Article

Discovery Medicine 2024; 36(187): 1641–1647
https://doi.org/10.24976/Discov.Med.202436187.150

Anti-Inflammatory Immunomodulatory Activity of Valacyclovir on the *in Vitro* Activated Mammalian Macrophages

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Published: 20 August 2024

Background: Aciclovir, often known as acyclovir, is a nucleoside analog that exhibits antiviral activity *in vitro* against human herpesvirus 6 (HHV-6), cytomegalovirus (CMV), varicella-zoster virus (VZV), and herpes simplex virus (HSV). Valacyclovir is an amino acid ester prodrug of acyclovir. We examined valacyclovir, which is also an anti-viral agent, for its effects on inflammation.
Methods: Mammalian Macrophages were activated by lipopolysaccharide (LPS) in the presence of a concentration range of Valacyclovir. Tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-12p40 enzyme-linked immunosorbent assay (ELISA) was performed to measure the production levels of these pro-inflammatory cytokines.
Results: Our results suggest that Valacyclovir had anti-inflammatory activity on the LPS-activated mammalian macrophages.
Conclusion: Valacyclovir has the potential to be utilized in the clinical setting as an anti-viral drug molecule with anti-inflammatory properties. Future studies are needed to further confirm its activities on different immune system cell types.

Keywords: anti-inflammatory activity; immunomodulatory activity; valacyclovir; macrophages; inflammation

Introduction

Acyclovir, also known as aciclovir, is a nucleoside antiviral drug, which targets DNA viruses in the herpes family. The active form of acyclovir is aciclovir triphosphate. Aciclovir triphosphate inhibits herpesvirus DNA polymerase, thus terminating viral DNA chain elongation, and stopping the viral DNA replication. Valaciclovir is an oral prodrug which is the L-valyl ester of aciclovir (acyclovir) [1]. Through rapid metabolization, it produces both L-valine and acyclovir which is the main antiviral ingredient. Several studies demonstrated strong *in vitro* action of valaciclovir against the herpes family viruses including varicella-zoster virus, herpes simplex virus (HSV)-2, and HSV-I in immunocompetent patients [1].

Anti-viral drugs can display high toxicity due to their interference with the host cell's functions. Numerous antiviral agents have teratogenic, carcinogenic, and immunosuppressive side effects in addition to acute toxicities such as bone marrow suppression, and thus these antiviral agents may be administered topically to reduce systemic toxicity [2]. When the host immune system functions normally, antiviral medications have a higher potential to exert their effects. Various approaches to fighting human viral infections combine treatments with immunomodulatory and antiviral medications [3].

In certain viral infections, the host's immune response appears to be abnormally weak or underactive such as the case with shingles outbreaks caused by the varicella-zoster virus. As a result, an immunosuppressant may worsen these diseases [4]. In numerous other instances, the host immune response can be excessively weak such as the influenza virus response in elderly individuals who have been vaccinated against influenza [5]. Other side effects of antiviral agents used in clinical practice such as ganciclovir, valganciclovir, and acyclovir include leukopenia, a condition resulting from an insufficient number of leukocytes. These chemicals were initially developed as prospective anticancer medications but later repurposed as antiviral agents due to their relatively suboptimal anticancer activity. However, studies investigating the effects of these antivirals on the immune system are very limited. Treatment with low doses of acyclovir has the ability to modify certain aspects of the T cell response to cytomegalovirus (CMV) [6]. Another study suggested that effective defense against HSV-1 replication in human macrophages and partial protection against the HSV-1 thymidine kinase-deficient strain are offered by encapsulated acyclovir in erythrocytes. As a result, when encapsulated acyclovir inhibits HSV-1 [7]. Due to the scarcity of studies on the effect of acyclovir as an immunostimulatory agent, we tested the immunostimulatory activity of Valacyclovir on non-activated mammalian macrophages along with its immunomodulatory activity on the lipopolysaccharide (LPS) activated macrophages.

Macrophages are the primary inflammatory cells of the immune system [8]. They can produce pro-inflammatory cytokines, but in some circumstances, they can also release anti-inflammatory cytokines and speed up the healing process of wounds [9]. They are essential candidates for understanding the immunostimulatory and immunomodulatory actions of the drug compounds in vitro due to their high capacity to produce cytokines and display antigens.

