Epstein-Barr virus infection and prognosis in haemophagocytic lymphohistiocytosis patients with underlying rheumatic diseases: a single-centre retrospective study

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Abstract

Objective

Haemophagocytic lymphohistiocytosis (HLH) with underlying rheumatic diseases (rHLH) is a lethal disease, in which Epstein-Barr virus (EBV) infection is a causative factor. Whether EBV infection is associated with treatment response and prognosis of patients with rHLH remains unclear. This study explored the clinical features of patients with Epstein-Barr virus-positive rHLH.

Methods

In this retrospective study, we included 137-patients and divided them into EBV-negative (n=116) and EBV-positive (n=21) groups. We compared the clinical characteristics, treatment responses, and prognoses between the two groups. Propensity score matching (PSM) was used to match patients between groups. Kaplan-Meier analysis was used to elucidate the relationship between the EBV-infected cell type and prognosis.

Results

EBV-positive patients were more likely to have relapsed or refractory rHLH. The survival time of the EBV-negative group was significantly longer than that of the EBV-positive group (p=0.012). Further analysis of EBV-infected lymphocyte subsets revealed a significant decrease in survival in the NK and/or T lymphocyte groups compared to the other cell types (p<0.01).

Conclusion

Patients with EBV-positive rHLH are more likely to experience relapse or refractoriness. For patients with rHLH, prompt testing of EBV-infected lymphocyte subsets should be performed upon EBV infection. An etoposide-based regimen is recommended for patients with EBV-positive rHLH, and rituximab may be effective in patients with refractory or relapsed rHLH with EBV-infected B lymphocytes. However, for patients with EBV-infected NK and/or T lymphocytes, treatment should be aligned with that for EBV-HLH.

Key words

macrophage activation syndrome, Epstein-Barr virus infection, haemophagocytic lymphohistiocytosis, rheumatic diseases

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Introduction

Haemophagocytic lymphohistiocytosis (HLH) is a fatal disease that primarily manifests with fever, pancytopenia, hepatosplenomegaly, hyperferritinaemia, coagulopathy (1). Based on the cause, HLH can be divided into primary and secondary HLH. Rheumatic disease-associated HLH is a type of secondary HLH also known as macrophage activation syndrome (MAS) (1, 2). The primary trigger factor of MAS is Epstein-Barr virus (EBV) infection (3), and EBV infection is associated with the development and activity of several rheumatic diseases (4). EBV is a causative agent of secondary HLH, resulting in high mortality rates (5). Currently, the empiric treatment for MAS is glucocorticoids, the recombinant IL-1 receptor antagonist (IL-1RA) anakinra, and/or intravenous immunoglobulin (IVIg). The first-line treatment for EBV-associated HLH (EBV-HLH) is the HLH-94 regimen, which may require haematopoietic stem cell transplantation (HSCT) for patients with refractory or relapsed EBV-HLH (5). There is a wide disparity in treatment and prognosis between MAS and EBV-HLH (6, 7). The 2-year survival rate of patients with EBV-HLH is less than 30% (7). Currently, diseases associated with EBV infection in infected B cells, such as PTLD and EBV-HLH, with only B lymphocytes in EBV infection, have a better prognosis because of the use of rituximab (8). Once EBV infects T lymphocytes or natural killer (NK) cells, it becomes an aggressive disease with high lethality.

Whether EBV-positive HLH patients with underlying rheumatic diseases (rHLH) have more severe clinical presentation or worse prognosis is unknown. Here, we retrospectively analysed 116 EBV-positive and 21 EBV-negative patients with rHLH to better understand the clinical features and treatment options for EBV-positive rHLH.

Materials and methods

Patients

We retrospectively analysed 145 patients who visited our centre with rheumatic disease-associated HLH between November 2015 and January 2021 based on the HLH-94 criteria (5), along with rheumatic diseases confirmation by a rheumatologist. Patients with defined connective tissue disease (CTD) were classified according to the current criteria established by the American College of Rheumatology (ACR). Undifferentiated CTD (UCTD) was diagnosed based on the framework proposed by Brent *et al*. (9). Of the 145, 77 patients had a clear history of rheumatic disease prior to HLH diagnosis, of whom one was excluded because of a history of EBV infection prior to the diagnosis of HLH. Next, 68 patients with HLH onset were screened for rheumatic diseases, of whom seven were excluded because of concurrent EBV infection (Fig. 1). Ultimately, 137 patients were enrolled in this study. The study was approved by the Ethics Committee of the Beijing Friendship Hospital.

Data collection

General information was collected from all patients, including sex, age, type and duration of rheumatic disease, and body temperature at the time of diagnosis. Laboratory examination data were collected, including routine blood examination, liver function, triglyceride levels, fibrinogen levels, serum ferritin levels, NK cell activity, soluble CD25 levels, liver and spleen size, haemophagocytosis, peripheral blood mononuclear cells (PBMC), serum EBV DNA, EBV-encoded RNA (EBER) of tissues, and EBV-infected lymphocyte subsets.

