**Nivolumab treatment for relapsed/refractory** **Epstein-Barr virus-associated hemophagocytic lymphohistocytosis in adults**

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**Key Points**

1. Nivolumab, as a monotherapy, led to complete responses in 5/7 r/r EBV-HLH patients and EBV clearance in 4 of them.
2. Nivolumab restored an anti-EBV program in CD8 T cells, revealed by scRNA-seq analysis.

**Abstract**

Epstein-Barr virus-associated hemophagocytic lymphohistocytosis (EBV-HLH) is a life-threatening hyperinflammatory syndrome triggered by EBV infection. It often becomes relapsed or refractory (r/r), given that etoposide-based regimens cannot effectively clear the virus. r/r EBV-HLH is invariably lethal in adults if without allogeneic hematopoietic stem cell transplantation. Here, we retrospectively analyzed the data of seven r/r EBV-HLH patients treated with nivolumab on compassionate use in West China Hospital. Six patients responded. Five of them achieved and remained in clinical complete remission for a median follow-up of 16 months (range 11.4-18.9 months). Importantly, both plasma and cellular EBV were completely eradicated in four patients. Single cell RNA sequencing analysis showed that hyperactive monocytes/macrophages and ineffective CD8 T cells with an unbalanced activation program were associated with the syndrome. Nivolumab treatment expanded PD-1+ T cells and restored the expressions of HLH-associated degranulation and co-stimulatory genes in CD8 T cells. Our data suggest that nivolumab, as a monotherapy, provides a cure promise for r/r EBV-HLH, presumably by restoring the unbalanced anti-EBV program of the immune system.

**Introduction**

Hemophagocytic Lymphohistiocytosis (HLH) is a fatal hyperinflammatory syndrome induced by inappropriate prolonged activation of lymphocytes and macrophages1. There are two categories of HLH: familial (FHL) and secondary (sHLH). FHL, caused by genetic mutations impairing the function of cytotoxic lymphocytes and NK cells, typically presents in infancy and early childhood. sHLH, triggered by infections, neoplasms and autoimmune diseases, is more common in adolescence and adult2. Epstein-Barr virus-associated HLH (EBV-HLH) is one of the most common subtypes of sHLH and usually exhibits poor prognosis and unsatisfactory treatment outcomes3.

To date, there is no standard treatment for EBV–HLH in adults. The most commonly used HLH-94/044,5 regimens comprised of etoposide and dexamethasone, can lead to early remission6,7, but often ends with rapid relapses8. The challenging dilemma for EBV-HLH treatment is that chemotherapies aim to eliminate the syndrome-causing hyperactive immune cells, while they also impair the capacity of patients’ immune system to clear EBV, the disease initiator. Current antiviral agents demonstrate marginal anti-EBV utility, which leaves allogeneic hematopoietic stem cell transplantation (allo-HSCT) the only potentially curative method for relapsed/refractory EBV-HLH (r/r EBV-HLH) so far9. Further, matched donors are limited and allo-HSCT also causes complications that influence patients’ long-term survival and life quality. Thus, better curative therapeutic strategies are on demand for r/r EBV-HLH.

The expression levels of programmed death protein-1 (PD-1) increase on the surface of CD8 T cells with EBV or other virus infections10,11. PD-1 inhibitors, approved for multiple cancers, have been shown to be effective for chronic viral infections in animal models12 and recently for progressive multifocal leukoencephalopathy (PML) patients with JC virus13. Interestingly, these antibodies also have high response rates in EBV+ gastric cancer14 and NK/T lymphoma15. A possible explanation is that enhanced immunity after PD-1 blockade helps to restrain cancer cells and also clear EBV infection. However, it is unknown whether PD-1 antibodies would be safe and effective for r/r EBV-HLH, a hyperinflammatory syndrome triggered by EBV infection.

**Method**

**Patients**

Here, we retrospectively analyzed the clinical data of seven r/r EBV-HLH patients treated with nivolumab as compassionate use in West China Hospital (ChiCTR 1900026232). All seven patients provided written informed consent to receive the off-label treatment with nivolumab and for blood sample collection. HLH was diagnosed according to the HLH-2004 diagnosis guidelines5. EBV-HLH was diagnosed using DNA polymerase chain reaction (PCR) testing with a threshold of 103 EBV-DNA copies/mL in plasma for confirmation of EBV infection1. FDG-PET/CT and bone marrow smear were performed to exclude malignancy-associated HLH. Next generation sequencing in a panel of 26 HLH-related genes was applied to exclude FHL (supplemental Table 1). r/r EBV-HLH was defined as persistent, recurrent or worsening symptoms after at least 6-week etoposide and dexamethasone-based chemotherapies. The clinical courses from estimated onset to nivolumab administration were more than three months to exclude infectious mononucleosis. Patients aged 15 years or older with r/r EBV-HLH and without available HLA-matched donors were included. This study has been approved by the Ethnic Committee of West China Hospital of Sichuan University.

