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Original Article

Effects of Alloferon versus Valaciclovir for Treating Chronic Epstein-Barr Virus Infection

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Abstract

Background and objectives: This study aimed to analyze the influence of alloferon on the Epstein-Barr virus (EBV) DNA copy number in saliva samples and the changes of natural killer (NK) cell content, cytotoxic activity of killer cells, and production of interferon alpha and gamma in patients suffering chronic EBV infection (CEBVI) at 6 weeks after therapy completion.

Methods: One hundred CEBVI patients (69 females and 31 males were divided into two groups: alloferon (n = 70; nine injections s/c, 1.0 mg every alternate day) and valacyclovir (n = 30; 500 mg two times/day, orally). The EBV DNA quantity in the saliva samples, the number of killer cells in the blood, and the cytotoxic activity of killer cells via spontaneous and induced expression of CD107a, a marker of degranulation, were determined after treatment with alloferon. The dynamics of interferon alpha and gamma production before and after alloferon therapy were also assessed.

Results: At 6 weeks after therapy completion, EBV DNA was not found in 38 (54.28%) patients in the alloferon group and in 9 (30.0%) patients in the valacyclovir group (p = 0.001). In addition, a reliable increase of the NK cell content and stimulation of cytotoxic activity of NK cells were detected in the CEBVI patients. Moreover, alloferon treatment did not lead to a reliable increase of interferon alpha or gamma production at 6 weeks aftertherapy completion.

Conclusions: Alloferon significantly reduces the EBV DNA copy number in saliva samples and induces the expansion of NK cells and cytotoxic activity of NK cells in CEBV patients. Alloferon also significantly affects the clinical complaints of CEBVI patients.

Introduction

In forming an antiviral immune response, an important role is given to certain cells—natural killer (NK) cells, which destroy virus-infected and tumorous target cells by their ability to directly destruct target cells and antibody-dependent cell-mediated cytotoxicity. The ability of cells to destroy other cells is carried out via perforin/granzyme-containing granules and extracellular ligands, including Fas ligands and ligands inducing apoptosis and exocytosis of cytolytic granules. During the release of cytolytic granules, containing perforin and granzymes, they hit the target-cell and launch apoptosis by means of caspase-mediated signaling pathways.[1] Healthy cells are able to inhibit activation of NK cells via the expression of molecules such as the main complex of histocompatibility class I (HLA-I), which interact with inhibitory receptors that are expressed on the surface of NK cells. NK cells express two main classes of HLA-I-specific inhibitory receptors: members of the killer-cell immunoglobulin-like receptor (KIR) superfamily and heterodimer of CD94/NKG2A.[2]

KIRs belong to the family of type-I transmembrane receptors, which are specific for the polymorphic molecules HLA-A, -B, and -C; however, NKG2A has the form of type-II transmembrane receptors of the C-type lectin-similar receptor family and

Keywords: Epstein-Barr virus; Alloferon; NK cells; Cytotoxic activity; Interferon.

Abbreviations: AMPs, antimicrobial peptides; CEBVI, chronic Epstein-Barr virus infection; CI, confidence interval; DNA, deoxyribonucleic acid; EBV, Epstein-Barr virus; HLA-I, main complex of histocompatibility class I; IFNα, interferon alpha; IFNγ, interferon gamma; KIR, killer-cell immunoglobulin-like receptor; NK, natural killer; PCR, polymerase chain reaction.

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binds HLA-E, nonclassical HLA, which is characterized by limited polymorphisms. KIR is characterized by a high level of polymorphisms, which can affect KIR/HLA interactions, meaning that certain combinations of KIR/HLA correlate with protection or susceptibility to a few human illnesses.[3] Inhibition of this receptor-mediated signal transmission is necessary protect against the high activity of NK cells. Some viruses suppress surface expression of HLA-I, thus hindering the presentation of viral antigens. Reducing the expression of HLA-I enables the identification and clearance of virus-infected target cells by NK cells.

Previously, we offered the concept of identification of target cells by NK cells via the absence of an inhibitory interaction with HLA-I, which is known as the "missing-self" hypothesis, and states that NK cells identify and destroy the cells of the "self" organism with low or compromised expression of HLA-I molecules. The receptors expressed on the target cells are able to ease activation concurrently with the identification of viral or stress-induced ligands on the target cells.[4]

Viral entry into a host cell occurs via binding with specific receptors, which either leads to fusion directly on the plasma membrane or penetration after clatrin- or caveolin-dependent endocytosis of viral particle. Finally, penetration of the virus might happen via micropinocytosis, which is nonspecific ingestion of extracellular material; but until now, it has not been identified as a strategy for penetration into NK cells. The penetration mechanism for the Epstein-Barr virus (EBV) is the expression of receptor CD21 and the coreceptor HLA-II after a cell-to-cell interaction.[5] A mechanism of penetration of a virus into NK cells is under discussion, since EBV habitually infects epithelial cells of the oropharynx and B cells via the interaction of glycoprotein gp350/220 with the receptor CD21 on the B-cell surface, and the consequent interaction of the viral complex of glycoprotein gp85-gp25-gp42 with HLA-II launches membrane fusion. NK cells can express the receptor CD21 while interacting with CD21-positive EBV-infected cells.[6] We also have demonstrated the CD21-independent mechanism of infecting NK cells by EBV.[7] Viruses are able to inhibit the cytotoxicity of NK cells or induce apoptosis, which can serve as an important strategy for evading from the immune response. Besides the influence of viruses on the production of effector cytokines, the expression of receptors on NK cells can vary, depending on the virus, leading to the change of polarization of NK cells and, finally, to the distortion of the control of other immune cells. Furthermore, the phenotype of the infected NK cells might be changed via a change of morphology or even conversion into malignant cells. The contamination of NK cells causes *de-novo* expression of receptors, which due to their nature, are not expressed in NK cells.[7] This means that every virus induces a proprietary phenotype that is often distorted toward the state of NK cells, which encourages infection persistence. The contamination of NK cells enables viral load increase.[8] Since NK cells are in the cohort of the first cells that react to viral infections, inhibition or distortion of a small subpopulation of NK cells affects the formation of the adaptive immune response to viral infections.[9]

NK cells are "naturally" cytotoxic and, unlike cytotoxic T cells, they do not require the preliminary action of an antigen. The cytolytic function of NK cells can be initiated by various processes, including degranulation and ligation of death receptors, which are critical for the rate of purification for both infected and dysfunctional cells.[10] Besides, stimulated NK cells produce interferon gamma (IFNγ) and tumor necrosis factor alpha and possess cytolytic functions, like the functions of cytotoxic $CD8^+$T lymphocytes.[11] Three main processes represent the molecular mechanisms that regulate the cytotoxicity of NK cells: (1) identification of target cells, (2) contact with the target cells and formation of the immunological synapse, and (3) induced death of target cells by NK cells. After identification, NK cells directly interact with the target cell via forming a lytic immunological synapse, which enables NK cells to induce the death of target cells via two main mechanisms.[12,13]

In human peripheral blood, NK cells are comprised of 5–15% of lymphocytes and consist of different stages of differentiation, which is determined by the expression of cell surface markers.[14] The role of NK cells with chronic EBV infection (CEBVI) is not completely understood. During EBV infection of B cells expressing lytic antigens, a portion of NK cells degranulate and proliferate. In the process of maturation, the NK cells consistently acquire specific markers, such as CD94, NKp46, CD56, and CD16. Based on the surface density of CD56, the NK cells of the blood mostly consist of two subpopulations: CD56brightCD16$^+$ and CD56dimCD16$^+$.[15] As a result of monokine stimulation, CD56brightCD16$^+$ NK cells produce a large number of cytokines and, after prolonged activation, acquire cytotoxicity. These cells are enriched in secondary lymphoid organs, where they differentiate into CD56dimCD16$^+$ NK cells, which make up ~90% of peripheral blood and express late markers.[16]

Currently, there is no single approach for the treatment of CEBVI, despite the fact that there are a number of specific antiviral drugs. In particular, acyclic nucleosides are widely used, such as acyclovir, valaciclovir (Valtrex), famciclovir (Famvir), and synthetic nucleoside analogs of guanosine like ganciclovir (Cymevene) and valganciclover (Valcyte).