Data on treatment regimens, including glucocorticoid- and etoposide-based regimens, were collected from all patients (6). The glucocorticoid-based regimen involved prednisone equivalent ≥2 mg/kg/day for 3 consecutive days, either alone or in combination with immunosuppressants (such as cyclosporine A, hydroxychloroquine, methotrexate, cyclophosphamide) or intravenous immunoglobulin (IVIG). The etoposide-based regimens included HLH-94, HLH-2004, and the doxorubicin-etoposide-methylprednisolone (DEP) regimen (10).

The efficacy of the treatment regimen was evaluated according to the criteria proposed by the United States HLH collaborative group (11). Refractory rHLH was defined as treatment with high-dose

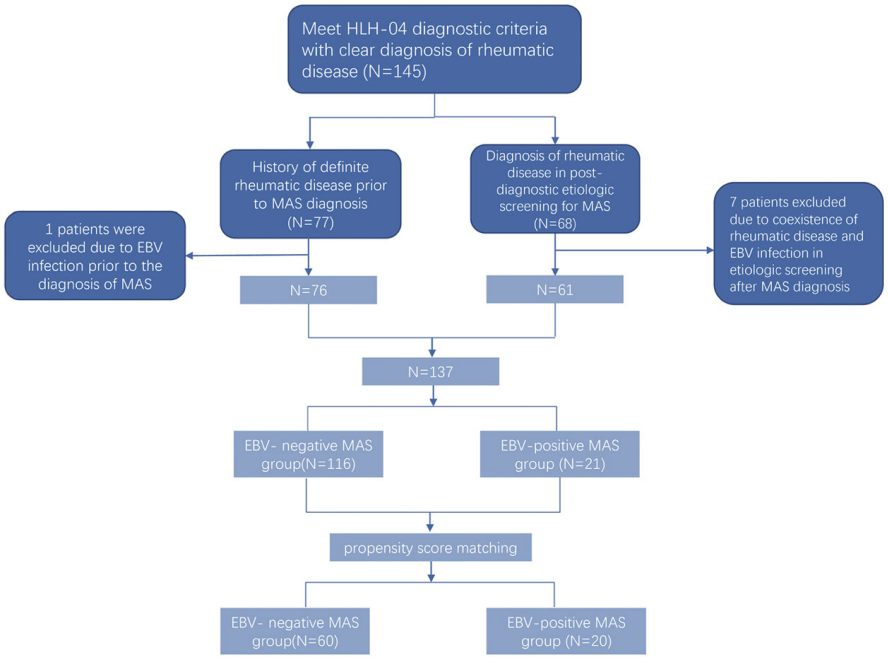

Fig. 1. Study flow chart.
EBV: Epstein-Barr virus; MAS: Macrophage activation syndrome.

glucocorticoids therapy (prednisone equivalent ≥2 mg/kg/day for 3 consecutive days without achieving at least partial response (PR). Relapsed rHLH was defined as meeting at least three HLH-2004 diagnostic criteria following the attainment of a complete response (CR) or PR with the initial treatment. If the patient has EBV-positive rHLH, the status of EBV DNA after treatment needs to be documented.

EBV positivity was defined as EBV DNA >500 copies/ml in serum or peripheral blood mononuclear cells. EBV-infected lymphocytes were analysed by magnetic bead sorting combined with RT-PCR to detect EBV DNA levels in CD4+T, CD8+T, CD19+B, and CD56+ NK cells. EBV-infected B (CD19+) cells are defined as EBV-DNA accumulating only in B (CD19+) cells or infecting B (CD19+) cells with 10 times the amount of EBV-DNA as compared to other cells. The EBV-infected NK and/or T cell type was defined based on the analysis of EBV-infected lymphocytes, suggesting that EBV infection involves Tor NK cells (CD4+ and/or CD8+ cells and CD56 + cells) or that T, B, and NK cells (CD4+ and/or CD8+ cells, CD19+ cells, and CD56 + cells) were involved but did not meet the criteria for EBV-infected B (CD19+) cells (12). NK cell activity was evaluated using flow cytometry to measure the cytotoxic activity of NK cells against the transfected fluorescent target cells.

Follow-up

By reviewing the medical records and telephone follow-ups, the survival conditions of all patients were recorded on August 24, 2022. The specific time and cause of death were recorded for the deceased patients.

Statistical analysis

All analyses were performed using Empower (R) (http://www.empowerstats.com; X&Y Solutions) and R (http://www.R-project.org). Normally distributed continuous variables are presented as means with standard deviations, and non-normally distributed variables are presented as medians with interquartile spacing. Categorical variables were summarized as absolute numbers with percentages. The Mann-Whitney U-test was performed for continuous variables, and chi-square analysis was performed for categorical variables. All factors with $p<0.10$ in the univariate analysis were retained in the multivariate model, and multifactorial logistic regression was used to analyse the relationship between EBV infection and relapsed or refractory rHLH. Cox proportional risk regression was used to analyse the relationship between EBV infection and prognosis of rHLH. Kaplan-Meier analysis with the log-rank test was used to analyse the differences in the incidence of deaths from different types of EBV infection.