**Treatment**

Patients received nivolumab, as a monotherapy, at a dose of 100-200mg via intravenous infusion every 3-4 weeks for induction therapy. Treatment would be stopped if Grade 3 or higher immune-related adverse events (irAEs) occurred or the disease continued to progress after four cycles of nivolumab infusions. When plasma EBV-DNA became undetectable, nivolumab was administrated at 100mg every three months for maintenance therapy up to one year.

Clinical and laboratory evaluations were performed weekly during induction therapy and before each infusion, including performance status, vital signs, plasma EBV-DNA copies, size of spleen and liver, complete blood count and levels of ALT, triglyceride, fibrinogen, LDH, sCD25 and ferritin. Intracellular EBV-DNA copies were quantified by qPCR in sorted B, T and NK cells.

**Assessment of Response**

We used the criteria for response assessment as previously described3,16. Complete response (CR) was defined as the resolution of all clinical signs and symptoms (clinical CR) and normalization of all the abnormal laboratory parameters including undetectable EBV-DNA in plasma (molecular CR). Partial response (PR) was defined as a minimal 25% improvement in ≧2 quantifiable symptoms and laboratory parameters, including >25% decrease of plasma EBV-DNA copies; >1.5-fold decrease of sCD25 levels; >25% decrease of ferritin and triglyceride levels; >100% increase of neutrophil numbers (>0.5 × 109/L if initial counts <0.5 × 109/L or > 2.0 × 109/L if initial counts 0.5–2.0 × 109/L) and >50% decrease of ALT levels in patients with liver dysfunction. Failure to achieve CR or PR was defined as no response (NR).

**Single Cell RNA Sequencing Analysis**

Single cell RNA sequencing (scRNA-seq) was performed with peripheral blood cells from patient 7 before (Day 0, T0), during nivolumab treatment (Day 7, T1; Day 21, T2), and when plasma EBV became negative (Day 76, T3). scRNA-seq libraries were generated following the recommended protocol of the 3’ scRNA-seq 10X genomics platform and using v2 chemistry by the manufactory and sequencing data were collected with Illumina NovaSeq 6000. Cellranger (version 3.0.0) was used for alignment to human genome hg19. The Seurat workflow was applied to classify cell subtypes and t-SNE was used to reduce dimensions17. Single cell data of individual subgroups were integrated to mimic bulk-RNA sequencing files by calculating the mean value of each gene expression, visualized by heatmap. KEGG pathways in CD8 T cells of different timepoints, enriched by ClusterProfiler, and gene networks were visualized by the cnetplot.enrichResult formula18.

**Data Sharing Statement**

scRNA-seq data have been deposited in the GEO database under accession number: GSE138504. Code used in this study is available on Github (<https://github.com/pangxueyu233/Nivolumab-treatment-for-r-r-EBV-HLH>). Original clinical data will be available by contacting the corresponding author T.L. (liuting@scu.ed.cn).

**Result**

**Patient Characteristics**

A total of seven patients (median age, 25 years; range, 15-36 years;), including four males and three females, with r/r EBV were enrolled (Table 1). All patients presented with typical aggressive HLH signs, including consistent high fever, life-threatening cytopenia, and high level of ferritin and sCD25. Their plasma EBV-DNA loads were >104 copies/ml (range 3.99×104/ml to 2.10×107/ml). Malignancies were excluded by FDG-PET/CT and/or bone marrow smear. Genetic tests were performed and no other HLH-related genetic mutations were detected but heterozygous mutations in UNC13D gene in two patients (patient 2 & 3). Though it has been reported that heterozygous UNC13D or other HLH-related mutations could contribute to late onset HLH19-21, neither of their parents with the same mutations were symptomatic.

The median time for primary chemotherapy was 19.6 weeks (range 6.3-72 weeks), after which, two patients did not respond to the chemotherapy and five patients experienced transient ameliorations, followed with rapid relapses (supplemental Table 2). The clinical courses, from the estimated symptom onsets to the first nivolumab infusions, were more than three months for all patients.