To our knowledge, this is the first study that will measure pro-inflammatory tumor necrosis factor-α (TNF-α), IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-12p40 cytokines' production levels by the macrophages in the presence of Valacyclovir. These pathways are essential for the processes involved in inflammation, therefore analysis of the differential immunostimulatory and immunomodulatory activities of the mammalian macrophages is important.

Materials and Methods

Cell Culture Work and Drug Preparation

Valacyclovir Hydrochloride (BP1194) was purchased from Sigma-Aldrich (Virginia Beach, VA, USA). Mammalian macrophage cells, J774A.1 (TIB-67, ATCC, Manassas, VA, USA) were obtained from ATCC and cultured under BSL2 aseptic conditions. Valacyclovir was resuspended in 1 mL sterile tissue culture grade dimethylsulfoxide (DMSO) (5.89569, Sigma-Aldrich) in 10 mg/mL concentration. All the cell lines were mycoplasma-free and the short tandem repeat (STR) analyses were performed.

Drug Administration and Viability Assay

The macrophages were grown in complete Roswell Park Memorial Institute 1640 (RPMI 1640) (R8758, Sigma-Aldrich) media that contains 10% fetal bovine serum (F7524, Sigma-Aldrich) with 1% antibiotics (100 µg/cm^3 penicillin and 100 µg/cm^3 streptomycin) (P4333, Sigma-Aldrich) [10,11]. The 24 well plates had 10^6 cells/1 mL in each well. The cells were incubated in a 37 °C 5% CO_2 humidified chamber and treated with the valacyclovir at concentrations of 1, 5, 10 and 20 µg/mL with or without 1 µg/mL of LPS (L5293, Sigma-Aldrich) for 24 hours [12]. Cells not treated with the compound nor LPS were designated as an untreated control group. The negative control group contained 10 µg/mL of Salicylic acid (247588, Sigma-Aldrich) along with 1 ug/mL of LPS and the positive control group contained 1 µg/mL of LPS only. The cells were stained with Trypan blue (93595, Sigma-Aldrich) and counted to determine cell viability.

Testing the Immunostimulatory and Immunomodulatory Activities

After incubation with Vacyclovir at different concentrations with or without 1 µg/mL of LPS for 24 hours, the supernatants were collected and analyzed to detect and quantify proteins including TNF-α (Cat no: 555212), IL-6 (Cat no: 555183), GM-CSF (Cat no: 555126) and IL-12p40 (Cat no: 555220) using enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol.

Statistical Analyses

Student *t-test* was performed using Graph Pad Prism Version V (Boston, MA, USA) and each data set had the results of at least three biologically independent experiments [10,11].

Results

Valacyclovir was Used at Non-Cytotoxic Concentrations

Cell viability was evaluated using Trypan blue assay after 24 hours of incubation with different concentrations of valacyclovir in the presence (Fig. 1A) and absence of LPS (Fig. 1B). According to the results, there was no difference in cell viability between the control and drug-treated groups, which suggest that these drug compounds were used at non-cytotoxic concentrations on the macrophages.

Valacyclovir was a Strong Anti-Inflammatory Compound

We tested the LPS immunostimulatory activity on macrophages in the presence of valacyclovir. Mammalian macrophages were treated with valacyclovir at doses of 1, 5, 10, and 20 µg/mL with and without LPS. The production levels of the cytokines TNF-α, IL-6, GM-CSF, and IL-12p40 were subsequently assessed by ELISA [13]. Our findings indicate that valacyclovir had no immunostimulatory action because the macrophages did not produce pro-inflammatory TNF-α (Fig. 2), IL-6 (Fig. 3), GM-CSF (Fig. 4), and IL-12p40 (Fig. 5) when valacyclovir was present alone in the absence of LPS. Thus, valacyclovir is not expected to activate macrophages and hence the immune system in the absence of any immunostimulating agent. However, in the presence of LPS, valacyclovir substantially reduced the production levels of pro-inflammatory cytokines in a concentration-dependent manner suggesting it could be a potent anti-inflammatory drug.

