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Short Communication

$CD38^{high}$/HLA-$DR^{+}CD8^{+}$ T lymphocytes display pathogen-specific expansion regardless of hemophagocytic lymphohistiocytosis

Lorenzo Lodi[*1,2], *Walter Maria Sarli*[*1,2], *Silvia Ricci*[1,2], *Laura Pisano*[2], *Silvia Boscia*[2], *Maria Vincenza Mastrolia*[3], *Sara Malinconi*[1], *Eleonora Fusco*[1], *Elena Sieni*[4], *Giuseppe Indolfi*[5,6], *Gabriele Simonini*[3,6], *Luisa Galli*[1,7] *and Chiara Azzari*[1,2]

[1] Department of Health Sciences, University of Florence, Florence, Italy
[2] Immunology Unit, Meyer Children's Hospital IRCCS, Florence, Italy
[3] Rheumatology Unit, Meyer Children's Hospital IRCCS, Florence, Italy
[4] Pediatric Hematology-Oncology Department, Meyer Children's Hospital IRCCS, Florence, Italy
[5] Hepatology Unit, Meyer Children's Hospital IRCCS, Florence, Italy
[6] Department NEUROFARBA, University of Florence, Florence, Italy
[7] Infectious Disease Unit, Meyer Children's Hospital IRCCS, Florence, Italy

The characteristic expansion of T $CD38^{high}$/HLA-$DR^{+}CD8^{+}$ lymphocytes observed in hemophagocytic lymphohistiocytosis (HLH) and macrophage activation syndrome (MAS) proved able to distinguish HLH/MAS from sepsis and systemic juvenile idiopathic arthritis. However, the performance of this marker in differentiating HLH/MAS from other pediatric febrile conditions with similar clinical onset and yet entirely different treatments remains unexplored. $CD38^{high}$/HLA-$DR^{+}CD8^{+}$ frequencies measured in the peripheral fresh blood of pediatric patients attended for suspicion of HLH/MAS were retrospectively recorded and clinical characteristics were retrieved. $CD38^{high}$/HLA-$DR^{+}CD8^{+}$ frequencies in HLH/MAS patients (15 patients; median: 22.0%, IQR: 11.0–49.0%) were compared with those who presented febrile conditions other-than-HLH (28 patients; median: 13.0%, IQR: 3.9–28.7%; $p = 0.24$). HLH and non-HLH patients were subsequently regrouped based on the presence of an identified infection (22 patients; median: 27.0%, IQR: 15.2-72.1%) and compared with those without infections (21 patients; median: 7.6%, IQR: 3.7–24.3%; $p = 0.0035$). $CD38^{high}$/HLA-$DR^{+}CD8^{+}$ percentages were significantly higher only in the infection group compared with the noninfection one, with a patent pathogen-specific expansion in Epstein–Barr virus primoinfection and visceral leishmaniasis regardless of the presence of HLH. $CD38^{high}$/HLA-$DR^{+}CD8^{+}$ frequencies do not appear as an HLH-specific marker as they naturally expand in other clinical situations that are common in childhood and may mimic HLH initial presentation.

Keywords: $CD38^{high}$/HLA-$DR^{+}CD8^{+}$ lymphocytes · Hemophagocytic lymphohistiocytosis · Macrophage activation syndrome

Introduction

Hemophagocytic lymphohistiocytosis (HLH) and macrophage activation syndrome (MAS) are rare, life-threatening hyper-acute manifestations due to detrimental overactivation of the immune system [1–3]. The diagnosis is based on the identification of at least five out of eight clinical and laboratory criteria as defined by the Histiocyte Society [4]. Specifically designed criteria are available for the diagnosis of MAS, the HLH associated with rheumatic diseases [5]. The differential diagnosis from conditions with a similar clinical presentation and yet entirely different treatments remains challenging, especially in the early stages of the disease.

Correspondence: Dr. Lorenzo Lodi
e-mail: lorenzo.lodi@unifi.it

[*] These authors contributed equally to this work.