In recent years, many studies have been published showing that insect and animal poisons are rich sources of antimicrobial substances and contain a wide range of active biological compounds with a well-defined chemical structure. Thus, antimicrobial peptides (AMPs) are a diverse group of oligopeptides with varying amounts (from 5 to more than 100) of amino acids that play an important role in the host's immune response when pathogens enter. Over 5,000 AMPs have been discovered or synthesized to date.[17] Natural AMPs can be found in both prokaryotes (*e.g.*, bacteria) and eukaryotes (*e.g.,* protozoa, fungi, plants, insects, and animals).[18,19] Antiviral AMPs neutralize viruses by integrating either into the viral envelope or by disrupting the attachment of viral particles to the surface of the cell membrane. Thus, AMPs are unable to compete with viral glycoproteins for binding to heparan sulfate receptors on the cell membrane. In addition, antiviral AMPs can penetrate the cell membrane and localize into the cytoplasm and organelles, causing changes in the gene expression profile of cells. This helps to block the expression of viral genes. In the event that AMPs interfere with viral replication, they interact intracellularly with the virion capsid, preventing its decapsidation; therefore, the viral nucleic acid cannot be released and transcribed.[20] Other mechanisms that are involved in the work of the innate immune system are also triggered: 1) induction of the expression of toll-like receptors that interact with the viral nucleic acid; 2) production of cytokines that stimulate the action of cytotoxic T cells and NK cells; 3) expression of MHC molecules in infected cells in order to present viral peptides to other cells of the immune system;[21] and 4) antiviral compounds can activate innate restriction factors encoded by the infected cell.[22]

The cationic peptides alloferon 1 and 2, consisting of 12 (HGVSGHGQHGVHG) and 13 (GVSGHGQHGVHG) amino acid residues, respectively, were isolated from the hemolymph of the firefly Calliphoravicina. Alloferon 2 corresponds to the trun-

cated form of alloferon 1 at the N terminus. Alloferon 1 is the most active; it has been shown to stimulate the natural cytotoxicity of human peripheral blood lymphocytes, induces the production of IFN in both mice and humans, and increase antiviral and antitumor resistance in mice.[23]

In 2003 in the Russian Federation, Alloquin-alpha ("BRANDPHARM", Moscow) was developed by an international team of scientists as a new type of antiviral medication (Reg. #002829/01 from 22 September 2003). The active moiety of the drug is the cytokine-like peptide alloferon (glystidine-glycine-valine-serine-glycine-histidine-glycine-glutamine-histidine-glycine-valine-histidine-glycine).

The objective of the present study was to determine the influence of alloferon on the EBV DNA copy number in saliva samples and the changes in killer cell content, cytotoxic activity of killer cells, and production of interferon alpha (IFNα) and IFNγ in patients suffering CEBVI at 6 weeks after therapy completion.

Methods

Patients

The clinical research in this study was carried out in compliance with the World Medical Association's Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects, 2013), the Protocol of the Council of Europe Convention on Human Rights and Biomedicine (1999), and articles 20, 22, 23 of the Act "On the Basics of Healthcare for the Russian Federation citizens" (21 November 2011; Fed. Statute No. 323-FZ; 26 May 2021 edition). In addition, it was conducted in accordance with a procedure approved by the local ethics committee under "Center of Dialysis of St. Petersburg", FRESENIUS MEDICAL CARE. All study participants had signed a voluntary informed consent form. Moreover, the present study was reported according to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines (Supplementary File 1).

This study included 100 patients suffering from CEBVI. The group consisted of 69 females and 31 males. The average age of the patients in alloferon group was 35.27 ± 1.24 years; while in the valacyclovir group, it was 33.34 ± 1.87 years. The average illness duration was 2.83 ± 0.86 years. Most of the patients often experienced exacerbation of chronic tonsillitis that was resistant to antibiotic therapy during childhood (alloferon group: 70%; valacyclovir group: 60%). There were also reports of infectious mononucleosis (alloferon group: 35.71%; valacyclovir group: 33.33%). All patients underwent differential diagnostics of CEBVI with other viral infections (human immunodeficiency virus, viral hepatitis, cytomegalovirus infection, and toxoplasmosis), helminth infestation, and autoimmune diseases, which are associated with EBV infection. The diagnosis of CEBVI was stated and confirmed in a lab during examination by relevant specialists at the preceding stage; after that, the patients were referred to an immunologist. The patients who had received antiviral or immune-modulating therapy in the preceding 6 months were not included in this trial.

CEBVI is known to have a prolonged duration and frequent relapses, with clinical and lab signs of viral activity similar to those of infectious mononucleosis, which are described in detail in the literature.[20]

The clinical methods used in this study included collecting the medical history, data on previous therapy, and the presence of comorbid conditions. During the initiation of the research, the patients did not indicate any other infections, chronic diseases, or changes of immune status system, which could affect the outcomes of this study. The clinical status of the patients was assessed in accordance with commonly accepted methodology including objective data and the patients' complaints during the examination. The registration of patients' complaints was conducted based on a subjective 3-point scale: 0, absence of symptoms; 1, mild symptoms; 2, moderate symptoms; 3, significant symptoms. All patients were divided into two groups for different therapy schedules. The alloferon group comprised 70 patients, who received alloferon therapy (9 injections s/c, 1.0 mg every alternate day). The drug was administered according to the official instructions of the manufacturer. Dose recalculation for the patient's weight was not carried out when the drug was administered. The subcutaneous injections were well tolerated by the patients, did not cause allergic reactions, and did not render hepatic-nephrotoxic actions on hemopoietic organs. The valacyclovir (control) group included 30 patients, who received a prolonged schedule of therapy with valacyclovir (500 mg, two times/day, orally), which is an acyclic nucleoside drug, for two months.

Polymerase chain reaction (PCR)

In order to confirm the viral etiology of disease, the patients were tested for the viral DNA via PCR in saliva samples, since as it is known that PCR of blood samples does not yield a positive result for infectious chronic forms. It has been reported that saliva sample testing is a more informative EBV DNA identification process for chronic and atypical forms of disease.[23–25] EBV spreads via saliva contact and penetrates through the epithelium lining the rhinopharynx. We have demonstrated that the level of infected B cells in the population varies between 5 and 3,000 for every 10^7 B-memory cells both in the peripheral blood (average value of $110/10^7$) and in the Waldeyer's ring (average value of $175/10^7$), *i.e.,* the virus evenly spreads around the ring.[26] Thus, the level of infected cells is analogous between the peripheral blood and the Waldeyer's ring, and only 1% of these cells are located in the peripheral blood. The virus consistently percolates into the oral cavity, where it mixes with saliva for roughly 2 min prior to every act of swallowing.

The patients were tested for the viral DNA by the PCR method (in saliva samples via PCR with hybridization fluorescence detection in real-time mode). The AmpliSence EBV/CMV/HHV6-screen-FL test system from the Central Scientific Institute of Epidemiology (Russia) was employed. The number of copies of EBV DNA per 1 mL of sample (NCDNA) was used to assess the viral load during the extraction of DNA from saliva. According to the manual, this indicator is calculated by the formula: NCDNA = CDNA × 100, where CDNA indicates the number of copies of viral DNA in the sample. The analytical sensitivity of this test system is 400 copies/mL.

Determination of cytotoxic activity

The cytotoxic activity of killer cells was assessed based on spontaneous and induced expression of CD107a, a lysosomal-associated membrane protein, whose expression on the cellular membrane of lymphocytes indicates degranulation of lysosomes. Assessment of CD107a expression was conducted after joint cultivation of peripheral blood mononuclear cells with the target cell line K562 (derived from human erythroleukemia). K562 cells express a number of ligands (MICA, MICB, ULBP2, and ULBP4) for the NKG2D receptor of cytotoxic lymphocytes. The interaction of NKG2D with one of these ligands leads to degranulation of lysosomes of NK cells, natural killer T (NKT) cells, and lymphoquine-activated $CD8^+$ T cells as well as the expression of CD107a on their mem-

Table 1. Dynamics of the EBV DNA copy number at 6 weeks after completion of antiviral therapy in patients with chronic CEBVI

Patient group	Number of copies/mL before therapy	Number of copies/mL after therapy	*p* value
Alloferon (*n* = 70)	321,873.65 ± 46,072.32, Mean: 286,900.00, 95% CI: 258,576.36–418,686.02	5,847.35 ± 2,020.39, Mean: 4,682.40, 95% CI: 4,503.53–22,430.8, 0.00 copies in 38 patients (54.28%)	0.0,001
Valacyclovir (*n* = 30)	273,837.25 ± 43,202.14, Mean: 238,321.58, 95% CI: 187,099.46–351,640.48	53,109.08 ± 28,828.32, Mean: 43,359.88, 95% CI: 27,720.27–628,486.02, 0.00 copies in 9 patients (30.0%)	0.001

CI, confidence interval; EBV, Epstein-Barr virus; DNA, deoxyribonucleic acid; CEBVI, chronic Epstein-Barr virus infection.

branes. Thus, the test assesses the ability of killer cells to participate in NKG2D-dependent cytolysis of killer cells.