Discussion

The antiviral agent acyclovir has efficacy against the herpes simplex virus for types 1 and 2, varicella-zoster virus, Epstein-Barr virus, and, to a lesser extent, cytomegalovirus. In general, acyclovir is quite well tolerated with few exceptions in which the ophthalmic adverse reactions are reported infrequently. However, it is challenging to determine whether these reactions are related to the medication or the underlying illness. Topical therapy only causes burning or stinging when applied, and in a small percentage

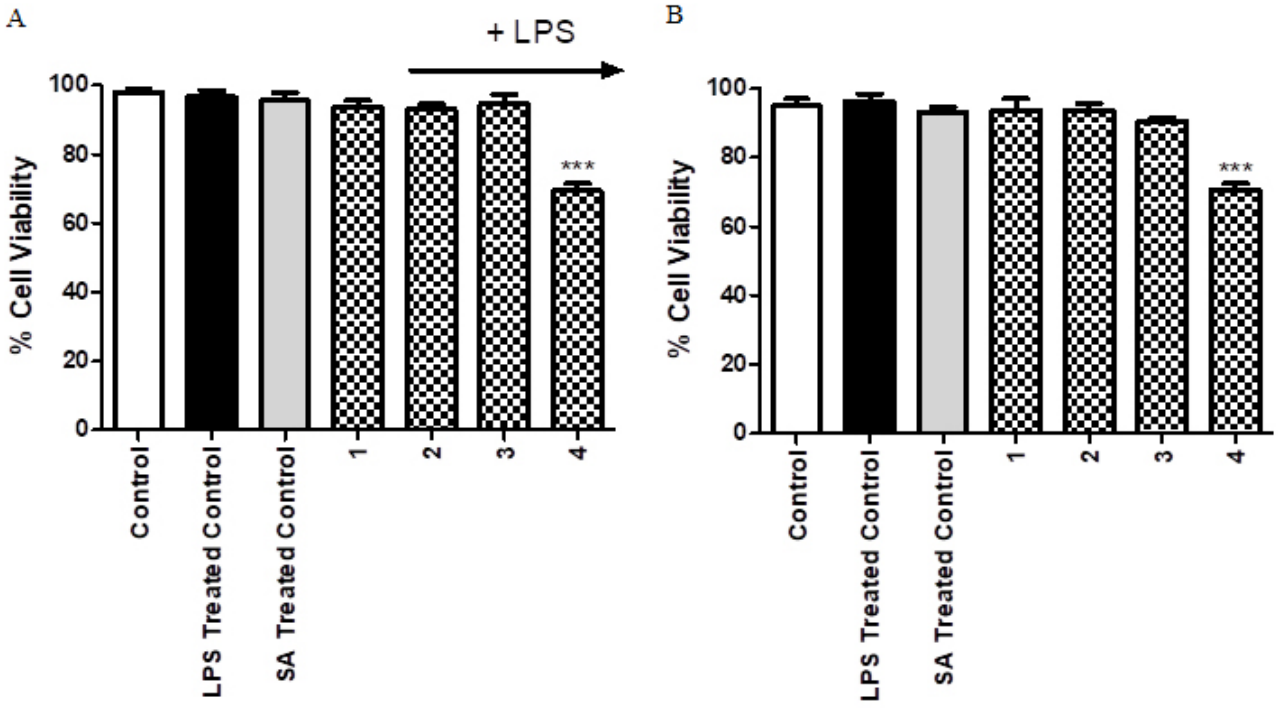

Fig. 1. The viability of lipopolysaccharide (LPS) stimulated (A) and unstimulated (B) mammalian macrophage cells stained by Trypan blue cells after the 24-hour incubation step. (*** $p < 0.0001$, N = 3). The cell counting was done in the presence of 1 (1), 5 (2), 10 (3) and 20 (4) µg/mL valacyclovir.