The plasma-soluble interleukin-2 receptor (sIL-2R) has proved to be a suitable marker to assess T-cell activation in HLH. However, sIL-2R is not an HLH-specific marker since other hyperinflammatory and immune regulatory disorders have T-cell activation profiles overlapping with HLH [6]. Moreover, sIL-2R levels may increase due to delayed renal or hepatic clearance that are not infrequent in HLH-like conditions. These limits, together with the restricted access to sIL-2R testing, reduce its use in clinical practice. Recently, novel flow cytometric markers have emerged, potentially supporting clinicians in the HLH diagnostic process [7, 8]. A strong correlation between activated T cytotoxic lymphocytes ($CD38^{high}$/HLA-DR^+CD8^+) frequencies and sIL-2R has been demonstrated, showing that this marker is capable of identifying T-cell activation in peripheral blood [9]. In fact, the characteristic expansion of $CD38^{high}$/HLA-DR^+CD8^+ observed in HLH has been shown to be able to distinguish HLH from sepsis where this population is barely represented [8]. $CD38^{high}$/HLA-DR^+CD8^+ T cells also showed increased frequencies both in MAS and secondary HLH when compared with systemic juvenile idiopathic arthritis (sJIA) [7].

Being easily accessible, the test is quickly gathering momentum, especially in pediatric settings where several febrile conditions beyond sepsis and sJIA may pose a diagnostic challenge when it comes to differentiating them from HLH/MAS. However, $CD38^{high}$/HLA-DR^+CD8^+ frequencies showed high variability within HLH/MAS, virtually ranging from 0 to 100% with median values around 50% [7]. Specific cut-off values have been identified for the differentiation of HLH/MAS from sepsis and sJIA [7, 8] but further data on the ability to differentiate HLH/MAS from other febrile conditions that may mimic the initial clinical presentation of HLH are currently lacking. In addition, when assessing the specificity of this marker for the early detection of HLH and its potential inclusion in the HLH diagnostic process, it should be noted that conditions other than HLH/MAS can result in a $CD38^{high}$/HLA-DR^+CD8^+ expansion [9–11]. This is particularly relevant when it comes to Epstein–Barr virus (EBV) infection, which is also the most common trigger of HLH and may benefit from an early differential between complicated EBV primoinfection and EBV-triggered HLH [12].

We thus aimed to retrospectively evaluate the performance of $CD38^{high}$/HLA-DR^+CD8^+ T cells in the early detection of HLH in febrile pediatric patients attended for suspicion of HLH/MAS presenting the first experience of test application in a real-life scenario of clinical practice.

Results and discussion

Between October 1, 2021, and October 1, 2023, a total of 43 patients (16 females; median age: 46.2 months, IQR: 19.6–120.1) attended Meyer Children's Hospital IRCCS for a suspicion of HLH/MAS had $CD38^{high}$/HLA-DR^+CD8^+ frequencies measured in peripheral blood. $CD38^{high}$/HLA-DR^+CD8^+ values, relevant clinical features, infectious agents, and discharge diagnosis are summarized in Tables 1 and 2.

$CD38^{high}$/HLA-DR^+CD8^+ frequencies in HLH- vs. non-HLH patients

The $CD38^{high}$/HLA-DR^+CD8^+ frequencies showed slightly higher median values in the HLH group compared with the non-HLH group with widely overlapping distribution ranges and high scattering. HLH group (15 patients; 5 females; median age: 18.9 months, IQR: 11.0–89.7) exhibited a median $CD38^{high}$/HLA-DR^+CD8^+ frequency of 22.0% (IQR: 11.0–49.0%; min–max: 0.4–89.0%), whereas non-HLH group (28 patients; females: 11; median age: 50.9 months, IQR: 29.3–130.2) showed a median $CD38^{high}$/HLA-DR^+CD8^+ frequency of 13.0% (IQR: 3.9–28.7%; min–max: 1.0–84.0%) with similar distribution ranges ($p = 0.24$; Fig. 1A). Given the debated classification of HLH secondary to visceral leishmaniasis (VL), a similar comparison was carried out excluding all those cases presenting with VL. Similar distribution ranges were confirmed between the two groups (HLH-non-VL median: 14%, IQR: 3.1–75.6 vs. non-HLH-non-VL median: 7.6%, IQR: 3.7–32.9%; $p = 0.690$). Among the HLH-group patients, 12/15 (80.0%) had a $CD38^{high}$/HLA-DR^+CD8^+ frequency value exceeding 10.15%, while among non-HLH-group patients, the positivity ratio was 15/28 (53.6%; $p = 0.1095$).