The studied drawn blood was transferred to a vacutainer with lithium heparin as an anticoagulant. Sample processing included the separation of a suspension of mononuclear cells of peripheral blood on a density gradient with consequent washing, cocultivation of MNPK and K562 at a ratio of 10:1 under conditions of a CO_2 incubator for 20 h in the presence of monoclonal antibodies and anti-CD3-FITC/CD(CD16$^+$56)-phycoerythrin and anti-CD-45PC5 (Beckman Coulter, Brea, CA, USA). To assess spontaneous cytotoxic activity, we added RPMI (Biolot) growth medium at the appropriate volume instead of K562 into the MNPK suspension.

The sample analysis was carried out using a Navios (Beckman Coulter) flow-through cytofluorometer. Accumulation was carried out for up to 1,000 events in a minimal population of cells (NK or NKT). The lymphocyte population was identified as CD45$^+$brightSSdim cells. We assessed the relative number of cells expressing CD107a (CD107a$^+$) in subpopulations of NK, NKT, and T lymphocytes. The stimulation index was calculated as the ratio of induced expression of CD107a to spontaneous expression.

Identification of NK cell number

Identification of the relative number of NK cells was carried out by means of multicolor flow cytometry as a part of research of subpopulation composition of lymphocytes in peripheral blood. Blood was drawn from the basilica vein and transferred to vacutainers containing ethylene diamine tetraacetate. Sample preparation was carried out in accordance with the manufacturer's instructions. We used the following monoclonal antibodies: anti-HLADR-FITC, anti-CD4-phycoerythrin, anti-CD3-ECD, anti-CD56-PC5.5, anti-CD25-PC7, anti-CD8-APC, anti-CD19-APC-AF700, and anti-CD45-APC-AF750. VersaLyse was used to lyse the erythrocytes. The samples were analyzed by a Navios flow cytofluorometer (device and reagents by Beckman Coulter). We accumulated 5,000 events in the lymphocytic region of CD45$^+$brightSSdim. NK lymphocytes were identified as CD3$^-$CD56$^+$CD45$^+$brightSSdim events. The absolute number of NK cells was calculated based on the complete blood count.

Identification of IFNα and IFNγ

Identification of the production rate (spontaneous, serous, and induced) of IFNα and IFNγ in the lymphocyte culture was conducted by means of a solid-phase enzyme-multiplied immunoassay using the test systems alpha-Interferon-IFA-BEST and gamma-Interferon-IFA-BEST (Vector Best, Novosibirsk Oblast, Russia). As an inductor of IFNα production, we used Newcastle disease virus (acquired at FGBU GISK named after L. A. Tarasevich, St. Petersburg, Russia) with an infectious titer of 8 lg EID/0.2 mL at 8 µL per cavity. As an inducer of IFNγ production, we used phytohemoagglutinin (PanEco, Moscow, Russia) at a dose of 10 µg/mL. The quantitative content of cytokines was identified in the serum and supernatant liquid after culturing the whole blood for 24 h via a solid-phase enzyme-multiplied immunoassay with the use of the test systems alpha-Interferon-IFA-BEST and gamma-Interferon-IFA-BEST (Vector Best). The manufacturer of the test systems provided reference values for the spontaneous, serous, and induced production of IFNα and IFNγ.

Statistical analysis

Statistical treatment of acquired data was conducted with IBM SPSS Statistics, 26 version (IBM Corp., Armonk, NY, USA). The results are presented as the mean± standard error. For the statistical treatment, we employed parametric (Pearson's method) and nonparametric (Spearman's method, tau (τ) Kendall) methods.

To verify compliance with the condition of independence of observations, we conducted linear regression analysis (with computation of the coefficient of determination (R squared) and the criterion of Durban-Watson) and dispersion analysis (analysis of variance) with computation of the criterion of Fisher (F) for verification of model significance. The standardized rate β with the 95% confidence interval (CI) was calculated. The critical significance level of difference of indicators was taken to be equal to 0.05.

Results

Alloferon therapy efficiency analysis

In all examined patients (*n* = 100), EBV infection was confirmed by PCR of their saliva samples. The PCR test was conducted prior to the start of alloferon therapy (*n* = 70) and valacyclovir therapy (*n* = 30) and at 6 weeks after the course of therapy completion. The results of the study are given in Table 1, demonstrating that the antiviral therapy efficiency in the alloferon group was significantly greater than that in the valacyclovir group ($p = 0.001$) ($p = 0.03$; Fisher's test).

NK cell content in peripheral blood

Further research was carried out of the NK cells in the peripheral blood prior to the start of therapy and at 6 weeks after therapy completion in both groups. The results are given in Table 2 and Figures 1–3.The data demonstrate that the contents of NK cells, NKT cells, and T cells in the peripheral blood reliably increased after the alloferon therapy, exceeding the initial values of the cell content before therapy. After the course of valacyclovir therapy, we detected a reliable reduction of the contents of all subpopulations of killer cells.

Dynamics of cytotoxic activity of NK cells

Next, we conducted an assessment of the dynamics of cytotoxic activity of NK cells in both groups before therapy and at 6 weeks after therapy completion (Tables 3, 4 and Figures 4, 5). From Table 3, one can see that the spontaneous and stimulated expression of CD107a, a marker of degranulation of cytotoxic granules, on NK cells at 6 weeks after completion of therapy by alloferon reliably grew but did not exceed the reference values; however, on NKT cells and T cells after therapy, the expression of CD107a reliably

Table 2. NK cell content (%) in blood before and at 6 weeks after the completion of therapy by alloferon or valacyclovir in CEBVI patients

Subpopulations of mononuclear cells in blood	Content of mononuclear cells (%) in blood before therapy	Content of mononuclear cells (%) at 6 weeks after therapy completion	*p* value
Alloferon group (*n* = 70)			
NK cells ($CD3^-CD16^+CD56^+$)	10.95 ± 0.78, Mean: 5.89, 95% CI: 9.53–12.58	12.33 ± 0.76, Mean: 5.78, 95% CI: 10.93–13.72	0.006
NKT cells ($CD3^+CD16^+CD56^+$)	6.97 ± 0.63, Mean: 4.74, 95% CI: 5.81–8.16	9.46 ± 0.65, Mean: 10.03, 95% CI: 7.60–12.25	0.0001
T cells ($CD3^+CD16^+CD56^-$)	2.97 ± 0.33, Mean: 2.35, 95% CI: 2.34–3.66	3.89 ± 0.31, Mean: 2.26, 95% CI: 3.30–4.50	0.0001
Valacyclovir group (*n* = 30)			
NK cells, ($CD3^-CD16^+ CD56^+$)	9.61 ± 1.31, Mean: 5.74, 95% CI: 5.89–12.46	6.94 ± 0.62, Mean: 3.89, 95% CI: 3.24–8.95	0.031
NKT cells ($CD3^+CD16^+CD56^+$)	6.94 ± 0.62, Mean: 4.86, 95% CI: 5.72–8.75	4.74 ± 0.56, Mean: 3.24, 95% CI: 3.64–6.20	0.04
T cells ($CD3^+CD16^+CD56^-$)	2.67 ± 0.49, Mean: 2.23, 95% CI: 1.71–3.07	1.21 ± 0.24, Mean: 1.01, 95% CI: 0.86–1.67	0.031

CEBVI, chronic Epstein-Barr virus infection; CI, confidence interval; NK, natural killer; NKT, natural killer T.