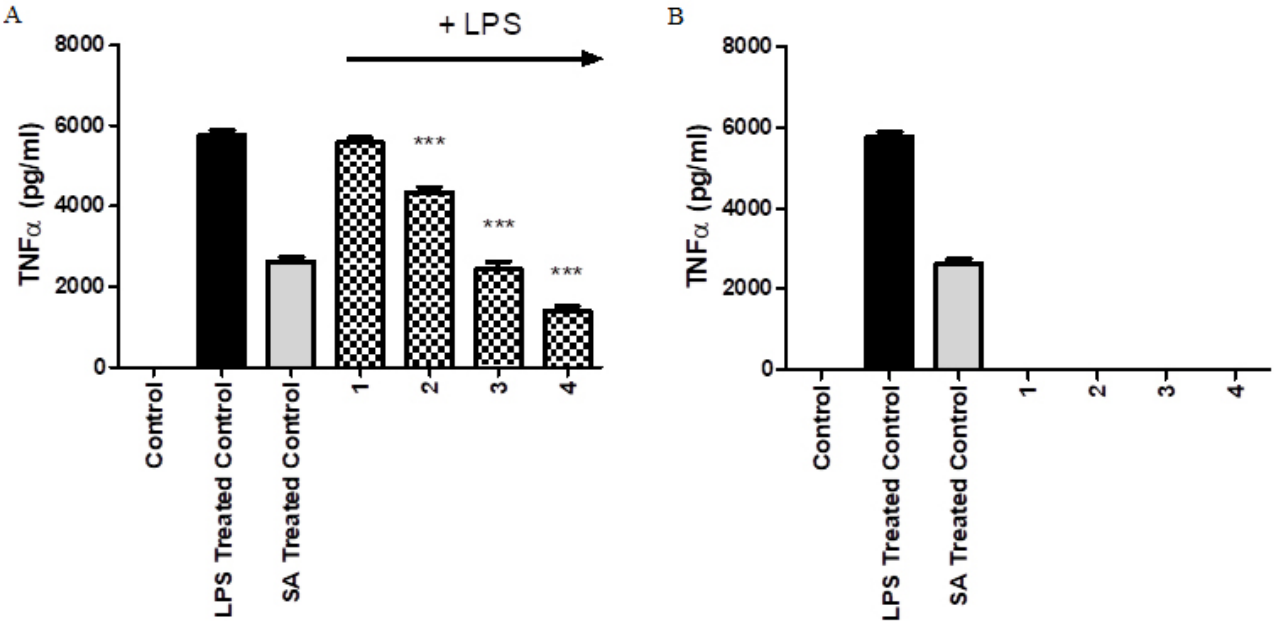

Fig. 2. Differential production levels of tumor necrosis factor-α (TNF-α). (A) Levels of TNF-α produced by macrophages during stimulation with LPS in the presence of 1 (1), 5 (2), 10 (3) and 20 (4) µg/mL valacyclovir. (B) Levels of TNF-α produced by macrophages when not stimulated with LPS in the presence of a range of drug molecule. (*** $p < 0.0001$, N = 3).

of patients, mild erythema or dryness. Inflammation and injection-site phlebitis are the side effects of intravenous acyclovir that are most commonly observed. The antiviral activity of acyclovir is comparable to that of other similar medications against herpes simplex virus. Viral thymidine kinase specifically phosphorylates them, producing the corresponding triphosphate, which competes with natural deoxyguanosine triphosphate (dGTP) to bind to viral DNA