**Efficacy**

Six out of seven patients responded to nivolumab (patient 1, 2, 3, 4, 6, & 7). Five of them (patient 1, 2, 3, 4 & 7) achieved clinical CR and another one (patient 6) had a transient response (supplemental Figure 1A). One patient (patient 5) had no response to the treatment with consistent fever and high levels of plasma EBV-DNA (supplemental Figure 1B). Four of the five clinical CR patients also achieved molecular CR. All five clinical CR patients have been remaining in remission for more than 40 weeks within a median follow-up of 16 months (range 11.4 -18.9 months). (Figure 1 & supplemental Table 3)

For five clinical CR patients, the median times and cycles from the first nivolumab infusion to clinical stabilization were 21 days (rang 2-59 days) and 1.6 cycles (range 1-3 cycles), respectively. Plasma EBV DNA copies decreased gradually. Four clinical CR patients reached molecular CR after a median of 15 weeks (range 10.7-21 weeks) and 4.5 cycles (range 3-6 cycles) of nivolumab. Patient 1, with prolonged intervals between infusions after clinical CR, failed to achieve molecular CR (Figure 2A).

Hemoglobin and platelet levels increased after treatment in all clinical CR patients. Cytopenia resolved around 6 weeks after the first nivolumab infusions (Figure 2B). Levels of HLH markers ferritin and sCD25 decreased and reached normal ranges in 5 clinical CR patients. Intracellular EBV-DNA in sorted B, T, NK cells was undetectable after molecular CR in three patients (patient 3, 4, 7), determined by qPCR (data not shown).

**Safety**

No Grade 3 or higher irAEs were recorded in all patients. During induction, persistent or slightly elevated fever was observed in the four of the five responding patients, lasting for about 1-2 weeks (Figure 2A). Transient deteriorating cytopenia and increased levels of ferritin or sCD25 were also detected in four patients. However, plasma EBV loads decreased at the same time in all of these patients, suggesting that these pseudo-progressions might be due to the active on-going anti-EBV immunity. Finally, all clinical symptoms resolved and laboratory parameters became normal without any other HLH-related medications. Patient 6 experienced EBV reactivation after two nivolumab infusions and discontinued the therapy.

During maintenance therapy, Patient 3 had transient and mild skin pruritus and lightening after 5 cycles of nivolumab. Otherwise, no treatment-related adverse events were observed.

**Single Cell RNA sequencing Analysis**

In order to explore the molecular and cellular mechanisms of nivolumab treatment for r/r EBV-HLH, scRNA-seq was performed with peripheral blood cells of patient 7 before (T0, Day 0), 1 week after treatment (T1, Day 7), after clinical CR (T2, Day 21) and when plasma EBV-DNA became negative (T3, Day 76). Cell types were identified according to their expressions of marker genes (Supplemental Figure 2).

Hyperactive monocytes/macrophages have been proposed to be major effector cells of HLH22. Our scRNA-seq analysis showed that more than 25% of monocytes/macrophages at T0 expressed abnormally high levels of active markers, such as TNF, IL1B and CD163. The expression levels of these genes and also the proportion of this population dramatically decreased at T1 and then on (Supplemental Figure 3). Consistently, there was a big population of abnormal CD8 T cells, indicated by high levels of IFNG and GZMB, at T0 and their percentages gradually reduced from T1 to T3 (Figure 3A). Interestingly, these cells also expressed high levels of PD-1 and LAG3, markers for exhausted T cells (Figure 3A-B, supplemental Figure 4). Notably, PD-1+ T cells expanded rapidly after the first nivolumab infusion, with high expressions of MKI67, implying being in active cell cycle. These cytotoxic T cells continued to expand at T2 but with reduced expressions of LAG3 and MKI67, correlated with further decrease of EBV-DNA copies. At T3, the cellular components and the gene signatures of the patient’s blood were similar to those in healthy adults23, further indicating molecular CR (supplemental Figure 2A-B and 4). Importantly, gene network analysis showed that T0 CD8 T cells highly expressed genes enriched in the EBV infection pathway, the T cell receptor signaling pathway and also the apoptosis pathway, consistent with the hyperinflammatory syndrome of the disease (Figure 3C). Surprisingly, multiple T cell activation pathways and the degranulation pathway were positively enriched in CD8 T cells after the first nivolumab treatment (T1), comparing to T0 CD8 T cells, suggesting that nivolumab restored the defective cytotoxicity of CD8 T cells (Figure 3D). Importantly, the expansion and activation of these PD-1+ CD8 T cells were correlated with dramatic reductions of intracellular EBV-DNA copy numbers in B and NK cells, suggesting their enhanced anti-EBV activity by nivolumab (Figure 3E).