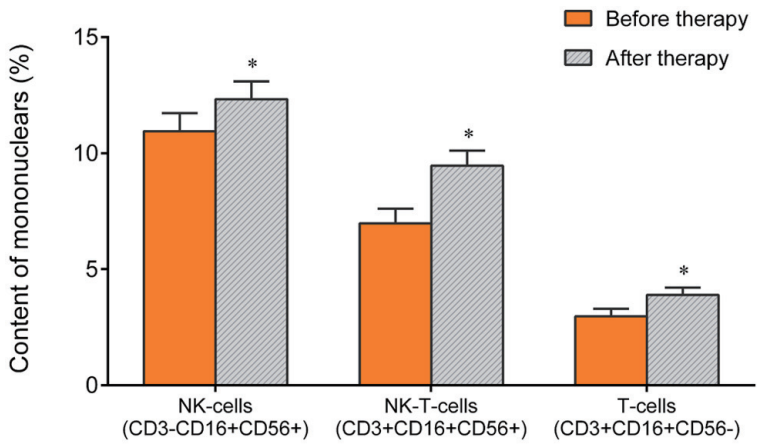

Fig. 1. NK, NKT, and T cell content (%) in blood before and at 6 weeks after completion of alloferon therapy in CEBVI patients (*$p < 0.05$). CEBVI, chronic Epstein-Barr virus infection; NK, natural killer, NKT, natural killer T.

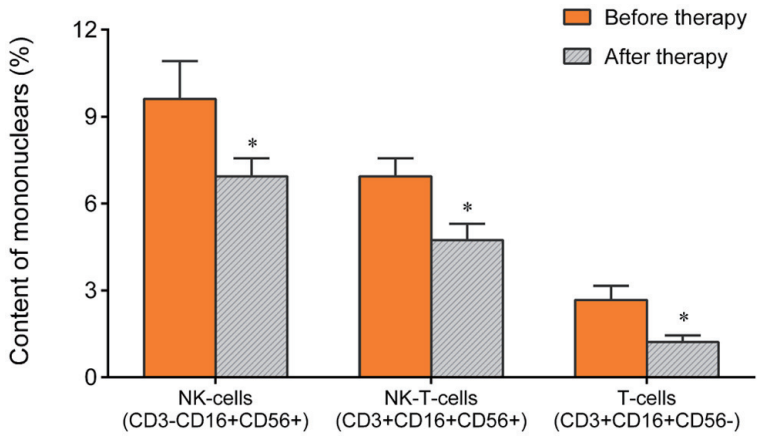

Fig. 2. NK, NKT, and T cell content (%) in blood before and at 6 weeks after completion of valacyclovir therapy in CEBVI patients (*$p < 0.05$). CEBVI, chronic Epstein-Barr virus infection; NK, natural killer, NKT, natural killer T.

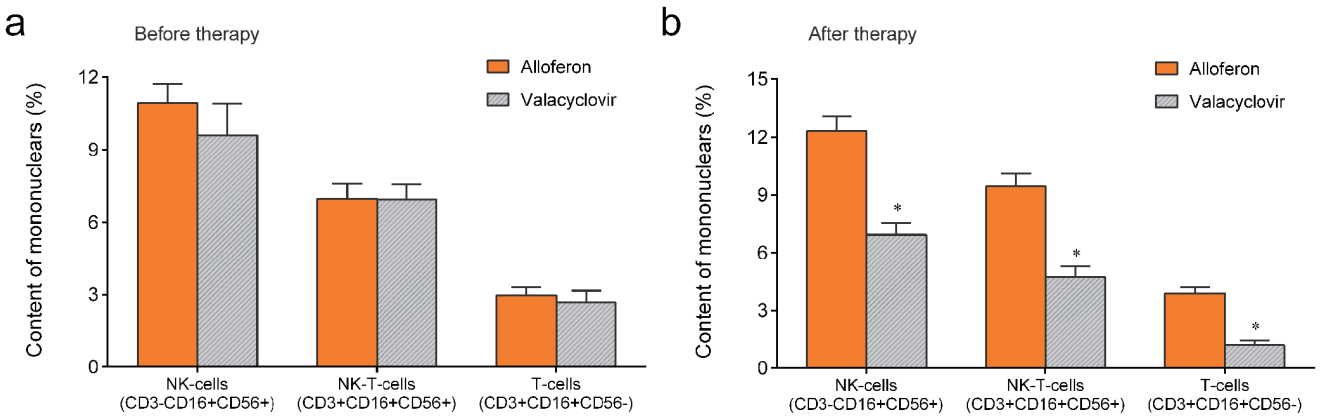

Fig. 3. NK, NKT, and T cell content (%) in blood before and at 6 weeks after completion of therapy by alloferon or valacyclovir in CEBVI patients (*$p < 0.05$). a) before therapy; b) after therapy. CEBVI, chronic Epstein-Barr virus infection; NK, natural killer; NKT, natural killer T.

increased, exceeding the reference values. Thus, alloferon therapy stimulates spontaneous and induced degranulation of killer cells.

As shown in Table 4, at 6 weeks after therapy completion by valacyclovir, the spontaneous and stimulated expression of CD107a on NK cells, NKT cells, T cells was reliably reduced. Thus, valacyclovir inhibits the spontaneous and induced degranulation of killer cells in CEBVI patients.

Dynamics of INFα and INFγ production

After a comparative analysis of the efficiency of two different schedules of CEBVI therapy, we carried out an analysis of the dynamics of INFα and INFγ production (spontaneous, serous, and induced) in lymphocyte culture before therapy and at 6 weeks after the completion of therapy by alloferon and valacyclovir. The results are shown in Tables 5 and 6.

From the presented data, at 6 weeks after therapy completion by alloferon, we observed a statistically unreliable tendency of reduction of serous, spontaneous, and induced IFNα production in the patients. The production of induced and serous IFNγ had an unreliable tendency to decrease at 6 weeks after therapy completion, and the production of spontaneous IFNγ was reliably lower than the initial level after therapy completion.

Table 3. Dynamics of cytotoxic activity of NK cells at 6 weeks after alloferon therapy completion in CEBVI patients

Expression of CD107a	Before start of therapy	At 6 weeks after therapy completion	Reference values	*p* value
Expression of marker of degranulation of cytotoxic granules CD107a on NK cells ($CD3^-CD16^+CD56^+$)				
Spontaneous	2.68 ± 0.38, Mean: 2.14, 95% CI: 1.82–4.32	3.99 ± 0.42, Mean: 3.77, 95% CI: 2.54–4.57	0.9–3.3	0.001
Stimulated	19.46 ± 1.18, Mean: 14.23, 95% CI: 13.07–21.68	21.28 ± 1.08, Mean: 19.33, 95% CI: 17.93–23.23	11.0–24.0	0.008
Index of stimulation	12.14 ± 0.96, Mean: 8.03, 95% CI: 6.75–13.54	13.37 ± 1.04, Mean: 8.85, 95% CI: 7.61–15.74	5.5–17.0	0.127
Expression of marker of degranulation of cytotoxic granules CD107a on NKT cells ($CD3^+CD16^+CD56^+$)				
Spontaneous	1.52 ± 0.16, Mean: 1.10, 95% CI: 1.21–1.84	2.23 ± 0.27, Mean: 1.80, 95% CI: 1.73–2.80	0.4–1.6	0.004
Stimulated	2.58 ± 0.26, Mean: 1.79, 95% CI: 1.34–3.13	323 ± 0.32, Mean: 2.25, 95% CI: 1.76–3.38	0.5–3.0	0.044
Index of stimulation	2.41 ± 0.29, Mean: 2.08, 95% CI: 1.87–3.03	3.98 ± 0.57, Mean: 2.89, 95% CI: 2.45–5.23	1.0–2.5	0.01
Expression of marker of degranulation of cytotoxic granules CD107a on T cells ($CD3^+CD16^+CD56^-$)				
Spontaneous	0.31 ± 0.031, Mean: 0.24, 95% CI: 0.26–0.38	0.71 ± 0.14, Mean: 1.07, 95% CI: 0.46–1.00	0.1–0.4	0.009
Stimulated	0.36 ± 0.044, Mean: 0.33, 95% CI: 0.28–0.45	1.45 ± 0.19, Mean: 1.43, 95% CI: 1.10–1.84	0.1–04	0.0001
Index of stimulation	1.16 ± 0.064, Mean: 0.44, 95% CI: 0.31–1.29	1.85 ± 0.18, Mean: 1.31, 95% CI: 0.83–2.20	≤1.0	0.001

CEBVI, chronic Epstein-Barr; CI, confidence interval; NK cells, natural killer; NKT, natural killer T.