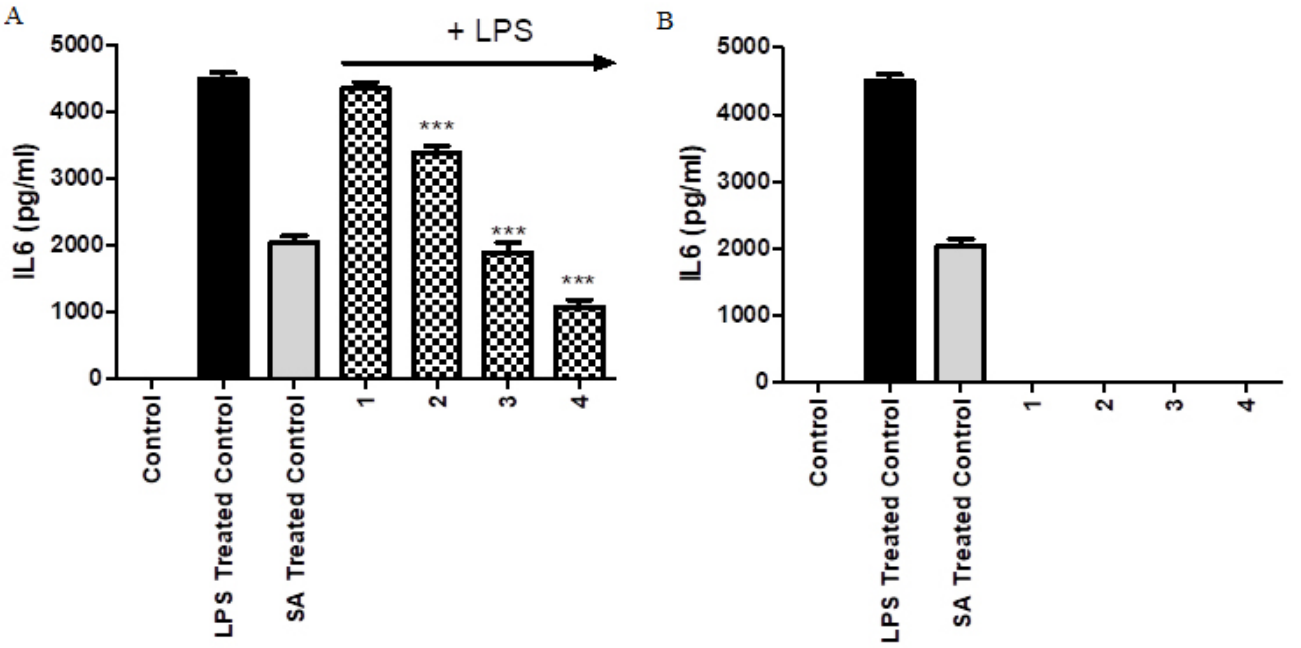

Fig. 3. Differential production levels of interleukin 6 (IL-6). (A) Levels of IL-6 produced by macrophages during stimulation with LPS in the presence of 1 (1), 5 (2), 10 (3) and 20 (4) µg/mL vasiclovir. (B) Levels of IL-6 produced by macrophages when not stimulated with LPS in the presence of a range of drug molecule. (*** $p < 0.0001$, N = 3).

