

Immunomodulatory potential and clinical efficacy of Allostatine against

Type 1 and 2 Herpes Simplex Virus (HSV) lesions

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It takes a fool to remain sane

- The Ark

In memory of Professor Sergey Ivanovich Chernysh (1951-2020), inventor of Allostatine, great scientist and good friend. A life spent to fight Viruses and Bacteria, killed by human idiocy while crossing the road.

Abstract

Allostatine is a peptide deriving from the Alloferon peptides, a “family of first line defense molecules” used by insects to fight infections. Allostatine has been reported to be particularly attractive as a potent antiviral and as a possible immunomodulatory adjuvant in vaccines, such as therapeutic cancer vaccines. To evaluate the ability of Allostatine to facilitate the killing a wide range of virus-infected target cells, as a result of triggering human natural killer (NK) and T lymphocyte (CTL), we assessed the effects of this peptide on both murine and human immune responses. In addition, in order to investigate Allostatine’s capabilities to modulate the immune system during HSV pathogenesis, clinical tests were carried on patients affected by skin and genital herpes resulting from HSV type 1 and type 2 infections. Some of these patients were experiencing relapsing herpes lesions for more than 4 years before the study. The efficacy in the treatment of cold sores and the elimination of genital herpes symptoms were evaluated by comparing to Acyclovir treatment.

For the first time we report that Allostatine significantly enhances both NK and CTL activities as well as macrophage functions by increasing nitric oxide (NO) release in mice. We also demonstrate that this peptide is selectively localized in the leukocyte cytoplasm and that it increases the expression of human NK markers NKp80, CD244, CD226 and CD25. Interestingly, we also show the peptide’s ability to increase IL-2-triggered and IFN- γ synthesis of CD3+/CD28+ activated T cells.

Our clinical test results provide evidence that Allostatine influences human T-lymphocytes activation markers. Allostatine also facilitates the full lesion recovery of HSV affected patients. A clear improvement of relapsing herpes lesions was observed at different anatomical locations in the majority of patients treated with the Allostatine-based product Allomedin. No adverse or toxic effect was observed.

Thus, we observed in murine and human *in vitro*, *ex-vivo* and *clinical* studies that Allostatine may be useful for modulating NK and T cell immune functions and promoting macrophage activities. In a clinical study a formulation based on Allostatine beneficially counteracted HSV-induced pathological features and relapses.

Introduction

Herpes Simplex Virus (HSV) is a commonly found human pathogen. An estimated 4.0 billion people are infected with Herpes Simplex Virus globally. The incidence rate of cold sores caused by the HSV1 virus, is the second largest worldwide, trailing only behind common cold. About 90% of the world population is infected with HSV1, with about 25-35% of the adults enduring recurrent spate of cold sores [Wald and Corey, 2007]. Genital herpes, an infection caused by HSV2, is one of the most common sexually transmitted diseases in the US [McQuillan et al., 2018]. The rate of occurrence of genital herpes varies across different countries in developing regions. Certain African nations dealing with the HIV epidemic are experiencing a high prevalence of HSV2 infection. The majority of people (approximately 80%) display no or very mild HSV2 symptoms that are easy to miss. In fact, the most common way to catch either strain of HSV is from someone who sheds the virus without any overt evidence of infection. This infection, however, can be dangerous under certain circumstances. If passed from a mother to a newborn, HSV2 can be fatal. In rare cases HSV1 can infect the brain, causing encephalitis, which is usually fatal; a growing body of facts shows that chronic brain infection with HSV1 makes a great contribution to Alzheimer's disease [Carter, C.J., 2008]. Furthermore, genital herpes also increases the risk of HIV infection by 40% [Looker et al, 2020]. According to WHO assessment, genital herpes is a cause of 10% of all suicides worldwide [Nissen et al., 2019]. HSV infection is lifelong and daily medication can be burdensome.

