clinical evaluation: Detailed history was taken concerning age, gender, parental consanguinity, age of disease onset, intake of immunosuppressive drugs, presence of infection and its site and symptoms, presence of underlying chronic illness, and similar cases in their family. Systematic clinical examination was performed for evaluation of HLH activity and/or underlying illness with emphasis on signs, type and site of infections, pallor, bleeding, fever, lymph node enlargement, hepatosplenomegaly, rash, joint swelling and neurological deficit. Underlying illness diagnosis was verified whether rheumatological disorder, malignancy or immunodeficiency. Patients were re-evaluated at week 9 of HLH induction therapy for αβ DN T-cell count in surviving patients.

2- Laboratory investigations included the following tests:

- Complete blood count (CBC) was done using the electronic counter (Sysmex XT-1800i, Canada). Peripheral blood was smeared and stained by Lishman's stain for white cell differential count. Results were compared to age related reference range.[6] Erythrocyte sedimentation rate (ESR) was measured by Westergren method. We also measured serum alanine transaminase (ALT), aspartate transaminase (AST), serum albumin, serum lactate dehydrogenase (LDH) and triglycerides (fasting for 10 hours) using NADH, Kinetic UV, IFCC rec, Spinreact Kit using Cobas (Roche, Germany). Serum concentration of fibrinogen (g/L) was measured by p-Nitrophenyl phosphate, Kinetic, DGKC, Spinreact Kit using a coagulameter (DADE Behring, USA). Serum ferritin (ng/mL) was measured using a coagulameter (Au680, Bechman Coulter, USA).
- Flowcytometric measurement of alpha beta double negative (αβ DN) T cells was done using EPICS XL™ flow cytometer (Beckman Coulter Inc, California, USA). αβ DN T cells were measured among patients' group at enrollment and by week 9 from the start of HLH induction therapy among survivors and were also measured among controls. Counts ≥ 2 % of T cells were considered elevated.[7]

Statistical Methods:

Data were analyzed using IBM SPSS Statistics version 23 (IBM Corp., Armonk, NY, USA), MedCalc version 15 (MedCalc Software BVBA, Ostend, Belgium). Normality of numerical data distribution was examined using the D'Agostino-Pearson test. Non-normally distributed numerical variables were presented as median and interquartile range and between-group differences were compared using the Mann-Whitney test. Paired numerical data were compared using the Wilcoxon signed ranks test. Categorical variables were presented as number and percentage and intergroup differences were compared using Fisher's exact test. Ordinal data were compared using the chi-squared test for trend. Receiver-operating characteristic (ROC) curve analysis was used to examine the value of αβ DN T-cells for discrimination between cases and controls, and for prediction of mortality among cases in active disease. A p value <0.05 was considered significant.

RESULTS

Ages of the enrolled patients ranged from 6 months to 13 years with mean±SD values of 4.4±3.6 years. They were 14 males (56%) and 11 females (44%) with mean age at disease onset of 3.5 years. In 17 patients, infection was the trigger of HLH, while it was malignancy in three (non-Hodgkin lymphoma in 2 patients and acute lymphoblastic leukemia in one patient), rheumatological disorders in two (juvenile idiopathic arthritis) and immune deficiency in three (Chediak-Higashi syndrome, Griscelli syndrome and severe combined immunodeficiency). Clinico-demographic and laboratory abnormalities of enrolled patients are depicted in table 1. Bone marrow aspirate and examination was done in 9 patients only (36%) and revealed evidence of hemophagocytosis in 5 (55%) of them.

At enrollment, 15 patients (60%) had mildly elevated αβ DNT cells (>2% of CD3+ T cells) with percentages ranging between 0.3 and 41.7%; median (IQR): 1.71 (1.25-2.12) and mean±SD : 3.42±8.06%. These values were significantly higher than those of the control group [median (IQR): 0.7 (0.4-0.8)], z= -4.483, p<0.001. αβ DN T cells percentage of >1.7% could discriminate between patients and controls with area under curve (AUC) of 0.870, sensitivity 76% and specificity 96 % (p<0.001) (figure 1).

Patients were put on induction therapy of HLH protocol 2004.[5] All patients received dexamethasone and 18 (72%), 15 (60%), 8 (32%) and 6 (24%) cases were co-treated with cyclosporine, etoposide, intravenous immune-globulin (IVIG) and intrathecal therapy (methotrexate with steroids) respectively. Upon re-evaluation by week 9 of HLH induction therapy, 8 out of the 25 enrolled patients (32%) survived through the study period. Survivors (n=8) and non-survivors (n=17) had comparable ages, initial clinical and laboratory findings and HLH treatment regimen received. Initial αβ DN T cells percentages were comparable between the 2 groups (z=-0.175, p=0.861). Among survivors, αβ DN T cells percentages were significantly lower by the end of induction therapy [median (IQR): 0.76 (0.45-1.17)] in comparison to initial levels [median (IQR): 1.71 (1.25-2.12)]; p=0.018 (figure 2).

Table 1. Main demographic, clinical and laboratory parameters of the studied patients.