Table 4. Dynamics of cytotoxic activity of NK cells before and at 6 weeks after completion of therapy by valacyclovir in CEBVI patients

CD107a expression	Before therapy	At 6 weeks after therapy completion	Reference values	*p* value
Expression of marker of degranulation of cytotoxic granules CD107a on NK cells ($CD3^-CD16^+CD56^+$)				
Spontaneous	2.74 ± 0.25, Mean: 2.24, 95% CI: 2.04–3.24	1.36 ± 0.12, Mean: 1.58, 95% CI: 1.13–2.39	0.9–3.3	0.001
Stimulated	18.34 ± 1.07, Mean: 10.02, 95% CI: 17.19–21.52	16.21 ± .02, Mean: 9.33, 95% CI: 14.06–19.16	11.0–24.0	0.01
Index of stimulation	13.12 ± 0.26, Mean: 8.03, 95% CI: 10.21–13.71	10.36 ± 1.03, Mean: 6.85, 95% CI: 7.44–11.07	5.5–17.0	0.001
Expression of marker of degranulation of cytotoxic granules CD107a on NKT cells ($CD3^+CD16^+CD56^+$)				
Spontaneous	2.62 ± 0.15, Mean: 1.54, 95% CI: 1.20–2.86	1.23 ± 0.25, Mean: 1.02, 95% CI: 0.81–1.74	0.4–1.6	0.004
Stimulated	2.53 ± 0.26, Mean: 1.79, 95% CI: 2.10–3.17	1.21 ± 0.31, Mean: 1.01, 95% CI: 0.80–1.68	0.5–3.0	0.04
Index of stimulation	2.46 ± 0.23, Mean: 2.06, 95% CI: 1.90–3.12	1.78 ± 0.53, Mean: 1.09, 95% CI: 0.76–2.44	1.0–2.5	0.01
Expression of marker of degranulation of cytotoxic granules CD107a on T cells ($CD3^+CD16^+CD56^-$)				
Spontaneous	0.31 ± 0.02, Mean: 0.24, 95% CI: 0.16–0.37	0.11 ± 0.02, Mean: 0.08, 95% CI: 0.05–0.16	0.1–0.4	0.001
Stimulated	0.38 ± 0.09, Mean: 0.33, 95% CI: 0.28–0.45	0.33 ± 0.02, Mean: 0.28, 95% CI: 0.26–0.39	0.1–0.4	0.01
Index of stimulation	1.40 ± 0.04, Mean: 1.04, 95% CI: 0.87–1.64	1.02 ± 0.21, Mean: 0.98, 95% CI: 0.83–1.28	≤1.0	0.01

CEBVI, chronic Epstein-Barr virus infection; CI, confidence interval; NK, natural killer.

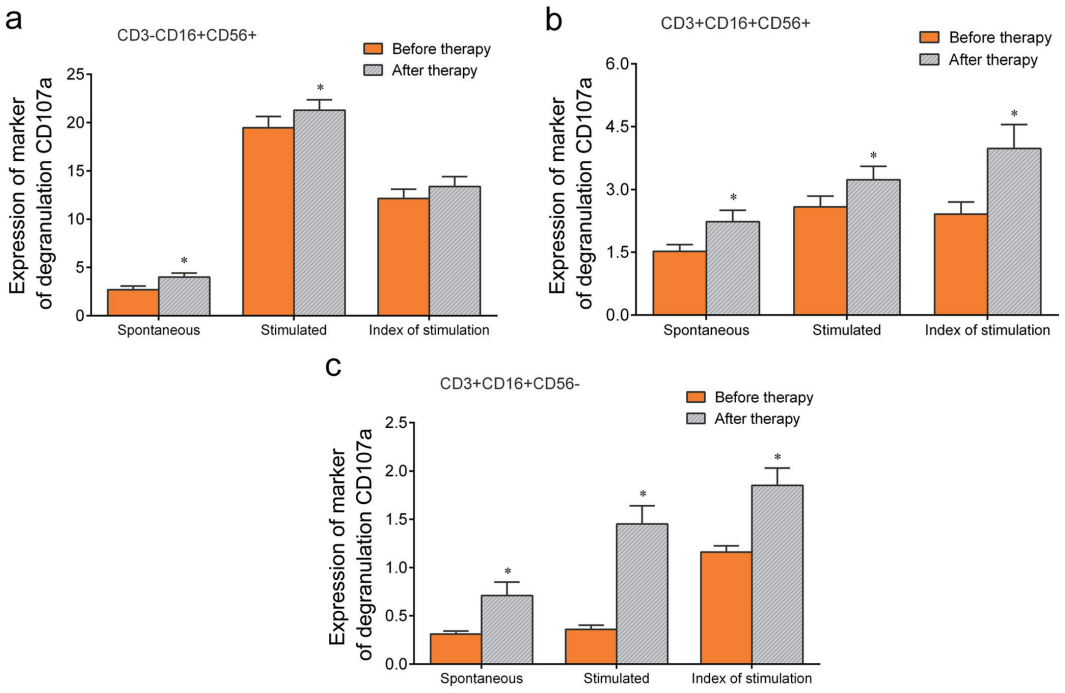

Fig. 4. Dynamics of cytotoxic activity of NK, NKT, and T cells before and at 6 weeks after alloferon therapy completion in CEBVI patients (*$p < 0.05$). a) Expression of marker of degranulation of cytotoxic granules of CD107a on NK-cells (CD3-CD16+CD56+); b) Expression of marker of degranulation of cytotoxic granules of CD107a on NK-T-cells (CD3+CD16+CD56+); c) Expression of marker of degranulation of cytotoxic granules of CD107a on T-cells (CD3+CD16+CD56–). CEBVI, chronic Epstein-Barr virus infection; NK, natural killer, NKT, natural killer T.

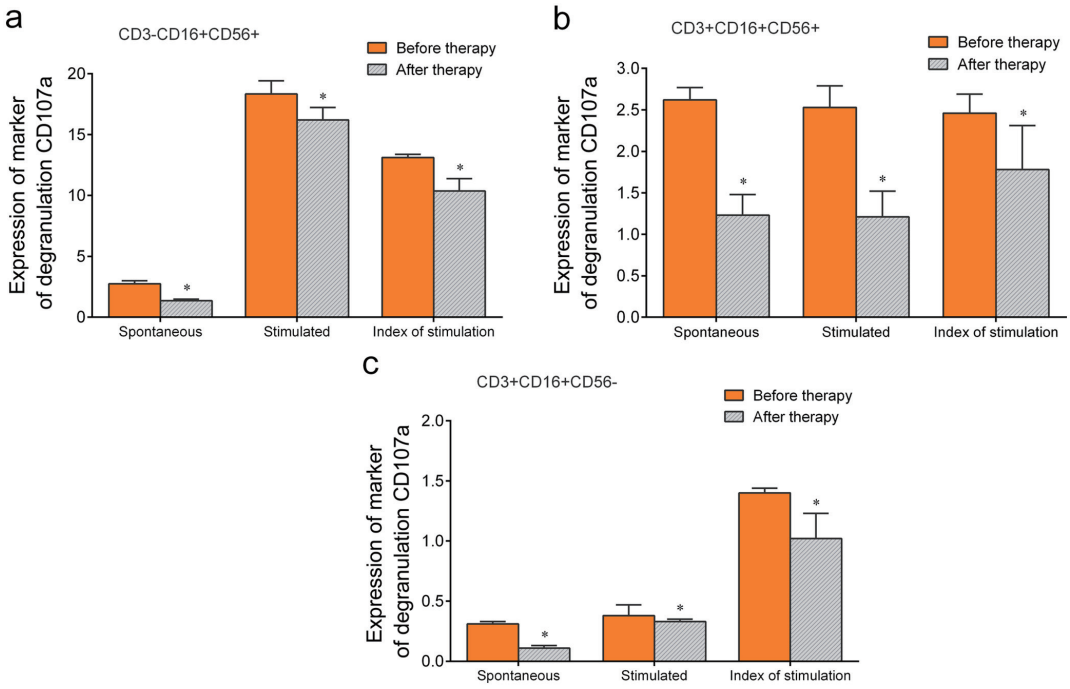

Fig. 5. Dynamics of cytotoxic activity of NK, NKT, and T cells before and at 6 weeks after completion of therapy by valacyclovir in patients chronically infected with Epstein-Barr virus (*$p < 0.05$). a) Expression of marker of degranulation of cytotoxic granules of CD107a on NK-cells (CD3-CD16+CD56+); b) Expression of marker of degranulation of cytotoxic granules of CD107a on NK-T-cells (CD3+CD16+CD56+); c) Expression of marker of degranulation of cytotoxic granules of CD107a on T-cells (CD3+CD16+CD56-). NK, natural killer; NKT, natural killer T.