Loss of function mutations of genes important for the functions of cytotoxic T and/or NK cells contribute to the HLH development24. Intriguingly, our data showed that the expressions of multiple of these genes, such as STXBP2, UNC13D, SH2D1A and CD27, despite of no mutations in patient 7, were repressed in CD8 T cells at T0 (Figure 3F). STXBP2 and UNC13D are involved in the cytotoxic granule releasing pathway and SH2D1A and CD27 are important for T cell co-stimulation. Thus, dysregulations of these genes might underlie the defective cytotoxicity of T cells to eradicate EBV. Consistently, the expressions of other critical costimulatory receptor genes and leukocyte degranulation genes, notably such as CD28, SLAMF1 and LYN, were repressed in T0 CD8 T cells. The expressions of these genes were upregulated in CD8 T cells at T1 and then synergistically downregulated at T2 and T3, when EBV was cleared and the syndrome resolved (Figure 3G).

**Discussion**

In this retrospective clinic data analysis, we found that nivolumab was highly effective in both disease control and EBV eradication for patients with r/r EBV-HLH. 71.4% (5/7) of the patients reached clinical CR with resolution of all the symptoms without relapse during the follow-up of up to one and half years. Importantly, EBV-DNA was undetectable in plasma and lymphocytes of 57.1% (4/7) of the patients. Remarkably, although HLH was generally thought to be a hyperinflammatory syndrome, nivolumab was well tolerated by r/r EBV-HLH patients and no drug related severe adverse effects were observed in all of the patients.

The current HLH-94/04 regimens use etoposide to target activated T cells and suppress inflammatory syndrome25. However, these immune cells are also the major effector cells to fight against EBV. Therefore, EBV cannot be effectively cleared by etoposide-based regimens, and quick relapses after chemotherapy are often recorded for patients with EBV-HLH8. Recent studies suggested that ruxolitinib and emapalumab would be effective for HLH control26,27. However, because EBV-HLH patients were not included in these trials, their potential effects for EBV-HLH remain to be tested. Theoretically, the main effects of these agents are to calm the cytokine storm while leave the real syndrome-initiator, EBV infection, untreated, so they may not be strong candidates for EBV-HLH as relapse seems to be inevitable. Rituximab-containing chemo-immunotherapeutic regimens have been suggested to improve clinical status in 43% EBV-HLH28. But their ability to clear EBV was limited, especially when other immune cells, such as T and NK cells, get infected. In contrast, in our study, five out of seven r/r EBV-HLH patients achieved CR and, more importantly, four of them had EBV completely eradicated in all types of lymphocytes with a monotherapy of nivolumab. These results strongly suggest that PD-1 inhibitor would provide a cure promise for r/r EBV-HLH.

It appears paradoxical to apply nivolumab, a PD-1 inhibitor, to treat EBV-HLH of hyperinflammation. Surprisingly, our scRNA-seq study showed that multiple HLH-related genes, such as CD27 and STXBP2, failed to be upregulated in hyperactive CD8 T cells in EBV-HLH, which might underlie the anti-EBV defects of immune system and suggests a novel mechanism for the onset of EBV-HLH. Nivolumab treatment expanded a subpopulation of cytotoxic T cells with high levels of PD-1 and LAG-3. High proportion of these cells became MKI67 positive after the first nivolumab infusion, indicating that these CD8 T cells were re-activated and proliferating from a previous exhausted population. Similar phenotypes were also observed in cancer patients after PD-1 or PD-L1 blockades29. Meanwhile, all of the dysregulated HLH-related costimulatory and degranulation genes were up-regulated in this population by nivolumab. The normalized cytotoxic activation program was correlated with EBV clearance. Interestingly, two patients with heterozygous mutations in UNC13D also responded to nivolumab and successfully reached molecular CR, suggesting immune cells with heterozygous hypomorphic mutations of HLH-related genes could also be functionally normalized by PD-1 inhibition. It would be interesting to test whether PD-1 inhibitors could be effective for adult onset FHL and other sHLH in future studies.

In conclusion, our study suggests that nivolumab could lead to a successful disease control of r/r EBV-HLH. In light of the serological and intracellular negative conversion of EBV-DNA, it might provide a cure promise for the disease without allo-HSCT. Larger systematically protocol-driven clinical trials and further mechanism studies are warranted.