To reduce the risk of selection bias inherent in retrospective observational studies, propensity score (PS) matching was used to match the patients between groups. PS was generated by a multivariate logistic regression model based on three variables: age, sex, and duration of autoimmune diseases. PS was performed in a ratio of 1:3 using the 'nearest method', with a caliper value of 0.05 without replacement. A two-sided $p<0.05$ was considered statistically significant.

Results

Baseline characteristics

Among 137 patients, 116 were diagnosed with rHLH and tested negative for EBV, whereas 21 were diagnosed with rHLH and tested positive for EBV. Of the 21 patients with EBV-positive rHLH, 21 exhibited EBV-emia and only 2 showed histologic EBER positivity at the same time. The baseline characteristics of the unadjusted and PS-adjusted groups are shown in Table I. In the unadjusted study group, patients with EBV-positive rHLH were older and had been suffering from rheumatic disease for a longer time. After PS matching, differences in baseline characteristics between the two groups were eliminated (Table I). Of the total patients, 42 (32%) were infected with CMV, whereas HHV-6, HHV-7, and parvovirus infections were observed in one patient each. Pneumonia was present in 50 patients, with 11 patients meeting the criteria for severe pneumonia. Additionally, three patients had bacteraemia, and three had skin and soft tissue infections.

Relapse or refractoriness

Before and after PS, the refractoriness or relapse of the EBV-positive rHLH group was significantly higher than that of the EBV-negative rHLH group ($p<0.01$; Table I). Univariate analysis conducted before PS revealed that age

Table I. Baseline characteristics of the unadjusted and PS adjusted populations.

	Before matching			After matching		
	EBV- negative MAS group (n=116)	EBV-positive MAS group (n=21)	*p*	EBV- negative MAS group (n=60)	EBV-positive MAS group (n=20)	*p*
Age at onset (y)	30.00 (23.00,43.25)	46.00 (30.00,54.00)	<0.01	38.00 (19.00,48.25)	45.5 (29.75,51.00)	0.13
Female gender	90 (77.59%)	17 (80.95%)	0.73	47 (78.33%)	16 (80.00%)	0.88
Type of disease			0.35			-
AOSD	58 (50.00%)	10 (47.62%)		25 (41.66%)	10 (50.00%)	
UCTD	24 (20.69%)	4 (19.05%)		13 (21.67%)	4 (20.00%)	
SLE	21 (18.11%)	3 (14.29%)		13 (21.67%)	2 (10.00%)	
Others	13 (11.20%)	4 (19.04%)		9 (14.00%)	4 (20.00%)	
Duration of rheumatic disease (m)	0.00 (0.00,7.50)	7.00 (4.00,24.00)	<0.01	0 (0, 5.50)	7.50 (4.25-33.00)	0.30
CNS involvement	10 (8.62%)	3 (15.00%)	0.37	5 (8.33%)	3 (15.79%)	0.35
Clinical manifestation						
Fever	115 (99.14%)	21 (100.00%)	0.67	59 (98.33%)	20 (100%)	0.56
Haemophagocytosis	83 (71.55%)	14 (66.57%)	0.65	47 (78.33%)	13 (65.00%)	0.23
Splenomegaly	80 (68.97%)	11 (52.38%)	0.13	40 (66.67%)	11 (55.00%)	0.35
Laboratory examination						
WBC (*10^9/L)	3.68 (2.12,8.26)	6.62 (3.23,10.84)	0.75	3.09 (2.03,7.21)	6.81 (3.74,10.90)	0.14
Neu (*10^9/L)	2.36 (0.93,5.53)	5.47 (3.51,9.57)	0.37	3.09 (2.03-7.21)	5.47 (3.51-9.57)	0.19
HGB (g/L)	92.00 (77.00,116.00)	93.00 (80.00,102.00)	0.66	91.50 (74.25-114.50)	94.50 (79.50-103.00)	0.93
PLT (*10^9/L)	93.00 (57.5,171.50)	114.00 (57.00,153.00)	0.82	78.50 (59.00-156.50)	114.00 (57.75-158.75)	0.31
ALT (u/L)	97.90 (33.00,289.50)	71.40 (23.00,193.00)	0.86	76.50 (27.25-239.00)	71.40 (34.75-217.50)	0.66
FBG (g/L)	2.12 (1.44,3.25)	2.49 (1.20,3.33)	0.43	1.99 (1.34-2.72)	2.49 (1.15-3.21)	0.32
TG (mmol/L)	2.16 (1.56,3.07)	2.81 (1.99,4.09)	0.67	2.50 (1.79-3.32)	2.78 (1.95-3.94)	0.83
SF (ng/mL)	5250.41 (1930.08,13403.38)	2352.00 (1650.0,10532.20)	0.68	6437.85 (2551.75-13854.90)	2176.00 (1612.50-5947.53)	0.52
sCD25 (pg/mL)	12324.00 (6644.00,18851.50)	7606.00 (6199.00,11291.00)	0.56	12326.50 (6581.50-19149.50)	8391.50 (5459.25-13510.25)	0.56
NK cell activity (%)	14.49 (13.10,15.84)	14.82 (13.92,17.76)	0.10	14.76 (13.11-15.95)	14.53 (13.89-17.63)	0.49
Induction therapy			0.23			
Glucocorticoid-based regimen	50 (43.10%)	12 (57.14%)		29 (48.33%)	12 (60.00%)	
Etoposide-based regimen	66 (56.90%)	9 (42.86%)		31 (51.67%)	8 (40.00%)	
Refractoriness or relapse	51 (43.97%)	18 (85.71%)	<0.01	27 (45.00%)	17 (85.00%)	<0.01