Furthermore, we conducted a study of the dynamics of INFα and IFNγ production before and at 6 weeks after valacyclovir therapy completion. The data are presented in Table 6.

From the presented data, at 6 weeks after the course of valacyclovir therapy, we detected a reliable reduction of the production of serous and induced INFα and a statistically unreliable reduction of the spontaneous INFα level. The production of serous and spontaneous INFα was reliably reduced after the completion of valacyclovir therapy. In addition, the production of induced INFα was also reduced after the completion of therapy in comparison to the initial level.

Dynamics of clinical complaints

The next step was to analyze the dynamics of the frequency of the main clinical complaints of the patients before and at 6 weeks after the therapy schedule completion in both groups. Table 7 and Figures 6–8 present the results after the applied therapy.

In the patients of the alloferon group, we noted reliable dynamics of clinical complaints such as subfebrile temperature, throat pain, asthenia, rear throat wall mucus running, stomatitis, pain in joints, irritability and tearfulness, and skin rash after therapy. The remaining complaints did not change. In the valacyclovir group of patients, there was a reliable reduction of only a few types of complaints, including throat pain, shivers, and hidrosis. The other complaints from patients remained at 6 weeks after the 2-month therapy course.

Predictive significance of the NK cell number

In order to determine the predictive significance of NK cells for the development of clinical complaints, we carried out an analysis

Table 5. Dynamics of IFNα and IFNγ production before the start and at 6 weeks after completion of alloferon therapy in CEBVI patients

Studied indicator	IFNα before therapy (pg/mL)	IFNα at 6 weeks after therapy completion (pg/mL)	*p* value
Spontaneous IFNα	5.20 ± 0.81, Mean: 3.32, 95% CI: 3.73–6.86	4.7333 ± 0.3224, Mean: 1.28, 95% CI: 4.06–5.33	0.553
Serous IFNα	4.53 ± 0.30, Mean: 1.24, 95% CI: 3.80–5.00	4.80 ± 0.42, Mean: 1.65, 95% CI: 3.93–5.60	0.546
Induced IFNα	501.96 ± 72.72, Mean: 524.41, 95% CI: 373.56–651.39	405.01 ± 48.02, Mean: 346.34, 95% CI: 314.38–503.18	0.206
Studied indicator	IFNγ before therapy (pg/mL)	IFNγ at 6 weeks after therapy completion (pg/mL)	*p* value
Spontaneous IFNγ	7.78 ± 2.18, Mean: 9.87, 95% CI: 4.05–12.52	3.42 ± 0.47, Mean: 2.12, 95% CI: 2.47–4.36	0.035
Serous IFNγ	8.15 ± 2.73, Mean: 10.04, 95% CI: 3.54–14.07	4.15 ± 0.48, Mean: 1.90, 95% CI: 3.23–5.07	0.161
Induced IFNγ	3,897.46 ± 1,103.75, Mean: 4,274.52, 95% CI: 2,045.17–6,404.91	2,584.53 ± 670.30, Mean: 2,488.89, 95% CI: 1,369.67–3,967.29	0.299

CEBVI, chronic Epstein-Barr virus infection; CI, confidence interval; IFNα, interferon alpha; IFNγ, interferon gamma.

Table 6. Dynamics of IFNα and IFNγ production before and at 6 weeks after the completion of valacyclovir therapy in CEBVI patients

Studied indicator	IFNα before therapy (pg/mL)	IFNα at 6 weeks after therapy completion (pg/mL)	*p* value
Spontaneous IFNα	4.00 ± 0.46, Mean: 1.50, 95% CI: 3.00–4.66	2.33 ± 0.31, Mean: 1.28, 95% CI: 2.00–3.00	0.051
Serous IFNα	6.59 ± 0.87, Mean: 4.19, 95% CI: 5.09–8.54	2.13 ± 0.13, Mean: 0.64, 95% CI: 2.00–2.41	0.001
Induced IFNα	464.15 ± 40.41, Mean: 181.18, 95% CI: 386.21–538.84	299.94 ± 28.69, Mean: 127.25, 95% CI: 242.31–358.41	0.001
Studied indicator	IFNγ before therapy (pg/mL)	IFNγ at 6 weeks after therapy completion (pg/mL)	*p* value
Spontaneous IFNγ	7.79 ± 2.18, Mean: 9.87, 95% CI: 4.00–12.20	3.42 ± 0.46, Mean: 2.12, 95% CI: 2.47–4.36	0.035
Serous IFNγ	5.22 ± 1.40, Mean: 6.13, 95% CI: 2.89–8.22	3.50 ± 0.45, Mean: 2.11, 95% CI: 2.47–4.37	0.035
Induced IFNγ	1,820 ± 492.21, Mean: 2,161.33, 95% CI: 950.05–2,877.36	1,649.61± 670.41, Mean: 2,989.98, 95% CI: 557.83–3,174.25	0.706

CEBVI, chronic Epstein-Barr virus infection; CI, confidence interval; IFNα, interferon alpha; IFNγ, interferon gamma.

by the linear regression method with computation of the determination coefficient R^2, utilization of the Durban-Watson criterion, and dispersion analysis (analysis of variance) with employment of the F criterion and calculation of the standardized rate (β) with the 95% CI. Significant results were acquired only from the group of patients receiving alloferon. The results of the F criterion and β coefficient, testifying the significance of the regression models, are as follows: (1) The NK cell content ($CD3^-CD16^+CD56^+$) in blood prior to alloferon therapy was associated with throat pain occurrence (F = 8.756, p = 0.004; β = 0.342, CI: 0.721–3.113, p = 0.004). (2) The NK cell content ($CD3^-CD16^+CD56^+$) in blood prior to alloferon therapy was associated with progressive sleep disorder development (F = 3.958, p = 0.024; β = 0.334, CI: 0.815–4.805, p = 0.006). (3) The NK cell content ($CD3^-CD16^+CD56^+$) in blood prior to alloferon therapy was associated with the development and progression of stomatitis (F = 4.170, p = 0.045; β = 0.244, CI: 0.035–3.072, p = 0.045). (4) The NK cell content ($CD3^-CD16^+CD56^+$) in blood prior to alloferon therapy affects the development and progression of irritability and tearfulness (F = 4.420, p = 0.039; β = 0.251; CI: 0.091–3.519; p = 0.030).

Thus, the linear regression analysis results indicate that the NK cell content ($CD3^-CD16^+CD56^+$) in the blood prior to therapy is a predictor of the development and progression of clinical complaints from CEBVI patients.

Predictive significance of the rate of induced IFNα production

In addition, linear regression analysis of the data of the patients receiving alloferon therapy indicated the following: 1) The rate of induced production of IFNα prior to alloferon therapy affects the expansion of NK cells ($CD3^-CD16^+CD56^+$) in blood (F = 6.737, p = 0.016; β = 0.461; CI: −1.707–56.983, p = 0.002). 2) After alloferon therapy, we saw some influence of the rate of induced IFNα on the expansion of NK cells ($CD3^-CD16^+CD56^+$) in blood (F = 5.453, p = 0.027; β = 0.398; CI: 14.158–78.815, p = 0.027). 3) The rate of induced production of IFNα prior to the start of alloferon therapy affects the spontaneous expression of CD107a, a marker of degranulation of cytotoxic granules, on T cells ($CD3^-CD16^+CD56^-$) (F = 9.130, p = 0.004; β = 0.390; CI: −0.018–76.874, p = 0.0004).