There is no drug presently available in the market that can permanently cure herpes infection, however, various anti-viral therapies are available to alleviate painful symptoms of the infection. Present therapies for Herpes infection include nucleoside analogs such as acyclovir, and other related drugs. These drugs act by stalling the synthesis of viral DNA. Major drugs available in the market include Zovirax, Famvir, Abreva, Denavir, and Vistide. However, these drugs are known to cause drug-related toxicity as a result of their interference with the natural metabolism of the human body. Also, with the virus subsequently developing resistance against the existing array of anti-virals, the need for innovative therapies for the treatment of herpes simplex infection, and the search for novel antiviral drugs continues. Therefore, there is a large potential for the herpes simplex therapeutics market in future.

NKs, CTL and macrophages are the major protective weapons used by the immune system to fight HSV1 and HSV2 virus infections; NK cells produce interferon- γ (IFN- γ) and promote IFN- γ production via T cells in response to many viral infections.

Alloferons were isolated from bacteria-challenged larvae of the blow fly *Calliphora vicina* and constitute a group of natural occurring peptides with strong antiviral and antitumoral abilities [Chernysh et al., 2002; Chernysh et al., 2012; Chernysh et al., 2013]. Along with other surgical maggots, *Calliphora v.* has a long history of medical use in wound and ulcer healing [Kruglikova A.A: & Chernysh S.I., 2013], with a first documented application back in the XVI Century by the French surgeon Ambroise Paré (1510-1590) [Whitaker et al., 2007]. *Calliphora* is well known to produce a series of potent antimicrobial substances after being challenged by bacterial infection. These antimicrobial peptides (AMPs) have primary structures similar to those described for other insects, such as insect defensin, diptericins, cecropins, and proline-rich peptides [Chernysh et al., 2002]. *Calliphora's* hemolymph possess a cytotoxic activity similar to the one of human NKs [Chernysh et al., 2004]. Particularly, two peptides of 13 and 12 amino acids have been identified in *Calliphora's* hemolymph which have been named Alloferon1 and Alloferon2, respectively [Chernysh et al., 2002]. Administration of a picomolar concentration of Alloferon1 to mice turned out into the stimulation of natural cytotoxicity as well as into antiviral and antitumoral activities *in vivo* [Chernysh et al., 2002; Chernysh et al., 2012].

Allostatine (also known as Alloferon3) is an “humanized” peptide which originated from rationally designed modifications of Alloferon1. By substituting two amino acids within the Alloferon1 sequence a pattern similar to human immunoglobulins was found. Allostatine-induced antiviral and antitumoral abilities have been demonstrated *in vitro* and *in vivo*. Particularly, the properties of Allostatine to act as a therapeutic cancer vaccine adjuvant has been demonstrated *in vivo* in DBA/2 mice vaccinated together with tumor antigen [Chernysh and Kozuharova, 2013].

Here, we present results on Allostatine efficacy against human HSV (cold sores and genital herpes) in patients.

Materials and Methods

Peptide

Diapharm Co, St. Petersburg has synthesized Allostatine, using the synthesis of solid phase and according to Fmoc/But strategy and purified by reverse phase HPLC with more than 98% as peptide final purity. It is a 13 amino acid linear peptide sequence His–Gly–Val–Ser–Gly–Trp–Gly–Gln–His–Gly–Thr–His–Gly and its empiric formula is C₅₆H₇₇N₂₁O₁₇ and its molecular mass is 1316 Da.

Allomedin® is a gel formulation product based on Allostatine, developed by the Company Allopharm Ltd., created by Prof. Sergey Ivanovich Chernysh. This compound is composed of Allostatine and water, carbopol, allantoin, phenyethanol, ethylhexyglyceol, sodium hydroxide as excipients, and it was developed to protect skin and mucous tunic against viral infections and registered in Russia and Countries belonging to the former USSR, as a skin care product.