Variable	Cases (n=25)
Age (years)	
Range	0.5 – 13
Mean±SD	4.4±3.6
Gender (No/%)	
Females	11 (44.0%)
Males	14 (56.0%)
Age of onset of HLH disease (years)	
Range	0.1 - 12.7
Mean±SD	3.5±3.5
Fever (°C)	
Range	38.0 –40.0
Mean±SD	39.1 ± 0.7
Duration of fever (days)	
Range	5.0 – 60.0
Mean±SD	22.1 ± 14.9
Underlying illness (No/%)	
• Infection	17 (68.0%)
• Malignancy	3 (12.0%)
• Immune deficiency	3 (12.0%)
• Rheumatological diseases	2 (8.0%)
Clinical findings (No/%)	
Fever ≥38.5 °C, >7days	22 (88.0 %)
Hepatomegaly	20 (80.0%)
Splenomegaly	18 (72.0%)
Lymphadenopathy	25 (100.0%)
Neurological manifestations	6 (24.0%)
Pancreatic affection	1 (4.0%)
Lab abnormalities	
• Hemoglobin <9 (g/dl)	19.0 (76.0%)
• Platelets < 100 (10^3/ul)	25 (100.0)
• ANC < 1 (10^3/ul)	16 (64.0%)
• Ferritin>500 (ng/ml)	25(100.0%)
• Fibrinogen <1.5 (g/l)	25(100.0%)
• Hypertriglyceridemia >265mg/dl	25(100.0%)
• Elevated LFTs	4 (16.0%)

ANC: absolute neutrophilic count; Lab: laboratory; LFTs: liver function tests; No: number; SD: standard deviation.

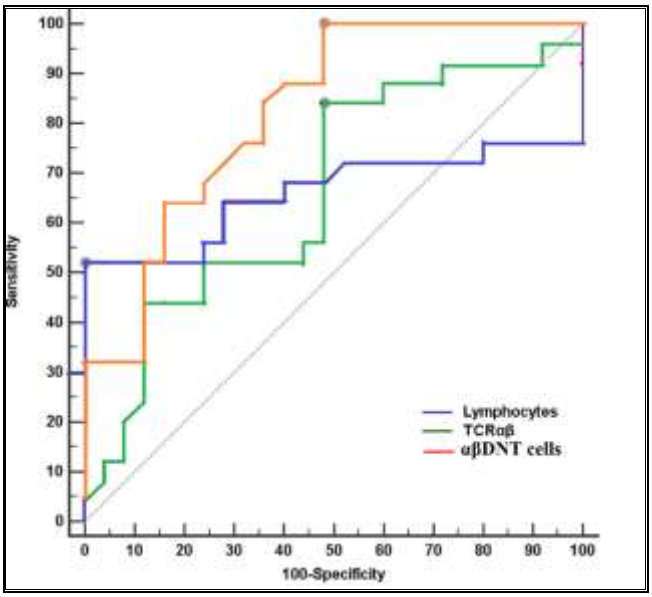

Figure 1. Receiver-operating characteristic (ROC) curves for discrimination between cases and controls in lymphocytes, TCRαβ and αβDNT cell counts.

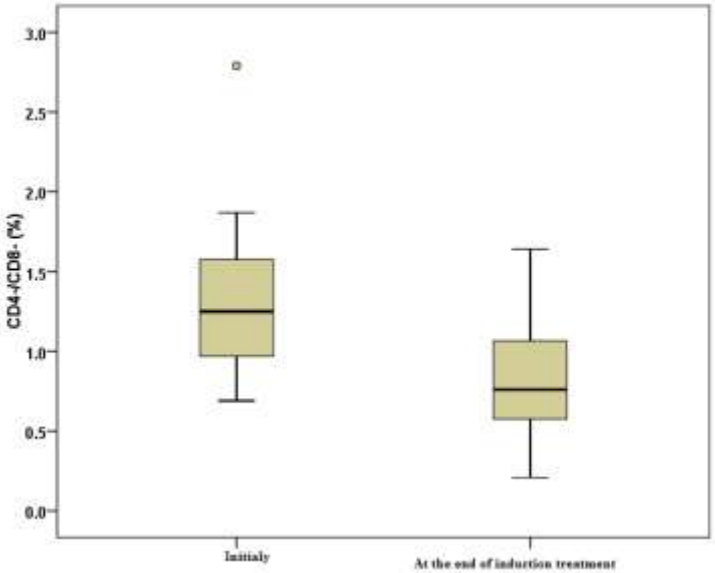

Figure 2. Box plot of the αβ DNT (%) before and after HLH therapy among survivors

DISCUSSION

Infection was the most common trigger among our patients diagnosed with HLH. This observation agrees with what has been previously reported that infection, especially viral, is the most common trigger of HLH activity and this applies to primary[8] and secondary HLH forms.[9,10] Common viruses in this domain include Epstein-Barr virus, cytomegalovirus and herpes simplex virus.[11] Also, other infections like fungal, parasitic or bacterial infections, including tuberculosis can trigger HLH activity. [12]

In the current study, immune deficiency was detected in three patients with Chediak-Higashi syndrome, Griscelli syndrome and severe combined immunodeficiency. None of these children survived through the study period despite receiving treatment. HLH with primary immunodeficiency has been characterized by poor prognosis requiring rapid clinical and genetic diagnosis of the PID as well as initiation of appropriate management, including allogeneic hematopoietic stem cell transplantation.[13]

Among our series, two patients had rheumatological diseases as a trigger for HLH. HLH secondary to rheumatological illness is

usually termed macrophage activation syndrome (MAS). MAS could be fatal and complicate various pediatric autoimmune diseases especially systemic juvenile idiopathic arthritis (SOJIA) and systemic lupus erythematosus (SLE). MAS tends to occur in the setting of active underlying disease**.**[14-16].