MAS patients displayed higher median values when compared with sJIA patients (Fig. 1C).

$CD38^{high}$/HLA-DR^+CD8^+ frequencies in infection- vs. noninfection-group patients

Infection group (22 patients; 10 females; median age: 21.1 months, IQR: 10.9–45.6; 10/22 with HLH) displayed a median $CD38^{high}$/HLA-DR^+CD8^+ frequency of 27.0% (IQR:15.2–72.1; min–max: 0.8–89.0%), while noninfection group (21 patients; 6 females; median age: 101.9 months, IQR: 46.4–148.8; 5/21 with HLH) had a median $CD38^{high}$/HLA-DR^+CD8^+ frequency of 7.6% (IQR: 3.7–24.3; min–max: 0.4–47.2; $p = 0.0035$; Fig. 1B). In order to remove a bias due to a slight over-representation of HLH patients recorded in the infection group (10/22; 45.5%) when compared with the noninfection group (5/21; 23.8%), a similar comparison of $CD38^{high}$/HLA-DR^+CD8^+ frequencies between infection- and non-infection-group patients was conducted excluding all HLH patient and the statistically significant difference was confirmed (infection-non-HLH median: 26.5%, IQR 9.0–67.7 vs. noninfection-non-HLH median: 5.4%, IQR, 3.6–25.0%; $p = 0.0321$). Again, a similar comparison between the infection and noninfection group was carried out excluding all VL and the statistically higher values of the infection group were confirmed (infection-non-VL median: 58.5%, IQR, 3.3–83.7 vs. noninfection-non-VL median: 7.6%, IQR 3.6–24.3%; p = 0.047).

The 81.8% (18/22) of infection-group patients presented a $CD38^{high}$/HLA-DR^+CD8^+ frequency higher than 10.15%, whereas among non-infection-group patients the percentage was 42.9% (9/21; $p = 0.0122$).

Among infection-group patients, 10 were diagnosed with VL, 7 with EBV-primoinfection, 1 with influenza, and 1 with HIV.

Table 1. Clinical and laboratory features of HLH patients.

HLH patients (15)	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15
INF/nINF	INF	INF	INF	INF	INF	INF	INF	INF	INF	INF	nINF	nINF	nINF	nINF	nINF
Sex assigned at birth	F	M	M	M	F	M	M	F	M	F	M	M	M	M	F
Age (months)	21	8	0	18	17	9	12	180	209	6	88	80	17	101	91
Diagnosis	Leishmania HLH	Leishmania HLH	Leishmania HLH	Leishmania HLH	Leishmania HLH	Leishmania HLH	Leishmania HLH	EBV HLH	EBV HLH	Influenza HLH	Primary HLH (XLP1)	Malignancy HLH	Malignancy HLH	Systemic JIA/MAS	Systemic JIA/MAS
Number of HLH/MAS Criteria	5	5	5	5	5	6	6	5	5	5	5	5	5	5	4
$CD38^{high}/HLA\text{-}DR^{+}CD8^{+}$ (% of $CD3^{+}CD8^{+}$)[a]	25.0	15.6	32.0	77.7	19.0	49.0	22.0	89.0	85.0	0.8	10.0	11.0	0.4	47.2	17.0
Clinical features at onset															
Fever	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Splenomegaly	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
Skin rash	–	–	–	+	–	+	–	–	–	–	–	–	–	+	+
CNS involvement	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Organ failure	–	–	–	–	–	–	–	–	–	Liver	Kidney	–	–	–	–
Laboratory features at onset[a]															
Bicytopenia	+	+	+	+	+	+	+	+	–	–	+	+	+	–	–
Neutrophils (cell/mmc)	451	742	357	429	642	470	243	453	1275	1764	883	106	8898	2296	1826
Platelets ($^{*}10^{3}$/mmc)	62	169	174	116	71	40	61	66	129	167	36	14	84	267	372
Hemoglobin (g/dL)	5.7	7.3	6.7	7.9	6.7	7.3	7.3	10.7	10.8	7.5	6.4	7.9	7.3	9	9.2
Ferritin (ng/mL)	5495	2942	25372	6945	1908	1195	3273	15120	3807	73191	20831	5930	1676	7454	1169
Tryglicerides (mg/dL)	308	404	348	416	714	242	473	261	413	549	936	103	266	429	259
Fibrinogen (mg/dL)	196	172	92	152	240	119	93	43	195	100	105	148	227	175	367
GOT (UI/L)	2128	188	824	378	53	106	252	846	130	4867	239	42	48	93	64
LDH (UI/L)	2092	1190	1450	1444	665	665	820	1561	1151	5319	1925	190	2966	734	369
Bone marrow hemophag.	n.a.	n.a.	–	–	n.a.	+	+	–	+	+	–	–	–	+	–