Ex-vivo experiments

Animals

All experiments on Balb/c mice (22–26 g) were performed in accordance with the Council of the European Communities (86/609/EEC; November 24th 1986). Directives regulating the welfare of experimental animals, and experiments were approved by the Life Sciences and Health Research Ethics Committee (cer-svs) of the Institute of Biotechnology (University of Monastir, Tunisia; ethical approval no. 2019/02/I/CER-SVS/ISBM; 9 January, 2019). In an accredited pathogen-free facility mice were housed under standard conditions. All the animals were provided ad libitum access to filtered water and standard rodent chow.

Isolation and culture of macrophages and splenocytes

Mice were sacrificed by cervical dislocation and primary splenocytes were extracted under sterile conditions, immersed in phosphate buffered saline (PBS, pH 7.4) (Gibco, BRL) and gently dissected with forceps. After exposure to lysis buffer (144 mM NH₄Cl, 1.7 mM Tris Base) to remove red blood cells, the cells were resuspended in complete RPMI-1640 medium (Gibco, BRL) containing 10% fetal bovine serum (FBS; Gibco), 1% penicillin (10,000 U/ml) and streptomycin (10,000 µg/ml) (GibcoBRL, Paisley, UK) after two washing steps. The cells were incubated at 37 °C at 5 % enriched CO₂ atmosphere. Other Balb/c mice were used in order to obtain peritoneal macrophages after PBS intraperitoneal

lavage. The extracted cells were resuspended in complete RPMI 1640 medium after washing steps. Cell viability was determined using the trypan blue exclusion technique.

Activity of natural killer (NK) and the cell-mediated cytotoxicity assay

Spleens prepared as described above were used as the source of effector cells; isolated splenocytes were plated at a density of 5×10^5 cells for NK activity at a concentration of 5×10^6 cells/ml per well in 100 μ l aliquot/well. These cells were exposed and stimulated by different concentrations of Allostatine (5,10,20 and 40 ng/ml) for 24 h. Subsequently, 100 μ l of target K562 cells and B16-F10 cells (5×10^4 cells/ml; yielding a 100:1 expected effector-target ratio) were added to the wells respectively for NK and CTL activity. Medium with 1% DMSO was used as a negative control. The plates were then incubated for 4 h and 24 h at 37°C in 5% CO₂ atmosphere respectively for NK and CTL activity. Forty micro-liters of MTT solution was added to each well. After incubation, the supernatants were removed by centrifugation and 100 μ l of DMSO was added to each well. The absorbance was measured at 540 nm using a microplate reader.

Target cell control, blank control and effector cell control were performed. NK cell activity was calculated as follows: NK activity or CTL activity (%) = $100 \times (\text{ODT} - (\text{ODS} - \text{ODE})) / \text{ODT}$, where ODT = optical density value of target cells control, ODS = optical density value of test samples, and ODE = optical density value of effector cells control

In vitro phagocytic assay on cellular lysosomal enzyme activity and NO measurement production

The cellular lysosomal enzyme activity has been determined by acid phosphatase activity. Briefly, a volume of 100 μ l of allostatine at different concentrations (5,10,20 and 40 ng/ml) were added to 100 μ l of a macrophage suspension (3×10^6 cells/ml) in flat-bottom, 96-well plates at 37 °C in humidified 5% CO₂ for 48 h. The medium was discarded and the macrophage monolayer in each well was solubilized with 20 μ l of 0.1% Triton X-100 (Biomatik Corporation, Cambridge, UK). Next 10 μ l of 100 mM p-nitrophenylphosphate (p-NPP) (AppliChem, GmbH, Darmstadt, Germany) solution and 50 μ l of 0.1 M citrate buffer (pH 5.0) were added. The plate was further incubated for 30 min, 150 μ l of 0.2 M borate buffer (pH 9.8) was used to terminate the reaction and the absorbance at 405 nm was measured. The percentage activity was calculated according to the following equation:

Lysosomal enzyme activity (%) = (OD sample-OD negative control)/ OD negative control)× 100.