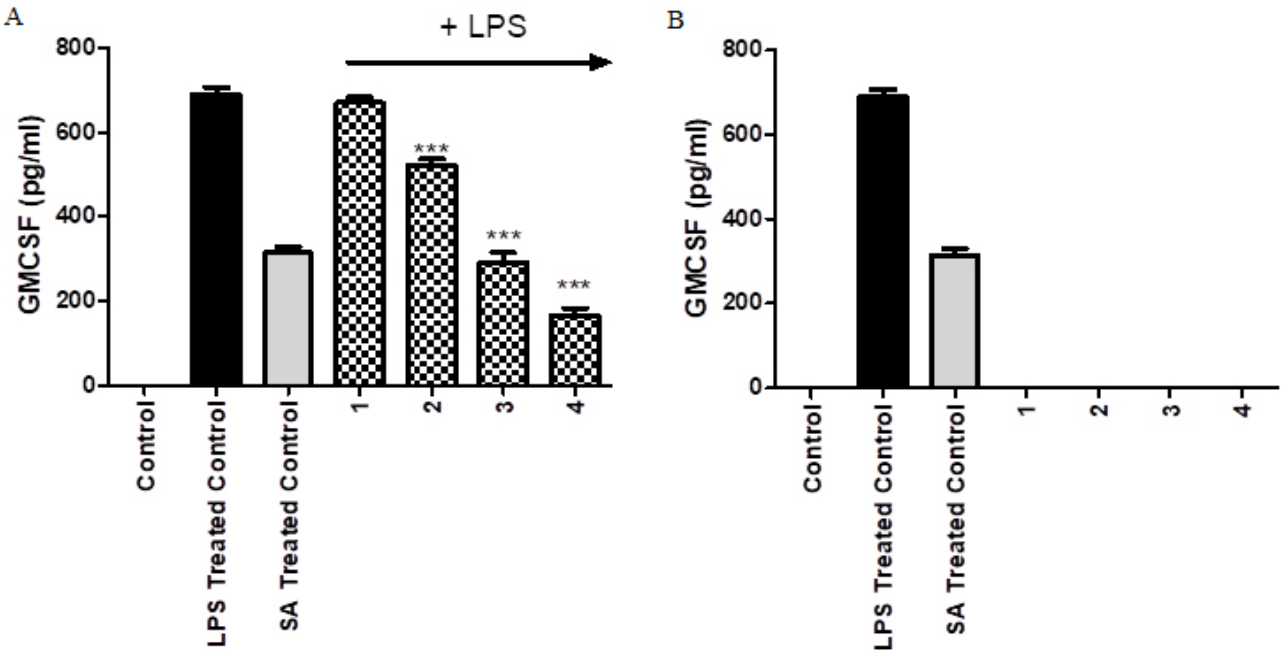

Fig. 4. Differential production levels of granulocyte-macrophage colony-stimulating factor (GM-CSF). (A) Levels of GM-CSF produced by macrophages during stimulation with LPS in the presence of 1 (1), 5 (2), 10 (3) and 20 (4) µg/mL vasiclovir. (B) Levels of GM-CSF produced by macrophages when not stimulated with LPS in the presence of a range of drug molecule. (*** $p < 0.0001$, N = 3).

polymerase. Acyclovir and analog medications are then added to the expanding DNA chain, which stops the expansion of DNA strands [14].

Antiretroviral therapy (ART) is the use of anti-human immunodeficiency virus (HIV) medications to treat individuals infected with HIV, but it is accompanied by side effects. Immune restoration disease (IRD) is one such side effect of ART, which happens when the immune system's regain of functionality is linked to an associated increase in inflammation, the discovery of underlying in-

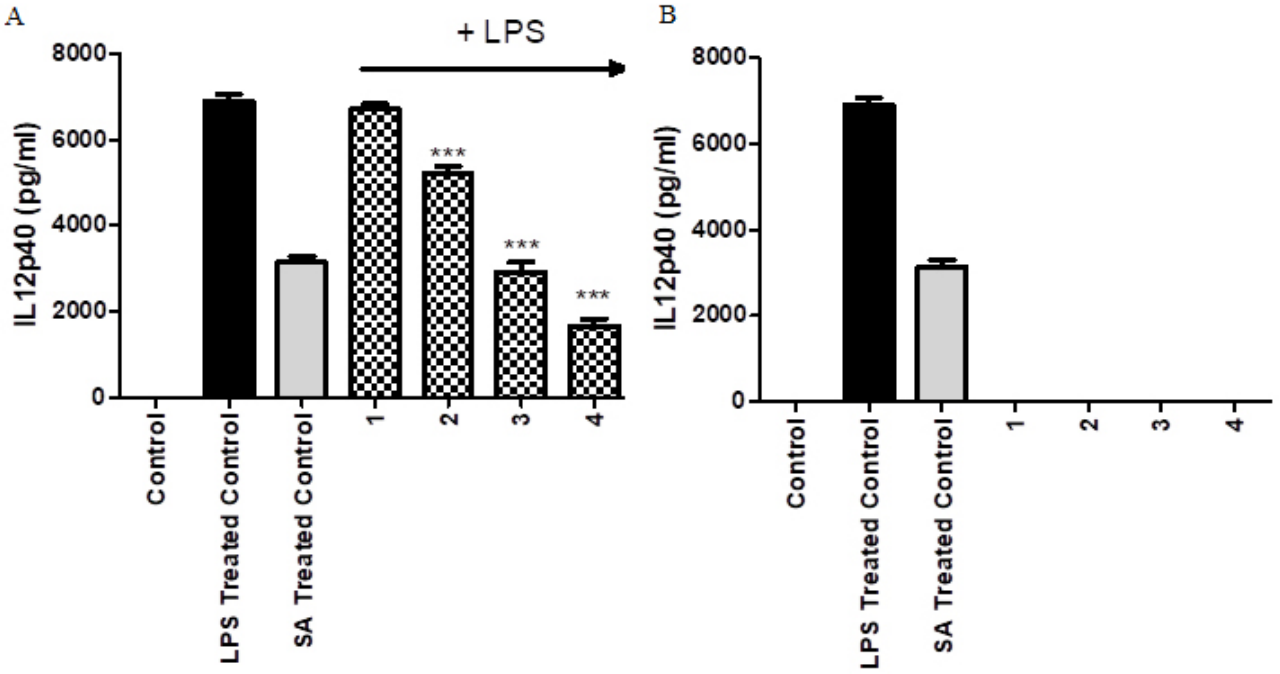

Fig. 5. Differential production levels of IL-12p40. (A) Levels of IL-12p40 produced by macrophages during stimulation with LPS in the presence of 1 (1), 5 (2), 10 (3) and 20 (4) µg/mL vasiclovir. (B) Levels of IL-12p40 produced by macrophages when not stimulated with LPS in the presence of a range of drug molecule. (*** $p < 0.0001$, N = 3).