Abbreviations: F, female; GOT, glutamic oxalacetic transaminase; Hemophag, hemophagocytosis; HLH, hemophagocytic lymphohistiocytosis; INF, infection group; Leishmania, visceral leishmaniasis; LDH, lactate dehydrogenase; M, male; MAS, macrophage activation syndrome; nINF, non-infection group; p, patient.
[a]The values reported represent the greatest alteration observed during the hospitalization for HLH.

Table 2. Clinical and laboratory features of nHLH patients.

Patients (28)	Sex assigned at birth	Age (months)	Discharge diagnosis	$CD38^{high}/HLA\text{-}DR^{+}CD8^{+}$ (% of $CD3^{+}CD8^{+}$)[a]	INF/nINF
P16	F	83	Primary EBV infection	57.0	INF
P17	M	20	Primary EBV infection	70.2	INF
P18	F	28	Primary EBV infection	84.0	INF
P19	F	100	Primary EBV infection	82.7	INF
P20	M	31	Primary EBV infection	60.0	INF
P21	M	13	Visceral leishmaniasis	29.0	INF
P22	F	44	Visceral leishmaniasis	23.9	INF
P23	F	10	Visceral leishmaniasis	14.0	INF
P24	F	70	HIV-AIDS	7.3	INF
P25	M	46	Sepsis (*S. pyogenes*)	18.9	INF
P26	M	5	Sepsis (*H. influenzae*)	2.0	INF
P27	M	27	Sepsis (*N. meningitidis*)	1.6	INF
P28	M	33	Kawasaki disease	1.5	nINF
P29	F	55	Kawasaki disease	5.5	nINF
P30	F	20	Kawasaki disease	12.0	nINF
P31	M	123	MIS-C	7.6	nINF
P32	M	39	MIS-C	38.0	nINF
P33	M	209	Systemic JIA	5.0	nINF
P34	M	30	Systemic JIA	3.6	nINF
P35	M	131	Systemic inflammatory syndrome	22.9	nINF
P36	M	161	Systemic inflammatory syndrome	25.7	nINF
P37	F	117	Systemic inflammatory syndrome	3.7	nINF
P38	F	149	Systemic inflammatory syndrome	1.0	nINF
P39	M	130	Systemic inflammatory syndrome	27.7	nINF
P40	F	46	B-cell acute lymphoblastic leukemia	5.3	nINF
P41	M	130	Diffuse large B-cell lymphoma	27.7	nINF
P42	M	174	Fever of unknown origin in XLP-2	3.1	nINF
P43	M	172	Fever of unknown origin in XLP-2	4.3	nINF

Abbreviations: AIDS, acquired immunodeficiency syndrome; EBV, Epstein–Barr virus; F, female; HIV, human immunodeficiency virus; INF, infection group; JIA, juvenile idiopathic arthritis; M, male; MIS-C, multisystem inflammatory syndrome in children; nHLH, non-hemophagocytic lymphohistiocytosis group; nINF, non-infection group; p, patient; XLP, X-linked lymphoproliferative syndrome.
[a] The values reported represent the greatest alteration observed during the hospitalization for HLH.