AOSD: adult-onset Still's disease; UCTD:undifferentiatied connective tissue disease; SLE: systemic lupus erythematous; CNS: central nervous system; WBC: white blood cell count; Neu: neutrophil count; HGB: haemoglobin concentration; PLT: platelet count; ALT: alanine aminotransferase; TG: triglycerides; FBG: fibrinogen; SF: serum ferritin; sCD25: soluble CD25; m: month; y: year.

at rHLH, duration of rheumatic disease >6 months, haemophagocytosis, initial treatment with etoposide, and EBV infection were associated with refractoriness or relapse of MAS patients with MAS. Furthermore, multivariate logistic regression analysis demonstrated that the probability of refractoriness or relapse in the EBV-positive rHLH group was significantly higher than that in the EBV-negative rHLH group (HR=4.24, 95% CI 1.08–16.67, p=0.04). These findings were also consistent with those of the post-PS analysis, with a corresponding HR of 5.11 (p=0.03). The detailed analysis results are presented in Table II.

Long-term outcomes

In this study, the median follow-up period was 715 days (quartile interval, 309–1330 days), and eight patients were lost to follow-up. Among the patients, 28 (19.3%) died, with 13 deaths attributed to HLH progression, five deaths due to primary rheumatic disease, nine deaths caused by severe infection, and one death resulting from gastrointestinal bleeding caused by HLH progression.

Univariate analysis conducted before PS revealed that age at onset, duration of rheumatic disease exceeding 6 months, neutrophil count less than 1.5×10^9/L, platelet count $<75\times10^9$/L, CNS involvement, EBV infection with natural killer (NK) and/or T cells, and ineffective induction therapy were correlated with poor prognosis in rHLH patients. In a multivariate COX model, age at onset (hazard ratio [HR] = 1.06, 95% confidence interval [CI] 1.03–1.09, p<0.01), EBV infection with NK and/or T cells (HR=7.02, 95% CI 1.74–28.38, p<0.01), and ineffective induction therapy (HR=2.52, 95% CI 1.04–6.07, p=0.04) emerged as independent prognostic factors (Table III). Single-factor Cox regression analysis after PS verified that age at onset, duration of rheumatic disease exceeding 6 months, and EBV infection with NK and/or T cells were significantly associated with poor prognosis. Further multivariate factor Cox regression analysis revealed that age at onset (HR=1.05, 95% CI 1.01–1.08, p<0.01), and EBV infection with NK and/or T cells (HR=7.68 95% CI 1.81–32.51, p<0.01) were independent risk factors for poor prognosis (Table III).

Kaplan-Meier analysis conducted before and after PS revealed that the overall survival (OS) of the EBV-positive

Table II. Univariate and multivariate analysis of associated factors for refractory or relapsed MAS.

Variable	Before matching				After matching			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
Age at onset, year	1.02 (1.00-1.05)	0.04	1.01 (0.98-1.03)	0.48	1.04 (1.01-1.07)	0.01	1.03 (0.98-1.06)	0.09
Female	1.44 (0.64-3.25)	0.38			1.50 (0.51-4.40)	0.46		
Duration of rheumatic disease >6 months	3.76 (1.80-7.82)	<0.01	2.88 (1.23-6.77)	0.01	3.42 (1.33-8.78)	0.01	1.68 (0.53-5.28)	0.37
CNS involvement	1.19 (0.38-3.73)	0.77			0.82 (0.19-3.54)	0.79		
Splenomegaly	0.69 (0.34-1.41)	0.31			0.51 (0.20-1.30)	0.17		
Haemophagocytosis	0.43 (0.20-0.92)	0.03	0.36 (0.14-0.83)	0.02	0.43 (0.15-1.26)	0.12		
Neu ≤1.5*10^9/L	0.67 (0.30-1.47)	0.32			0.42 (0.15-1.22)	0.11		
HGB ≤90 g/L	1.76 (0.89-3.48)	0.10			2.33 (0.94-5.75)	0.07	1.71 (0.56-5.21)	0.34
PLT ≤75*10^9/L	0.92 (0.47-1.81)	0.81			1.57 (0.64-3.84)	0.32		
SF ≥5000 pg/mL	1.02 (0.48-2.16)	0.96			0.75 (0.31-1.80)	0.59		
TG ≥3 mmol/L	0.91 (0.44-1.90)	0.81			0.93 (0.36-2.39)	0.89		
FBG<1.5g/L	1.62 (0.77-3.44)	0.21			1.30 (0.51-3.32)	0.59		
CMV infection	1.97 (0.94-4.13)	0.07	1.80 (0.76-4.24)	0.18	2.61 (0.94-7.27)	0.07	2.34 (0.71-7.71)	0.16
EBV infection	**7.65 (2.13-27.4)**	**<0.01**	**4.24 (1.08-16.67)**	**0.04**	**6.93 (1.83-26.15)**	**<0.01**	**5.11 (1.17-22.29)**	**0.03**
Initial treatment including etoposide	0.35 (0.17-0.70)	<0.01	0.32 (0.14-0.71)	<0.01	0.32 (0.13-0.81)	0.02	0.41 (0.14-1.16)	0.09

Neu: neutrophil count; HGB: haemoglobin concentration; PLT: platelet count; TG: triglycerides; SF: serum ferritin; FBG: fibrinogen; CMV: cytomegalovirus.