αβ DN T cell percentages were significantly elevated among our HLH patients with a median value of 1.7% compared to controls (value). In addition, αβ DN T cells percentage was elevated in 15 patients with levels >2% of CD3+ T cells. These observations need to be further validated using molecular studies and cell cultures with cytokines assay. It may emphasize that mild elevation of αβ DN T cells is not specific for ALPS and should be interpreted cautiously even in presence of lymphoproliferation.

αβ DN T cells are legitimate components of the normal immune system that normally do not exceed 2% of T cells. Fas-mediated apoptosis actively removes normally existing αβ DN T cells from the periphery while impaired Fas-mediated apoptosis leads to accumulation of these cells, manifesting as lymphoproliferation. Both regulatory and pathogenic functions have been attributed to αβ DN T cells. The excessive activation of CD8+ T lymphocytes and macrophages in HLH leads to chronic stimulation of CD8+ cytotoxic lymphocytes and the release of cytokines.[17] Although there is no direct and reproducible evidence for this concept, yet it might explain the link between the HLH uncontrolled inflammation and the mild increase in αβ DN T cells.

In support of the above findings, ***Abolghasemi et al. (2015),*** reported a 14-years old boy with HLH diagnosis who had elevated αβ DN T cells and normal level of vitamin B12. A diagnosis of HLH was considered since he met five of 8 criteria including pancytopenia, >38.5 °C fever, splenomegaly, hyperferritinemia and hypo-fibrinogenemia. Treatment of HLH was initiated according to the HLH-2004 protocol. The authors also noted that the patient's spleen had atypical T-cell hyperplasia with αβ DN T cells, consistent with the diagnosis of ALPS. He was then treated with prednisone and azathioprine which led him into remission. [18]

HLH is known to have a high mortality rate whether primary or secondary. In Japan, among 57 HLH patients who underwent HSCT, survival rate was 65.0% for FHL and 85.7% for EBV-HLH. [19, 13] Another cohort study on 116 HLH Chinese children (mean age at diagnosis 27.5 months), had a fatality rate of 26 %, half of them died in the first 30 days of diagnosis.[20] We revaluated our patients by week 9 of HLH induction therapy in the 8 children who survived through the study and observed that the median αβ DN T percentages were significantly lower compared to initial levels (p=0.018), indicating a possible correlation of these cells to the degree of inflammation. αβ DN T cells had been described to be one of the major producers of IL-17, a well-known proinflammatory cytokine,[21] which may indicate a pathogenic role for αβ DN T cells. ***El-Sayed et al[22]*** in their prospective study which included 21 female patients with juvenile SLE (10–17 years old) found that the αβ DN T cells percentage values correlate with SLE disease activity. [22] Steroid therapy was reported to induce a significant decrease in the number and proportion of αβ DN T cells.[23]

Initial αβ DN T cell percentages were comparable among survivors and non-survivors in our series. To the best of our knowledge, no previous studies addressed the relation between αβ DN T cells and HLH activity or prognosis. However, αβ DN T cells are reported to have prognostic value in other disorders. ***Licciardi et al[24]*** in their study on 39 patients with oligoarticular JIA, reported that low synovial αβ DN T cells (<1.8%) at disease onset is linked to longer free-disease survival. Also, their patients who relapsed during the follow-up showed higher αβ DN T cells at diagnosis as compared with non-relapsed patients.

Detailed comparison between survivors and non-survivors in our study revealed comparable clinical and laboratory data except for initial platelet counts which were significantly lower among survivors. This finding is indeed limited by the sample size and might point to the presence of other poor prognostic parameters in the non-survivors including poor adherence to treatment or influence of the original disease. Although, low platelet count was described as a poor prognostic factor associated with high mortality in several studies**,** [25-27] ***Oto et al***[28]in their study on 34 children with HLH, denied any relation between the platelet count and HLH prognosis.

In conclusion, this pilot study denoted mild αβ DN T cell elevation in HLH patients that was associated with HLH activity and to some extent with severity. This may denote that αβ DN T cell overexpression is not ALPS specific. Our conclusions are indeed limited by the sample size and short follow up duration. Also, the consecutive enrollment of the sample led to uneven distribution of patients according to HLH triggers and this can hinder the power of analysis. Further wider scale

studies may increase our insight into this condition. Also, an investigation at the molecular level could be more informative in terms of elucidating the relation between ALPS and HLH.

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