VL triggered full HLH in seven patients. $CD38^{high}/HLA\text{-}DR^{+}CD8^{+}$ frequencies were elevated in both groups with similar percentage values in VL-HLH (median: 25.0%, IQR: 20.5–40.5, min–max:15.6–77.7) and VL without HLH (median: 23.9%, IQR: 19.0–26.5, min–max: 14.0–29.0; Fig. 1C). EBV-primoinfection was responsible for the highest $CD38^{high}/HLA\text{-}DR^{+}CD8^{+}$ frequencies recorded in all study group with similar values in EBV-HLH patients (median: 87.0%, IQR: 86.0–88.0, min–max: 85.0–89.0) and non-HLH EBV-primoinfection patients (median: 70.2%, IQR: 60.0–82.7, min–max: 57.0– 84.0; Fig. 1C). In particular, one patient with steroid-refractory EBV-HLH was treated first-line with ruxolitinib and it was observed that, while $CD38^{high}/HLA\text{-}DR^{+}CD8^{+}$ frequencies were progressively increasing during partial remission and successive relapse obtained with high dose glucocorticoids, a progressive decrease was observed immediately after the introduction of JAK-inhibitor therapy that successfully led to HLH remission (Fig. 1D) [13]. The patient with influenza-driven HLH and predominant hepatic involvement showed normal frequencies of $CD38^{high}/HLA\text{-}DR^{+}CD8^{+}$ cells (Fig. 2). The febrile patient with HIV infection and profound AIDS-related lymphopenia showed a slightly increased proportion of $CD38^{high}/HLA\text{-}DR^{+}CD8^{+}$ cells (7.3%). Two patients with sepsis were confirmed to have normal percentages of $CD38^{high}/HLA\text{-}DR^{+}CD8^{+}$ cells while one showed increased proportions (18.9%).

Utility and constraints of $CD38^{high}/HLA\text{-}DR^{+}CD8^{+}$ as an HLH/MAS marker

The lower values observed in sepsis and sJIA compared with those of HLH/MAS further corroborate the utility of this marker in discriminating between these conditions. On the one hand, $CD38^{high}/HLA\text{-}DR^{+}CD8^{+}$ depletion was expected in sepsis, which is characterized by a known suppression of the adaptive immune system affecting the proliferative and effector functions of cytotoxic T cells in the context of a global lymphopenia [14]. On the other hand, highly heterogeneous data obtained from patients