Table III. Cox proportional risk regression analysis of indicators associated with prognosis.

Variable	Before matching				After matching			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
Age at onset, year	1.06 (1.70-8.05)	<0.01	1.06 (1.03-1.09)	<0.01	1.06 (1.02-1.09)	<0.01	1.05 (1.01-1.08)	<0.01
Female	0.52 (0.24-1.15)	0.10			0.63(0.24-1.63)	0.34		
Duration of rheumatic disease >6 months	3.04 (1.44-6.42)	<0.01	1.24 (0.51-3.05)	0.64	2.44 (1.01-5.89)	0.05	1.18 (0.43-3.25)	0.74
CNS involvement	3.28 (1.33-8.09)	0.01	1.91 (0.63-5.79)	0.25	2.12 (0.71-6.34)	0.18		
Splenomegaly	0.97 (0.24-1.05)	0.07	0.52 (0.20-1.32)	0.17	0.38 (0.16-0.92)	0.11		
Haemophagocytosis	0.97 (0.44-2.14)	0.94			0.67 (0.27-1.69)	0.40		
Neu ≤1.5*10^9/L	2.34 (1.11-4.92)	0.03	1.54 (0.62-3.82)	0.35	1.45 (0.58-3.64)	0.43		
HGB ≤90 g/L	1.54(0.75-3.25)	0.26			1.66 (0.68-4.08)	0.27		
PLT ≤75*10^9/L	2.42 (1.13-5.17)	0.02	1.68 (0.70-4.03)	0.24	1.97 (0.80-4.81)	0.14		
SF ≥5000 pg/mL	1.95 (0.91-4.16)	0.09	1.62 (0.68-3.82)	0.27	1.17 (0.49-2.80)	0.73		
TG ≥3 mmol/L	1.14 (0.52-2.53)	0.74			1.07 (0.43-2.69)	0.89		
EBV infection type								
No	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
B	**1.99 (0.59-6.77)**	**0.27**	**1.57 (0.37-6.70)**	**0.54**	**1.72 (0.48, 6.18)**	**0.41**	**1.19(0.33,4.40)**	**0.78**
NK and/or T	**14.47(4.64-45.05)**	**<0.01**	**7.02 (1.74-28.38)**	**<0.01**	**13.93 (4.00, 48.55)**	**<0.01**	**7.68(1.81,32.51)**	**<0.01**
ND	**3.42 (1.00-11.63)**	**0.05**	**0.65 (0.13-3.17)**	**0.59**	**2.16 (0.48, 9.74)**	**0.32**	**1.58(0.35,7.21)**	**0.56**
Ineffective induction therapy	2.52 (1.20-5.29)	0.01	2.52 (1.04-6.07)	0.04	2.04(0.85, 4.91)	0.11		

Neu: neutrophil count; HGB: haemoglobin concentration; PLT: platelet count; TG: triglycerides; SF: serum ferritin; FBG: fibrinogen; CMV: cytomegalovirus.

rHLH group was worse than that of the EBV-negative rHLH group (p=0.00041 *vs*. p=0.012) (Fig. 2A-B).

EBV lymphocyte subsets in all EBV-positive rHLH were further examined. Among the 21 patients with EBV-positive rHLH, 11 were predominantly infected with B cells, four were predominantly infected with NK and/or T cells, and six were infected with unknown cell types (Online Supplementary Table S1). Upon grouping based on EBV infection type, it was observed that the overall survival, as determined by Kaplan-Meier analysis, was significantly worse for the group with NK or T cell-infected group before and after PS compared to the other three groups (p<0.01) (Fig. 2C-D). However, the EBV-infected B lymphocyte group showed no significant decrease compared with the EBV-negative MAS group.

Treatment of EBV-positive MAS patients

Among the 11 MAS patients with predominantly EBV-infected B lymphocytes, 9 initially received glucocorticoid treatment, and only 2 did not experience relapse or refractoriness. Two patients were initially treated with the etoposide-based regimes, and one relapsed. One patient treated with the Ru-DEP regimen did not experi-