Nitrite concentration (NO₂) released by macrophages was determined using Griess reaction in cell culture supernatants. Briefly, 100 µl of supernatant from each sample was mixed with 100 µl of Griess reagent (equal volumes of 1 % sulfanilamide and 0.1 % naphthylethylenediamine dihydrochloride in 2.5 % phosphoric acid), and incubated at room temperature for 15 min. Absorbance was then measured at 540 nm using a microplate reader and compared with a standard curve of sodium nitrite

Purification of human peripheral blood mononuclear cells (PBMCs)

Venous peripheral blood samples were obtained after informed consent from healthy human volunteers between the ages of 18 and 60 years. Mononuclear cells were obtained using BD Vacutainer CPT (Cat. No. 362780) according to the protocol recommended by the manufacturer, and then transferred to the medium.

Evaluating of NK cell phenotype changing during cytotoxicity test

The mononuclear fraction was obtained from the venous blood of each donor using a cell preparation tube (362753 BD, USA) in accordance with the manufacturer's instructions. NK cells (CD3⁻ / CD16⁺ / CD56⁺) were isolated from the mononuclear fraction using magnetic separation (ThermoFisher 11349D Dynabeads™ Untouched™ Human NK Cells Kit) according to the manufacturer's protocol, resulting NK cell concentration was adjusted to > 90%. Using an automatic cell counter (BioRad), the number of NK cells obtained was determined. The isolated cells were cultured in RPMI 1640 medium containing 5% inactivated autologous serum and antibiotics (Sigma-Aldrich A5955 Antibiotic Antimycotic Solution 100×). A portion of NK cells was preincubated overnight in the allostatine-containing medium. NK cells were further mixed with target cells (K562), at a E:T ration of 1:10. In accordance with the experimental scheme, the resulting mixture was separated by volume and placed in the well of a U-shaped plate for the 18 hour incubation in a CO₂ thermostat at +37 °C. After incubation, changes in the expression of the following markers were evaluated: CD25 (Becton Dickinson: anti-Human CD25, 562442), NKp80 (Miltenyi Biotec: anti-Human NKp80-FITC,

130-094-843), CD 226 (BioLegend: anti-Human CD226_FITC 338303) and CD 244 (Miltenyi Biotec: anti-Human CD244 (2B4)-PE, 130-099-071).

Visualization of allostatine interaction with human leucocytes *in vitro*

10 µg / ml fluorescein-labeled allostatine was added to fresh human blood. After an hour of incubation at +37 °C, erythrocytes were lysed using BD FACS Lysing Solution (Cat. No. 349202) according to the manufacturer's recommendation. The remaining fraction leukocytes was washed with PBS, fixed with 2% paraformaldehyde in PBS, placed on glass and enclosed in Diamond Antifade Mountant with DAPI (Invitrogen™ ProLong™, Cat. No. P36966). The resulting preparations were analyzed with a Leica TCS SP5 confocal scanning microscope.

The effect of Allostatine on the production of cytokines by activated human T cells *in vitro*

Polyclonal activation of T cells was performed using beads coated with CD3 / CD28 antibodies (Thermo Fisher Scientific, Dynabeads Human T-Activator CD3 / CD28, Cat. No. 11131D), according to the manufacturer's recommendations. The response was evaluated by the production of IFN-γ and IL-2 cytokines by cells. For detection of cytokine producing cells, the protocol recommended by Becton Dickinson (BD) was used, IFN-γ was stained using BD Fast Immune Anti-Human IFN-γ-FITC, Cat. No. 340449, IL-2 - BD FastImmune Anti-Human IL-2-PE, Cat. No. 340,450.