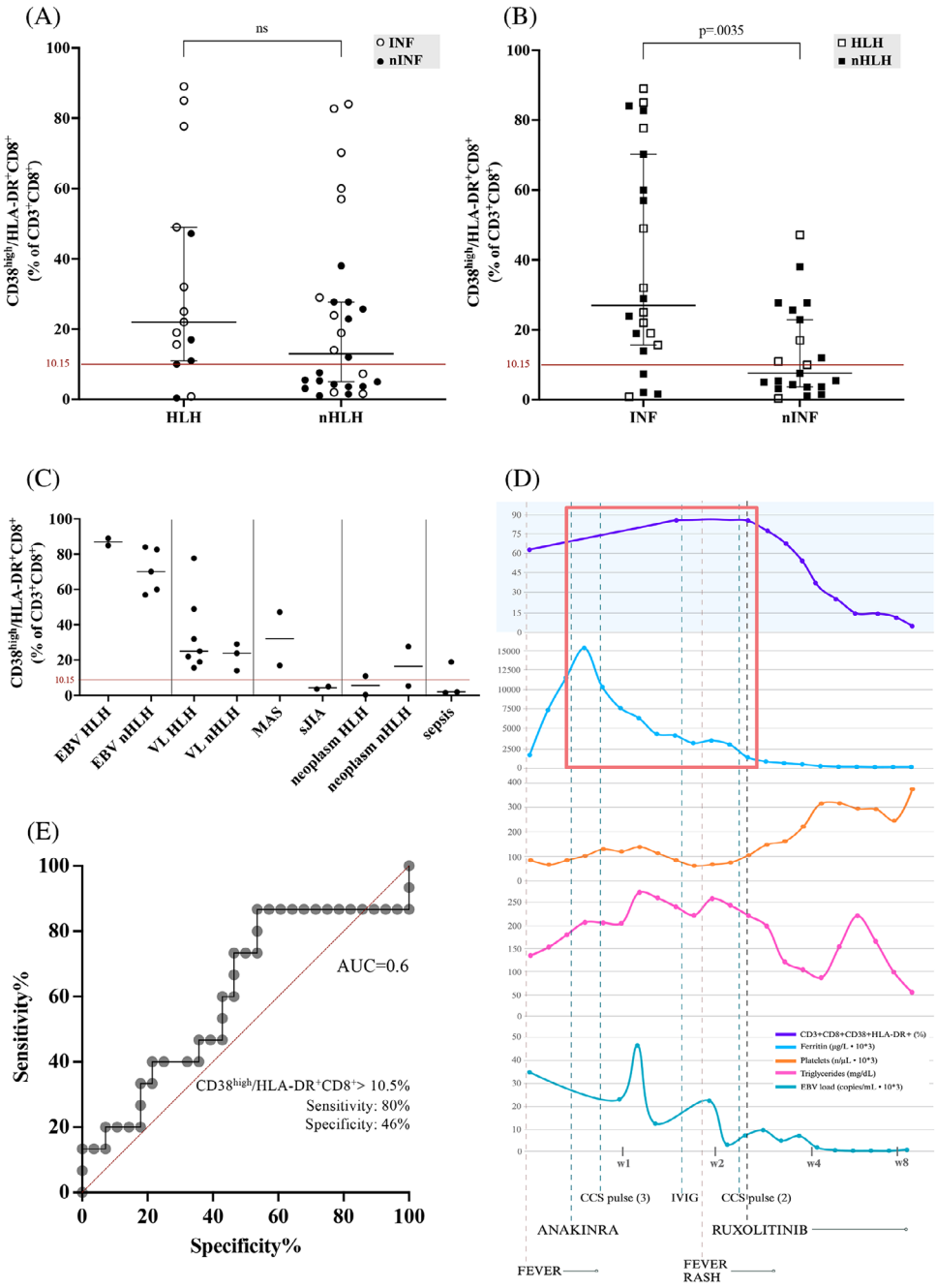

Figure 1. CD38^{high}/HLA-DR^{+}CD8^{+} frequencies display pathogen-specific expansion. (A) Frequencies of CD38^{high}/HLA-DR^{+}CD8^{+} (expressed as percentages of the CD3^{+}CD8^{+} cells) in pediatric patients with HLH/MAS and conditions other than HLH (empty dots represent patients with an identified infection) and (B) in the same patients' sample regrouped based on the presence or absence of a specific infection (empty squares represent patients with HLH). Error bars represent the median with 95% confidence intervals. Differences between groups were assessed using the Mann–Whitney test. (C) Frequencies of CD38^{high}/HLA-DR^{+}CD8^{+} in pediatric patients with EBV-HLH and EBV-primoinfection, VL-HLH and VL, sJIA and MAS, malignancy-HLH and malignancy, and sepsis. (D) Temporal trends of CD38^{high}/HLA-DR^{+}CD8^{+}, HLH biomarkers, and plasma EBV load in patient 8 during EBV-HLH. The red box highlights the divergence in serum ferritin and CD38^{high}/HLA-DR^{+}CD8^{+} frequency trends during partial remission and successive relapse obtained with high-dose glucocorticoids. (E) ROC analysis of CD38^{high}/HLA-DR^{+}CD8^{+}, comparing pediatric patients with HLH-related (15 patients) and non-HLH-related (28 patients) persistent fever. AUC, area under the curve; CCS, corticosteroids; EBV, Epstein–Barr virus; HLH, hemophagocytic lymphohistiocytosis group; INF, infection group; IVIG, intravenous immunoglobulins; MAS, macrophage activation syndrome; nHLH, non-hemophagocytic lymphohistiocytosis group; nINF, noninfection group; ROC, receiver operating characteristic; sJIA, systemic juvenile idiopathic arthritis; VL, visceral leishmaniasis.