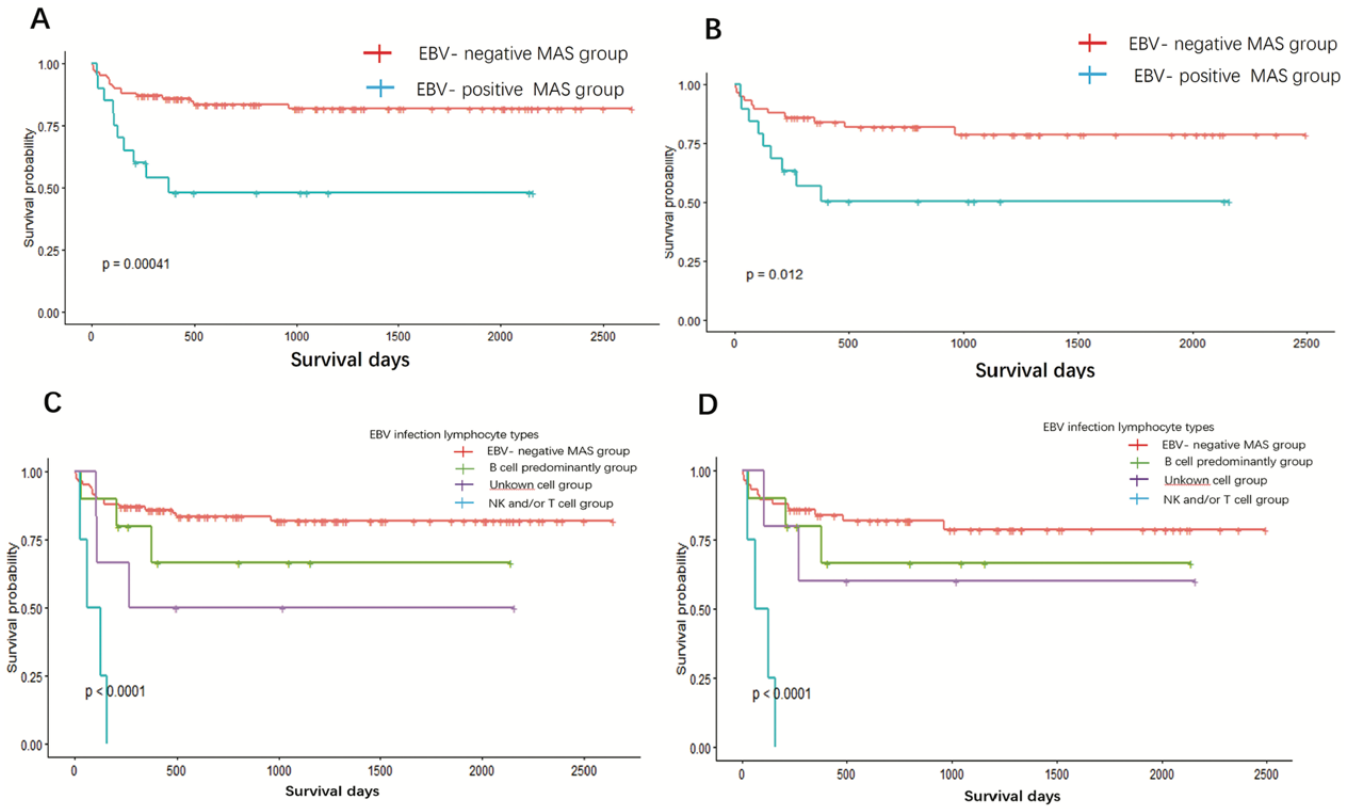

Fig. 2. Kaplan-Meier curves of MAS patients with and without EBV infection
A: before PSM. **B**: after PSM. **C**: Kaplan-Meier curves of MAS patients with different EBV-infected lymphocyte subsets - **C** before PSM, **D** after PSM. PSM: propensity score matching.

ence relapse. Four patients with EBV-infected B lymphocytes were treated with a rituximab (a CD20 monoclonal antibody that can specifically eliminate EBV in B lymphocytes)-based regimen. Two of these patients achieved long-term survival, whereas the other two died due to MAS progression (Suppl. Table S1).

Three of the four patients with MAS and EBV-infected NK and/or T cells were treated with an etoposide-based regimen, and all tested positive for EBV. One patient who underwent HSCT died because of post-transplantation relapse. Three patients who did not undergo HSCT eventually succumbed to HLH progression (Suppl. Table S1).

Characteristics of patients with EBV positive and rheumatic disease in aetiological screening

We summarised the treatment and prognosis of seven patients who were previously excluded because of both positive EBV DNA and rheumatic disease during screening for the onset of MAS. Among these patients, five were primarily infected with B cells and three showed relief after treatment with glucocorticoids, while two experienced refractory MAS that improved after receiving salvage therapy. All five patients eventually tested negative for EBV DNA and survived throughout the follow-up period. In contrast, two patients with EBV-infected NK and/or T cells showed refractory MAS with the continuous presence of EBV DNA. One of these patients was eventually diagnosed with CAEBV, whereas the other died of HLH relapse after undergoing HSCT (Suppl. Table S2).