Allostatine-mediated immunostimulation and study design for clinical trials

Ethic statement

The Permission for carrying out the Allostatine clinical trials No. 544 was received from Russian Ministry of Health on November 18, 2008, and also the excerpt from the protocol No. 12 of the meeting of the Ethics Committee was received in July 15, 2008 (Moscow, Russia). Patients were screened before the study. If the Patient met the inclusion / exclusion criteria, he was included in the study, given unique blind-coded patient identification number (UINP) and randomized. All the selected patients signed an informed consent to agree to participate in the experience of clinical use.

The effect of subcutaneous injection of Allostatine on human T-lymphocytes

In order to determine the response of the whole T-cell population, healthy volunteers, after informed consent, received a subcutaneous injection of 1 mg Allostatine. Immediately prior to injection, 12 hours and 5 days after injection, the proportion of T cells (Miltenyi Biotec VioBlue labeled anti-Human CD3, Cat. No. 130-098-165) in peripheral blood expressing CD25 (with BD BV605 labeled anti-Human CD25, Cat. No. 562661) and HLA-DR (with BD APC labeled anti-Human HLA-DR, Cat. No. 560744) activation markers were determined.

Clinical study on patients suffering from Herpes simplex virus

Allostatine (Allomedin gel) safety and therapeutic efficacy in patients experienced relapsing herpes lesions

The clinical study characterizing the safety and therapeutic efficacy of Allomedin (a gel formulation based on Allostatine for topical use) was evaluated in 104 patients suffering from Herpes simplex virus (81 females, 23 males). The clinical effect of Allomedin was compared to treatment effect of acyclovir for cold sores and the elimination of genital herpes symptoms. All these patients experienced relapsing herpes lesions at different anatomical locations for more than 4 years before the study. The age of the patients was varied from 18 to 60 years.

Efficacy and safe use of Allostatin (Allomedin gel) in patients with chronic recurrent herpes simplex lesions.

We evaluated the efficacy (therapeutic equivalence) of topical application of Allomedin cosmetic gel for skin care. The degree of reduction of objective and subjective symptoms was studied in patients suffering from recurrent Herpes Simplex Virus lesions compared to the effectiveness of therapy with Zovirax, cream and Fenistil Pentsivir cream.

Also, we examined the safety, side effects and tolerability of local application of the “Allomedin” cosmetic gel in relieving Herpes Simplex Virus relapses by registering adverse reactions (AR) and serious adverse reactions (SAR).

This evaluation included 156 patients of both sexes (80 men and 76 women) from 18 to 80 years old (mean age 40.7 ± 2.1 years) with a clinically established diagnosis of chronic recurrent herpes simplex lesions.

Inclusion criteria

In a first study, patients of both sexes, ranging from 18-60 years of age, with a medical diagnosis of recurrent (>3 relapses in the past 4 years) herpes simplex lesions, clinically and in laboratory confirmed, were selected for the study.

For the second clinical evaluation, 156 patients aged from 18 to 80 years old, 80 men and 76 women, were selected. These patients were clinically diagnosed with chronic recurrent herpes simplex.

Exclusion criteria

Patients who refused to participate in the study and/or the ones who violated the offered protocol were excluded from the study. Excluded were also patients with somatic diseases in the stage of decompensation (Cardiovascular diseases, chronic pulmonary, renal and hepatic failure). Individuals reporting putative intolerance to the components of drugs were also not admitted (e.g., fly puncture allergies).

Alcoholism and drug addiction in the anamnesis, mental illness that does not allow an assessment of the effectiveness of therapy were considered as cause of exclusion as well as the presence of active tuberculosis, systemic connective tissue diseases, HIV infection, acute respiratory viral infections, acute and chronic hepatitis, acute bronchitis and pneumonia. Patients who previously used drugs with antiviral and immunomodulatory activity and women during pregnancy and lactation, as well as women with a wish to become pregnant were excluded. Also, patients who participated in another clinical trial in the last 3 months were not admitted.