Discussion

The elimination of virus-infected cells is carried out by means of the cytotoxic activity of NK cells.[27] The distinctive feature of NK

Table 7. Frequency of clinical complaints (%) from patients with chronic infection of Epstein-Barr virus before and after 6 weeks of therapy with alloferon (*n* = 70) or valacyclovir (*n* = 30)

Complaint	Frequency of clinicalcomplaint (%)			
	Before alloferon therapy	After alloferon therapy	Before valacyclovir therapy	After valacyclovir therapy
Subfebrile temperature	5,000	24.28 (p = 0.001)	76.66	66.66 (p = 0.054)
Lymphadenitis	24.28	20.00 (p = 0.084)	53.33	46.66 (p = 0.086)
Throat pain	50.00	40.00 (p = 0.001)	86.66	53.33 (p = 0.001)
Asthenia	64.28	50.00 (p = 0.001)	80.00	66.66 (p = 0.054)
Shivers	32.85	15.71 (p = 0.006)	66.66	33.33 (p = 0.001)
Hidrosis	68.57	57.14 (p = 0.001)	93.33	53.33 (p = 0.001)
Mucus dripping	40.00	24.28 (p = 0.001)	70.00	63.33 (p = 0.052)
Stomatitis.	24.28	12.85 (p = 0.001)	36.66	33.33 (p = 0.054)
Pain in joints	17.14	8.57 (p = 0.036)	33.33	26.66 (p = 0.054)
irritability and tearfulness	74.28	65.71 (p = 0.001)	70.00	66.66 (p = 0.058)
Skin rash	70.00	54.28 (p = 0.001)	53.33	46.66 (p = 0.086)

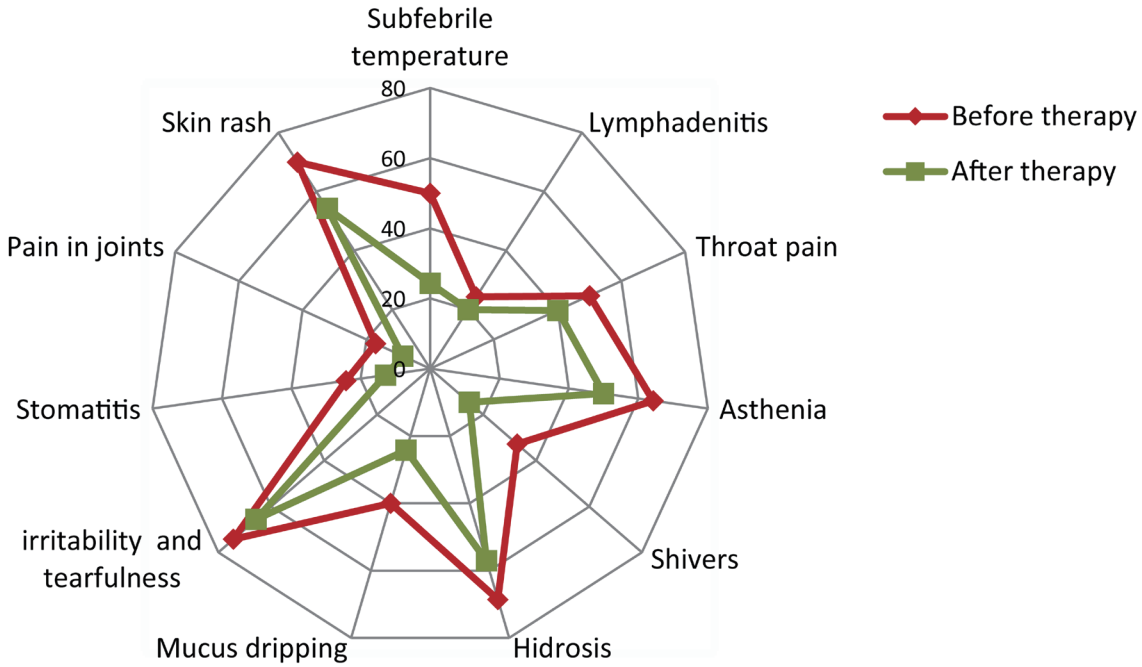

Fig. 6. Frequency of clinical complaints (%) by patients chronically infected with Epstein-Barr virus before and at 6 weeks after alloferon therapy.

cells' activation is degranulation, *i.e.,* a release of lytic content of granules. The internal surface of granules is covered with CD107a (lysosome-associated membrane protein 1), a glycosylated protein, which appears on the cell surface as a consequence of fusion of lysosomes with the plasma membrane. The degranulation leads to the expression of CD107a on the cell surface and depletion of intracellular perforin. After degranulation, CD107a is exposed on the surface of cytotoxic lymphocytes, protecting the membrane from perforin-mediated damage.[28] All resting killer cells upon receipt of signals for degranulation are able to express surface CD107a and mediate cytotoxicity. Polarization and degranulation of cytolytic granules are two stages of cytotoxicity of NK cells, which are controlled by certain signals emanating from various receptors. Neither polarization nor degranulation is sufficient for the effective lysis of target cells. The ability of NK cells to destroy the virus-infected cells takes place until the depletion of NK cells, which is likely related, in part, to the

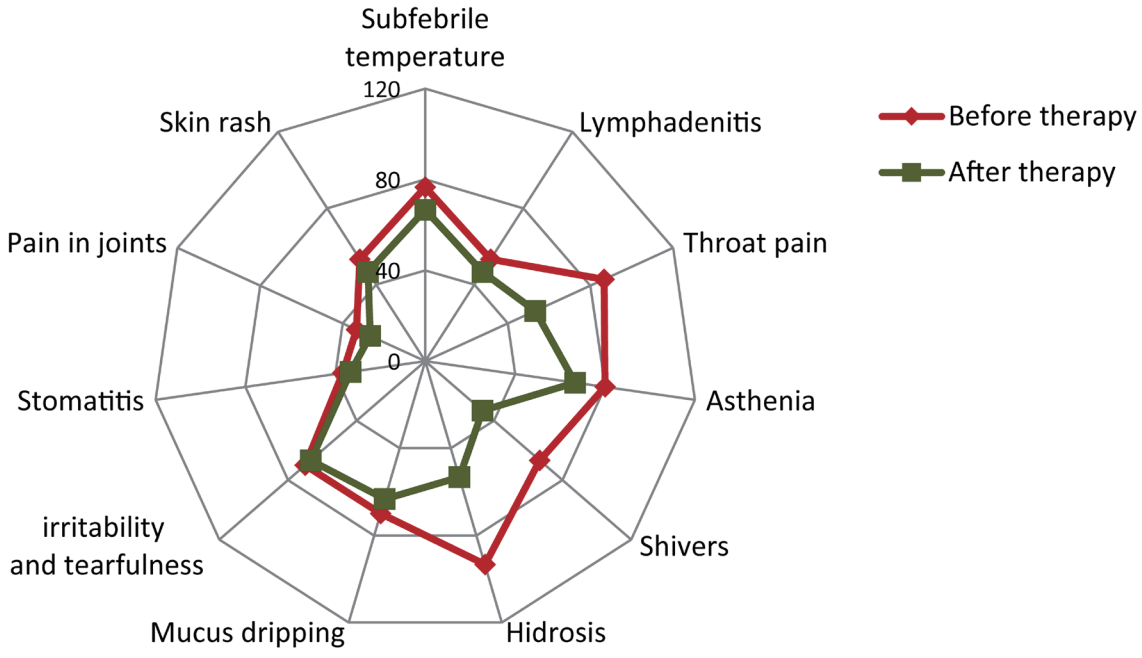

Fig. 7. Frequency of clinical complaints (%) by patients chronically infected with Epstein-Barr virus before and at 6 weeks after valacyclovir therapy.