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**Authorship**

Contributions: T.L., Y.L., and C.C. designed and supervised this study. T.N., X.S., J.L., Y.G., L.X., and Y.W. recruited and treated the patients. X.P., P.L., W.J., and X.C. performed scRNA-seq analysis. P.L., X.P., C.C., Y.L. and T.L. analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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**Table 1. Clinical and laboratory manifestation on presentation and after relapse/refractory disease**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Case** | **Sex** | **Age (yrs.)** |  | **Initial Presentation** | | | | | | | **At Relapse/Refractory Disease** | | | | | |
| **Onset Symptoms** | | **WBC**  **(109/L)** | **Ferritin**  **(ng/ml)** | **sCD25**  **(U/ml)** | **PlasmaEBV -DNA (/ml)** | **Genetic**  **Defect** | **Symptoms** | | **WBC**  **(109/L)** | **Ferritin**  **(ng/ml)** | **sCD25**  **(U/ml)** | **PlasmaEBV -DNA (/ml)** |
| **1** | M | 29 | Fever, Cytopenia Splenomegaly | | 0.94 | >2000 | >7500 | 3.40E+05 | ND | Fever, Cytopenia  Splenomegaly | | 0.27 | 1430 | >7500 | 1.20E+04 |
| **2** | M | 23 | Fever, Cytopenia, Splenomegaly, Liver Dysfunction | | 0.57 | >2000 | 4344 | 2.37E+05 | UNC13D  Heterozygous c754-8C>T | Fever, Cytopenia Splenomegaly, Liver Dysfunction | | 0.89 | >2000 | 6406 | 6.19E+04 |
| **3** | F | 20 | Fever,  Cytopenia | | 1.26 | >2000 | 2030 | 7.36E+05 | UNC13D  Heterozygous  c1232G>A | Fever,  Cytopenia | | 0.88 | 1190 | 1214 | 2.65E+02 |
| **4** | F | 15 | Fever, Cytopenia, Respiratory Failure | | 1.07 | >2000 | >7500 | 2.10E+07 | ND | Fever, Cytopenia | | 1.32 | >2000 | 751 | 5.51E+02 |
| **5** | M | 35 | Fever | | 3.82 | 1794 | 4816 | 3.99E+04 | ND | Fever | | 7.3 | 755.9 | 997 | 1.04E+04 |
| **6** | M | 36 | Fever, Cytopenia | | 0.64 | 1604 | >7500 | 1.56E+05 | ND | Fever | | 2.89 | 169.6 | 434 | 8.80E+01 |
| **7** | F | 16 | Fever, Cytopenia | | 1.35 | >2000 | >7500 | 5.16E+04 | ND | Fever, Cytopenia | | 2.85 | 303.8 | 1884 | 2.80E+02 |

**Figure Legends**

**Figure 1. Swimmer’s plot of time on treatment for seven r/r EBV-HLH patients.**

Dash lines indicating the ends of induction and starts of maintenance treatment and full lines indicating the cassations of nivolumab treatment.

Abbr: ED, Etoposide + Dexamethasone; GED, Gemcitabine + Etoposide + Dexamethasone; CsA, Cyclosporin A; L-Asp, L-Asparaginase; CR, complete response; PR, partial response.

**Figure 2. Dynamics of clinical and laboratory parameters after nivolumab infusions in patients with clinical complete response**

(A) Dynamics of body temperature (black lines) and plasma EBV-DNA copy numbers (blue lines) in clinical CR patients (n=5) during nivolumab induction therapy. (B) Levels of white blood cells, platelets and hemoglobin in peripheral blood of clinical CR patients. (C-D) Levels of sCD25 (C) and ferritin (D) in peripheral blood of clinical CR patients.

**Figure 3. Single cell RNA sequencing analysis of peripheral blood cells of patient 7 before and after nivolumab infusions.**

(A) Upper, violin plots showing the expressions of IFNG, GZMB and LAG3 in CD8 T cells at different time points. Lower, pie charts showing the ratios of IFNG, GZMB and LAG3 positive CD8 T cells at different time points. (B) Expression levels of PD-1 projected on t-SNE graphs of T0 and T1 peripheral blood cells. (C-D) Regulatory networks of signature genes in CD8 T cells at T0 (C) and T1 (D), analyzed with ClusterProfiler. Sizes of circles for enriched pathways indicate the numbers of genes enriched in these pathways and colors of dots for each gene indicate their relative expression levels. (E) Line chart showing intracellular EBV-DNA copies in NK, B and T cells. (F) Heatmap showing the expression levels of HLH-related genes in CD8 T cells. (G) Heatmaps showing the expression levels of GO leucocyte degranulation genes (left) and Costimulatory receptors genes (right) in CD8 T cells.