Discussion

HLH is a lethal hyperinflammatory disease characterised by abnormal activation of histiocytes and cytotoxic T cells, resulting in a cytokine storm, haemophagocytic activity, and multiple organ damage (1). HLH can be categorised into primary and secondary HLH, with secondary HLH, also known as MAS, caused by rheumatic diseases (2). It has been established that infections trigger MAS (3). Among patients with MAS with juvenile idiopathic arthritis, approximately one-third of cases are triggered by infectious factors, with EBV being the most common pathogen (13). However, the role of EBV in the occurrence of MAS in patients is often a source of confusion. Since EBV can also cause HLH, the presence of EBV infection in patients with MAS raises questions about whether EBV plays a major role in the development of HLH or is merely an inducer of MAS. Furthermore, the prognoses of MAS and EBV-HLH differ significantly, with MAS having a relatively good prognosis for secondary HLH and a lower mortality rate than infection and tumour-related HLH (14). However, the prognosis for EBV-HLH is poor. The 1-year survival rate of patients with EBV-HLH is only 25%, and that some patients require HSCT to be cured (5).

The current first-line treatment regimen for rHLH consists of glucocorticoids (GCs), recombinant IL-1 receptor antagonists (IL-1RA), anakinra, and/or intravenous immunoglobulin (IVIg) (15). In contrast, the first-line

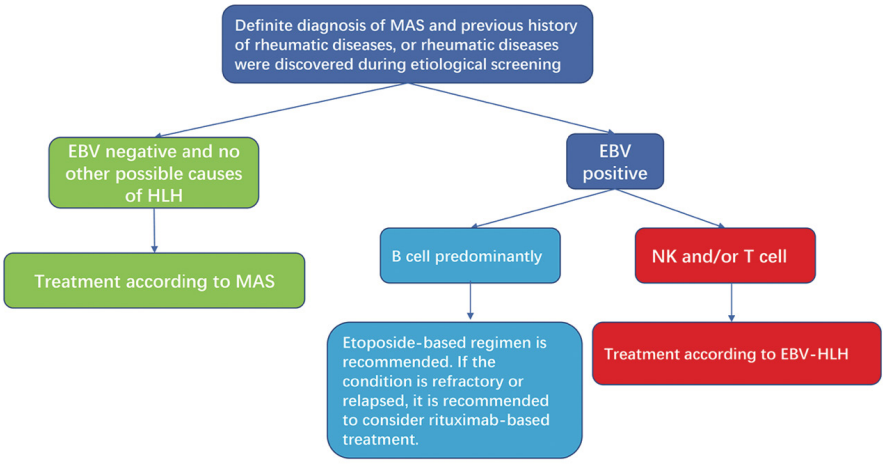

Fig. 3. Stratification and treatment of MAS patients by EBV-infected lymphocyte subsets.

treatment for other etiologies of HLH, such as primary HLH, EBVHLH, and malignancy-associated HLH, is the HLH-94 regimen, which incorporates etoposide and dexamethasone, and has significantly improved survival (16, 17). However, the early introduction of cyclosporine A into the HLH-04 protocol, which is based on the HLH-94 regimen, did not yield improved outcomes (18). These studies predominantly involved paediatric patients with primary HLH; however, the current treatment regimens for adult HLH are also supported by these findings. Considering the marked differences in treatment and prognosis, it is particularly important to diagnose the aetiology and identify the prognosis of patients with EBV-positive rHLH. Previous research has distinguished between EBV-associated HLH and non-EBV-associated HLH based on the frequency of the CD3+HLADR+ subset in PBMCs, as well as the levels of interferon-gamma (IFN-γ) and sCD25 (19). Although some studies have explored the differentiation of HLH etiology based on cytokine levels (20, 21), the metrics employed in these studies remain suggestive rather than definitive. This study is the first to investigate the prognosis of patients with EBV-positive rHLH, providing evidence for the etiological diagnosis and treatment selection of these patients.

Based on the results of our study, we determined that patients with EBV-positive rHLH were more likely to experience relapse or refractoriness. Therefore, we recommended an etoposide-based regimen for these patients. In our cohort, ~15.4% of patients with MAS were complicated with EBV infection, and only two patients showed positive tissue EBER, while the others showed EBV-emia. Therefore, analysing EBV-infected lymphocyte subsets in the peripheral blood can be a valuable method for determining the type of EBV infection and aiding in the diagnosis and treatment of these patients.

In this study, most patients with MAS who had predominantly EBV-infected B cells were treated with an etoposide-based regimen to achieve MAS remission. Three patients who experienced relapsed MAS were relieved after receiving a rituximab-based regimen, resulting in the clearance of EBV. Unfortunately, one patient died of gastrointestinal bleeding due to MAS relapse. One patient with persistently positive EBV DNA died due to MAS progression. Gomez *et al*. reported a patient with AOSD complicated with EBV triggering HLH who was relieved after receiving the HLH-2004 regimen (22). Schäfer *et al*. reported a patient with MAS in AOSD in whom EBV was the trigger factor; however, MAS could not be relieved after treatment with the HLH-94 regimen, and EBV clearance and disease remission were achieved after treatment with rituximab (23). Unfortunately, the patients did not undergo testing for EBV-infected lymphocyte subsets. This study confirmed that rituximab is an effective regimen for treating EBV-HLH with only B lymphocytes infected with EBV (12). Furthermore, there have been many successful cases of rituximab use for MAS treatment (24-26). In terms of prognosis, there was no significant difference in the rHLH of EBV-infected B cells compared to that of EBV-negative patients (Table III). This may be attributed to the fact that EBV clearance can be achieved in most patients with appropriate treatment of EBV-infected B lymphocytes. Therefore, when patients with MAS are complicated by EBV infection dominated by B lymphocytes, the primary treatment is to control MAS. Rituximab-based treatments may achieve good results in patients with relapsed or refractory disease.