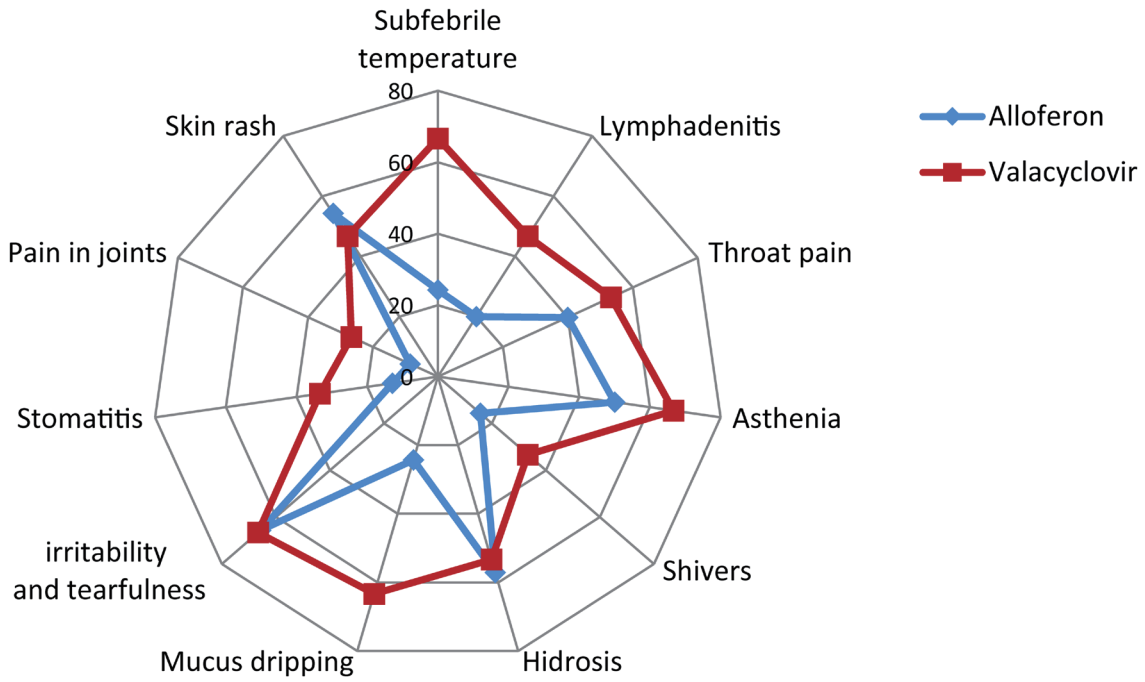

Fig. 8. Frequency of clinical complaints (%) by patients chronically infected with Epstein-Barr virus at 6 weeks after therapy completion in the alloferon and valacyclovir groups.

depletion of cytolytic granules.[29]

The analysis of the NK cell degranulation results indicates that they correlate to the standard cytotoxicity results. That is, the expression of CD107a can be a sensitive marker for determining the cytotoxic activity.[30] Our work detected a reliable increase of the spontaneous and induced expression of CD107a on $CD3^-CD16^+CD56^+$, $CD3^+CD16^+CD56^+$, and $CD3^+CD16^+CD56^-$ cells at 6 weeks after the completion of alloferon therapy. Thus, alloferon induces the spontaneous and induced degranulation of NK cells, *i.e.*, the cytotoxic activity of cells in the CEBVI patients. The work by Lee *et al.* indicates the explicit antiviral activity of alloferon via an increase of the regulation of the cytotoxicity of NK cells, which is mediated by enhanced secretion of perforin/granzyme.[31] The data from these authors and the results acquired by us fully confirm the effectiveness of alloferon at stimulating the cytotoxic activity of NK cells.

The expansion of NK cells depends on the number of free viral antigens. In our study, we did not detect a correlation between the general quantity of NK cells and the viral load in the saliva sample; these findings do not coincide with an earlier published study in which a correlation was detected between the number of NK cells and the level of EBV DNA in whole blood.[32] In addition, Williams *et al.* have indicated that an increased viral load reversely correlates to the percentage of NK cells in the peripheral blood; the Pearson's coefficient (r) was −0.87 ($p < 0.001$).[33] Since we conducted our study in patients with CEBVI, assessment of the EBV DNA content was carried out only in saliva samples. Detect EBV DNA in blood samples at this stage is not possible due to the chronic stage of infection, when replication of EBV happens in the oropharynx and viral shedding into the saliva can be observed for a long time.[34] Lytic replication may not only be responsible for the expansion of prior-differentiated subpopulations of the NK cells, but apparently it is also a target of NK cells. Thus, EBV infection causes expansion of NK cells in the blood, which precedes the peak of the T-cell response and induces lengthy differentiation, mainly of $CD16^+NKG2A^+KIR^-$NK cells, which witness an early phenotype of differentiation.[35] In our study, the NK cell content prior to the start of the alloferon therapy did not exceed reference values. At 6 weeks after alloferon therapy, we noted a reliable growth of the NK cell content; thus, the medication acted on the expansion of NK cells. The linear regression data indicate that the content of $CD3^-CD16^+CD56^+$cells in the blood prior to therapy is a predictor of the development and progression of clinical complaints of the CEBVI patients.

The linear regression method indicated that the rate of production of IFNα prior to alloferon therapy affects the expansion of $CD3^-CD16^+CD56^+$ cells in the blood and the spontaneous expression of CD107a on $CD3^-CD16^+CD56^-$ cells. After alloferon therapy, we saw continuing action of induced production of IFNα only on the expansion of $CD3^-CD16^+CD56^-$ cells. For the first time, the role of IFNα in boosting the production of cytokine cytotoxicity of NK cells was shown by Portales *et al.* in their work, demonstrating the enhanced expression of perforin and granzyme by the NK cells in patients who were receiving a therapy of pegylated IFNα2b.[36] Later, we published the results of a study on the influence of the signaling of type-I IFN on amplification of the cytotoxic response by NK cells on herpes virus infection. In an experiment using NK cells from healthy donors, it was shown that NK cells treated with IFNα significantly increased their cytotoxicity, increasing the portion of dead cells by approximately 20% in comparison with NK cells not treated by IFNα.[37] It is known that compound [3–13] alloferon manifests strong antiviral activity, inhibits the replication of human herpesviruses, and induces the production of IFN in humans. Research by Chernysh indicates that at 2 h after the injection of alloferon, the IFN content becomes 2–2.5 times greater than the usual background level, remains at an elevated level for 6–8 h, and returns back to initial values at 24 h. The author suggests that the medication acts as an inducer of IFN and stimulates a secondary immune response that exceeds the primary response in the same or other IFN-producing

cells, reflecting a cascade-like reaction.[38] Based on the data, one can assume that the expansion mechanism of $CD3^{-}CD16^{+}CD56^{+}$ cells and enhancement of the spontaneous expression of CD107a on $CD3^{-}CD16^{+}CD56^{-}$ cells are concomitant, *i.e.*, by way of action of alloferon itself and periodic short-lasting boosts of IFNα production during the course of therapy.

The analysis of clinical complaints demonstrated reliable positive dynamics in patients after alloferon therapy. We noted an improvement in the general physical condition of the patients and an increase of their working efficiency, which were not observed under standard antiviral therapy by acyclic nucleosides. The medication injections were well tolerated by the patients.

Future directions

Based on previously obtained data on the effect of alloferon, it can be assumed that the drug stimulates the cytotoxic activity of cells. Indirectly, this assumption is based on the revealed positive dynamics of spontaneous and induced expression of the NK cell degranulation marker. We also demonstrated that alloferon increases the content of NK cells after a course of therapy.

Nevertheless, it would be interesting to study the specific mechanisms leading to the activation of NK cells. In this regard, it is necessary to investigate the secretion of perforin and granzyme under the influence of alloferon. It is also important to estimate the duration of the effect of alloferon therapy on the cytotoxic activity of NK cells (*e.g.*, after 3–6 months). It is advisable to continue studying the effect of alloferon on the cytotoxic activity of NK cells.

Conclusions

The results of this study demonstrated that alloferon can be used as an effective antiviral agent, which was confirmed by a significant decrease in the viral load. The course of therapy with alloferon reduces the manifestations of clinical complaints in patients with CEBVI and is well tolerated by the patients, without causing negative side effects.

Supporting information

Supplementary material for this article is available at https://doi.org/10.14218/ERHM.2022.00119.

Supplementary File 1. Epidemiology (STROBE) guidelines.

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Conflict of interest

The authors have no conflicts of interest related to this publication.

Author contributions

Study concept and research design, statistical processing of data, and research supervision as well as responsibility for integrity of all parts of the article (RIA); material gathering and processing, data analysis and interpretation, and script composition (RIA and RTS); lab research (KAA); editing (RTS and KAA); and text writing and editing as well as further revision for important intellectual content (RIA, RTS, and KAA). All authors have made substantial contributions to this study and approved the final version to be published.

Ethical statement

The clinical research carried out in compliance with the WMA Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects, 2013) and the Protocol of Council of Europe Convention on human rights and biomedicine 1999 and articles 20, 22, 23 of the Act "On the basics of healthcare for the Russian Federation citizens" dated November 21, 2011 Fed. Statute №323-FZ (May 26, 2021 edition). The clinical study was conducted in accordance with a procedure approved by the local ethical committee under LLC "Center of Dialysis of St. Petersburg" FRESENIUS MEDICAL CARE. All study participants had signed voluntary informed consent.

Data sharing statement

The statistical code and dataset used in support of the findings of this study are included within the article.

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