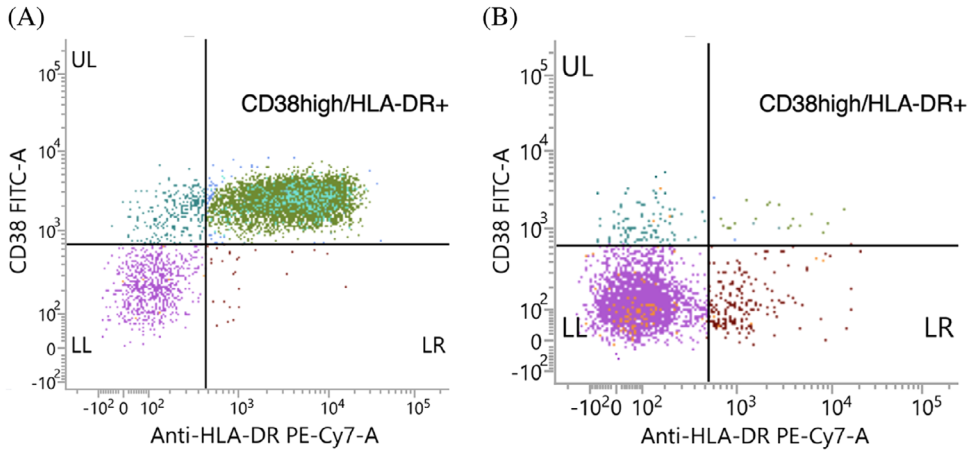

Figure 2. CD38^{high}/HLA-DR^{+}CD8^{+} expand in specific infections regardless of HLH. CD38^{high}/HLA-DR^{+}CD8^{+} cells, gated on CD3^{+}CD8^{+} T cells. Fig. 2A depicts a patient with EBV-HLH with elevated CD38^{high}/HLA-DR^{+}CD8^{+}. Fig. 2B depicts a patient with influenza-HLH with normal CD38^{high}/HLA-DR^{+}CD8^{+}. EBV, Epstein–Barr virus; HLH, hemophagocytic lymphohistiocytosis.

with EBV, VL, influenza, and *HIV* suggest that the CD38high/HLA-DR^{+}CD8^{+} increase may be pathogen-specific and not exclusively related to infection or HLH/MAS. A cut-off of 10.50% (similar to previously established cut-offs [7]) exhibited a sensitivity of 80% and a specificity of 46%, with an area under the receiver operating characteristic (ROC) curve of 0.6 (95% CI, 0.43–0.79; $p = 0.24$). These findings proved a substantial limitation of this marker in the identification of HLH/MAS in pediatric patients with an HLH-like initial clinical presentation (Fig. 1E). This is dependent on the expansion of CD38^{high}/HLA-DR^{+}CD8^{+} observed in EBV-primoinfection and VL irrespective of the presence of HLH and the lack of expansion observed in some HLH (e.g. influenza-induced HLH).

Data limitations and perspectives

We acknowledge a significant limitation in this study due to the absence of sIL-2R data which, however, reflects the limited availability of this test in the majority of clinical settings, casting this experience in the real-life scenario of clinical practice. Despite fulfilling HLH criteria and responding to HLH-related treatment, we should take into consideration that those patients without CD38^{high}/HLA-DR^{+}CD8^{+} expansion likely did not have elevated sIL-2R and may have been improperly labeled as having HLH. However, the CD38^{high}/HLA-DR^{+}CD8^{+} expansion in non-HLH conditions remains patent and in agreement with recently published data [9].

Relying on nonspecific T-activation markers alone, like CD38^{high}/HLA-DR^{+}CD8^{+}, in the absence of appropriate clinical and laboratory context, may lead to mislabeling such conditions as HLH, leading to misdiagnoses.

The HLH-2004 criteria, designed for familial HLH, may overestimate HLH incidence when the underlying primary disease involves substantial splenomegaly, fever, and cytopenia, as observed in VL, a notorious mimic of HLH. However, our results were confirmed even when excluding all VL patients and it should be highlighted how the confirmed expansion of CD38^{high}/HLA-DR^{+}CD8^{+} in VL [15] may enhance misdiagnosis, especially in nonendemic regions resulting in inappropriate treatments and, consequently, higher mortality rates.

Moreover, it should be noted that organ-limited HLH (e.g. isolated-CNS, kidney, or liver) may present with T activation that is restricted to the affected tissue and not show sIL-2R or CD38^{high}/HLA-DR^{+}CD8^{+} modifications in the peripheral blood [16, 17]. This could have been the case of patients 10 and 11 whose HLH manifestations were predominant in the liver and the kidney respectively.

Conclusion

In conclusion, we showed the results of a first experience of CD38^{high}/HLA-DR^{+}CD8^{+} application in a real-life clinical-practice scenario confirming that CD38^{high}/HLA-DR^{+}CD8^{+} frequencies, while valuable to differentiate HLH/MAS from sepsis and sJIA, do not emerge as an HLH-specific marker, as they seem to naturally expand in other clinical situations that are common in childhood and may mimic the HLH initial presentation. It is generally accepted that in a complex disease like HLH, no single parameter is going to be highly sensitive and specific. So more than the quest to identify a single absolute cut-off for CD38^{high}/HLA-DR^{+}CD8^{+} as an HLH-specific marker, it might be useful to interpret their results in a broader context together with clinical course and other supportive laboratory tests. This study also identifies a potential role of CD38^{high}/HLA-DR^{+}CD8^{+} reduction as an early marker of therapeutic response in those HLH cases where found expanded. We acknowledge that the conclusions of this study are preliminary due to the limited sample size, which is inherent to the rarity of the disease in daily clinical practice. This issue becomes more pronounced when attempting to form disease-specific subgroups. Thus, larger patient cohorts are required to define the specific role

of this lymphocyte population in the HLH diagnostic and therapeutic process.