EBV infection is widespread among individuals and persists in memory B cells (27). However, EBV reactivation can occur when the body's immune function is weakened, particularly cellular immunity (28). Patients with rheumatic diseases often experience EBV reactivation, which is associated with the development of various autoimmune diseases such as SLE, RA, and SS (4, 29). The reactivation of EBV leads to the expression of cleaved genes and replication of the viral genome, resulting in the production and release of new infectious viruses. These viruses can infect epithelial cells, B cells, T cells, and natural killer cells. The mechanism by which EBV spreads from B cells to NK and/or T lymphocytes remains unclear. Nevertheless, once EBV infection reaches NK and/or T lymphocytes, it may give rise to EBV-HLH, chronic active EBV infection, and NK/T-cell lymphoma, which exhibit aggressive characteristics (28). Our findings indicate that NK-and/or T-lymphocyte-predominant EBV infection is a risk factor for mortality in patients with rHLH. In this study, four patients succumbed to their condition despite receiving ineffective treatment with the HLH-94 regimen, ruxolitinib, and/or PEG-asparaginase in combination with the DEP regimen. These patients continued to test positive for EBV DNA, even after treatment. Therefore, once EBV-infected T and/or NK cells are detected in MAS patients, the management principle should still follow EBVHLH, regardless of the

long-term history of rheumatic disease. Research on patients with CAEBV has revealed that EBV infection of the full spectrum of the haematopoietic system, including both lymphoid and myeloid lineages, and HSCT, is the only way to achieve EBV clearance (30). It has been hypothesised that patients with rHLH and EBV-infected NK and/or T cells may also have coexisting CAEBV. Unfortunately, these patients have not been tested for EBV infection, specifically in myeloid cells. Nevertheless, for patients with EBV-infected NK and/or T cell-refractory or relapsed rHLH, HSCT is recommended as soon as possible after controlling the storm of inflammatory factors by chemotherapy and achieving long-term clearance of EBV. Based on our findings, we recommend stratification of MAS patients by EBV-infected lymphocyte subsets, and the specific recommendations are shown in Figure 3.

This study is limited in terms of the small number of cases of EBV-positive MAS, which requires caution when drawing definitive conclusions. Another limitation is that some patients may not have undergone EBV DNA testing at the time of rheumatic disease diagnosis. This could lead to the misdiagnosis of some patients with CAEBV as having rheumatic disease, thereby potentially exaggerating the impact of EBV on MAS in this study.

Conclusion

EBV infection is linked to refractoriness or relapse and poor prognosis in patients with rHLH, particularly when EBV infects NK and/or T cells. When a patient is diagnosed with EBV-positive rHLH, prompt testing for EBV-infected lymphocyte subsets is crucial. If EBV mainly infects B-lymphocytes, it can be managed using routine MAS treatment. Etoposide-based regimens are recommended and rituximab may be effective in patients with refractory or relapsed disease. However, if EBV infects NK and/or T lymphocytes, the treatment approach should align with that for EBV-HLH. This conclusion also holds true for patients with HLH onset, EBV infection, and rheumatic disease identified during aetiologic screening.

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Identification and validation of fibroblast-related biomarkers in rheumatoid arthritis by bulk RNA-seq and single-cell RNA-seq analysis

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Abstract

Objective

Rheumatoid arthritis (RA) is an autoimmune disorder characterised by chronic inflammation of the synovium, resulting in joint destruction, disability, and a shortened lifespan. Fibroblasts play a crucial role in the progression of RA, therefore, the identification of fibroblast-related biomarkers may provide novel insights for therapeutic intervention.

Methods

We employed single cell analysis to identify distinct cellular subtypes. Following the identification of fibroblast cells, we conducted high-dimensional weighted gene co-expression network analysis to isolate modules closely associated with these fibroblasts. We then extracted differentially expressed genes between RA and normal samples from the training set, which comprised GSE55235 and GSE55457. Protein-protein interaction network was used to prioritise the top 40 fibroblast-related differential expression genes. Then three machine learning methods – least absolute shrinkage and selection operator, support vector machine recursive feature elimination, and random forest – were utilised to identify fibroblast-related biomarkers that are highly correlated with RA. After validating these findings using an external dataset (GSE77298), we developed a diagnostic model based on the identified biomarkers. Finally, we performed western blot analyses to confirm the expression levels of these biomarkers.

Results

Two fibroblast-related biomarkers, AIM2 and PSMB9, were successfully identified and validated, demonstrating a strong association with RA. The nomogram developed from these biomarkers exhibited excellent performance in diagnosing and predicting patient outcomes.

Conclusion

This study not only identified and rigorously validated two fibroblast-related biomarkers for RA, but also provided valuable insights into the early diagnosis of the disease and the formulation of patient management strategies.

Key words

rheumatoid arthritis, fibroblast-related biomarkers, machine learning, single-cell RNA analysis