fectious agents, and the potential reactivation of latent viral diseases [15–17]. There is an approximately 5-fold increased risk of varicella-zoster virus infection reactivation, increased CMV retinitis, and rare cases of herpes simplex virus (HSV)-associated encephalitis in the first 3–4 months at the start of ART administration [18]. Immune reconstitution inflammatory syndrome (IRIS), the most severe type of IRD, is defined by a significant deterioration in clinical status even in the presence of diminishing virus loads. Increased levels of proinflammatory cytokines, specifically the production of IL-6, are linked to tuberculosis and IRIS and found to be a predictive marker for IRD [16,19]. In our study, we found that upon treatment with valacyclovir (an ART agent), the levels of IL-6 dropped significantly indicating valacyclovir has anti-inflammatory properties. A recent study by Nason *et al.* [20] sought to find the link between the increased production of cytokines, including TNF-α and IL-6 and the reactivation of CMV and herpes simplex 2, however, the link was not determined. Therefore, it is important to study the effect of valacyclovir on the production of these pro-inflammatory cytokines. According to our results, valacyclovir exerted an anti-inflammatory effect since the levels of these cytokines were reduced.

GM-CSF is a crucial growth factor and a pro-inflammatory cytokine that is produced by activated T cells and macrophages, among other immune cells. Critical processes that aid the immune system in combating infections are facilitated by the GM-CSF signaling pathways. Specifically, GM-CSF induces macrophages to become activated to an M1-like pro-inflammatory phenotype *in vitro* [21,22]. This results in the production of chemokines for leukocyte recruitment [23] such as C-C motif chemokine ligand-2 (CCL2), CCL24, CCL5, and CCL1, and cytokines for pro-inflammatory actions upon stimulation such as TNF-α, IL-6, IL-12p70, IL-23, and IL-1β [24].

GM-CSF improves innate antiviral immunity by increasing cell viability, maturation of the other immune cells, and antigen presentation capacity. Although research has been done on the antiviral properties of GM-CSF against respiratory viruses such as influenza virus and respiratory syncytial virus (RSV), the anti-inflammatory effects of antiviral treatments remain to be clarified. Moreover, the excessive inflammation created by GM-CSF production might hinder tissue function [25]. In this study, we showed that when stimulated with an agent like LPS, the levels of GM-CSF were decreased under the influence of valacyclovir suggesting an anti-inflammatory effect of this drug. Valacyclovir did not exhibit an immunostimulatory effect in the absence of LPS. Additionally, the drug behaved like an immunomodulatory agent in the presence of an immunostimulant agent such as LPS.

Although our results suggest that valacyclovir has anti-inflammatory activities on the activated mammalian macrophages, there are some limitations to our studies. First, more immune cell types, including the primary cells, should be tested to further support our results. Moreover, this drug molecule should be tested on animal models of

inflammation to assess its immunomodulatory activity *in vivo*. Furthermore, intracellular signaling pathways can be analyzed both at protein and gene expression levels to decipher the molecular mechanism of its immunomodulatory activity.

Conclusion

Acyclovir, an antiviral drug used in the clinical setting for many years, is one of the most effective medications against viruses including herpes simplex. However, the anti-inflammatory effects of the drug are not well known. In this study, we showed that, when immune cells such as macrophages are treated with acyclovir, no cytokines were produced in the absence of an immunostimulant. However, when the cells were stimulated with LPS, acyclovir showed an anti-inflammatory effect. In the clinical setting, this information can be useful to prevent the overuse of anti-inflammatory molecules during anti-viral treatment with acyclovir.

Availability of Data and Materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

EA conceptualized the study, conducted the experiments, analyzed the data, wrote the manuscript, read and approved the final version of the manuscript, and agreed to be accountable for all aspects of this work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The author declares no conflict of interest.

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