Materials and methods

$CD38^{high}$/HLA-$DR^{+}CD8^{+}$ frequencies measured in pediatric patients attended at Meyer Children's Hospital IRCCS from October 1, 2021, to October 1, 2023, for suspicion of HLH/MAS (i.e. persistent fever of unknown origin and 2 or more other diagnostic criteria including splenomegaly, bilinear cytopenia, hyperferritinemia, hypertriglyceridemia, hypofibrinogenemia, hemophagocytosis) [4, 5] were retrospectively recorded. Flow cytometric analyses were performed on peripheral fresh blood samples collected during the hyper-acute clinical phase. HLH patients were sampled when meeting HLH or MAS diagnostic criteria [4, 5] and prior to any immunosuppressive treatment. Blood samples were stained for surface markers with anti-CD3 PE (clone SK7), anti-CD8 PerCP-Cy5.5 (clone SK1), anti-HLA-DR PE-Cy7 (clone L243), and anti-CD38 FITC (clone HB-7; BD Bioscience), and incubated for 15 min at room temperature. After red blood cell lysis with BD Pharm Lyse Lysing Buffer samples were acquired on BD FacsLyric (BD Biosciences) and analyzed with BD FacsSuite software v1.5. Results are expressed as the percentage of $CD38^{high}$/HLA-DR^{+} cells of the total $CD3^{+}CD8^{+}$ cells. CD38 expression was considered high, in agreement with previous literature, in the case of high cytofluorometric fluorescence (Fig. 2). In the case of multiple measurements taken over different time points and across several days during hospitalization and follow-up, only the highest frequency recorded was included. An extensive infectious work-up was carried out. The frequency of $CD38^{high}$/HLA-$DR^{+}CD8^{+}$ cells was compared among patients diagnosed with HLH/MAS and treated accordingly versus those diagnosed with febrile conditions mimicking HLH/MAS initial presentation, not fulfilling the specific criteria (non-HLH), and who then recovered without an HLH-specific treatment. An additional comparison of $CD38^{high}$/HLA-$DR^{+}CD8^{+}$ cell frequencies was performed between those diagnosed with an infection *vs* those without identified infections (non-infection). Based on previous literature data [7], $CD38^{high}$/HLA-$DR^{+}CD8^{+}$ cell frequency was considered to expand above 10.15% and the test was considered positive. Positivity rates were compared in the patient groups identified (HLH vs. non-HLH and infection vs. noninfection).

Data were expressed as median and interquartile ranges. Differences between groups were assessed using Fisher's exact test for categorical variables and Mann–Whitney test or unpaired *t*-test with Welch's correction for continuous variables respectively nonparametric or parametric. A ROC curve analysis was used to evaluate previously defined cut-offs and eventually identify an optimal value to distinguish HLH/MAS from other pediatric febrile conditions virtually mimicking the initial clinical presentation of HLH. For original data, please contact the corresponding author.

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Ethics Statement: The study was conducted according to the Declaration of Helsinki. Specific approval by the local Institutional Review Board was not required because all analyses included in this study were performed as part of routine clinical activity according to Good Clinical Practice.

Patient Consent Statement: Prior to data collection, written informed consent was acquired from the parents or legal guardians of the patients for data sharing. Patients' data were retrospectively retrieved from their medical records.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Abbreviations: **EBV**: Epstein–Barr virus · **HLH**: hemophagocytic lymphohistiocytosis · **MAS**: macrophage activation syndrome · **ROC**: receiver operating characteristic · **sIL-2R**: soluble interleukin-2 receptor · **sJIA**: systemic juvenile idiopathic arthritis · **VL**: visceral leishmaniasis

Full correspondence: Dr. Lorenzo Lodi, Viale Pieraccini 24 Firenze, 50139 Italy.
e-mail: lorenzo.lodi@unifi.it

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