Acceptable Concomitant Therapy:

Concomitant therapy for acute or chronic diseases could be continued in accordance with the doctor's appointments. Information about concomitant drugs (trade name, dosage or dosage changes, indication, start date, termination date) was noted in the Patient's Individual Registration Card (IRC).

Non-acceptable Concomitant Therapy:

Any other antiviral drugs (except Allomedin, Fenistil Pentsivir, Zovirax) were not allowed for use during the study.

At the start of the trial treatment (1st day) and on the 3rd, 5th, 8th, 10th day of treatment, complaints and the clinical picture of the disease (presence of subjective symptoms, localization of the process, size of lesions, presence of vesicles, hyperemia, edema, erosions, crusts), were evaluated. The data obtained were fixed in the Individual Registration Card (IRC) of the patient.

The severity of the sign (symptom) were evaluated on a 3-point verbal analogue scale as:

- 0 - no symptom,
- 1 - weak manifestation
- 2 – moderate, acceptable
- 3 - pronounced manifestation.

The treatment consisted of Allomedin application onto the surface of herpetic lesions 2 to 3 times a day for 3 to 5 days.

At the 5th (final) visit (on day 10), the Investigator and the Patient carried out a subjective assessment of the effectiveness of the treatment (good effect, satisfactory or unsatisfactory).

Evaluation of the effectiveness of the use of gel "Allomedin" and reference products was determined as follows:

- good effect - marked improvement: arresting the manifestations of the disease, the lack of new manifestations and relapses;
- satisfactory effect - incomplete relief of disease manifestations;
- unsatisfactory effect - lack of effect from Treatment or worsening of subjective and objective symptoms.

Safety assessment

During and at the end of the Trial, adverse reaction (AR) scores were collected, and estimated for the possible connection with Allomedin, and estimated for the possible connection with other drugs.

Assessment of severity of adverse events:

- Light - not progressive, causing minimal discomfort;
- Medium - significantly, but not completely violating the usual daily activity;
- Severe - reactions that completely or very significantly disrupt daily activity

The degree of possible reliability of the association of adverse reaction (AR) with the studied drugs was determined as follows:

- No connection - there is clear evidence of a lack of association of adverse events (AE) with the Allomedin
- Doubtful connection - there is no clear temporary connection with the use of the product, there are other factors (drugs, diseases, chemicals) that may cause them
- Possible connection - related in time with the use of the product, can be explained by the presence of associated diseases or taking other drugs or chemical compounds, reactions to withdrawal of the product and repeated use are unknown
- Probable connection — associated in time with the using drug, hardly ever to be associated with comorbidities or other factors, decreases withdrawal of the drug, the response to repeated using is not known.

Statistical analysis

Assays were performed three times as expressed in the procedure. All data are represented as mean \pm standard deviation. Graphs were drawn using GraphPad Prism® software (Graph-Pad Prism® 6.07, GraphPad Software, Inc., CA, USA). One-way analysis of variance was used to examine the differences between sets of data. A P-value less than 0.05 were considered as significant.

Results

Allostatine improves murine NK and CTL cells activities

Natural Killer cells (NKs) represent the first innate line of defense against many pathogens, while MHC-restricted cytolytic T cells (CTL) are required for specific pathogen eradication as an adaptive cellular response. The examination of cytotoxic activity of splenic NK and CTL cells against NK sensitive tumor cells (K562 line) and melanoma cells (B16-F10) respectively, showed that, compared with the control cells, Allostatine treatment was able to significantly enhance NK and CTL cells activities at a concentration of 10 and 20 ng/mL. Our results showed a maximum increase at 20 ng/mL (81.69%) of the Allostatine dose (Fig. 1A). Also, cells treated

with increasing doses of Allostatine displayed a significant dose-dependent cytotoxicity versus those incubated with B16-F10 target cells only. (Fig.2B) The highest activity (54.21%) was obtained with a dose of Allostatine of 20 ng/mL.

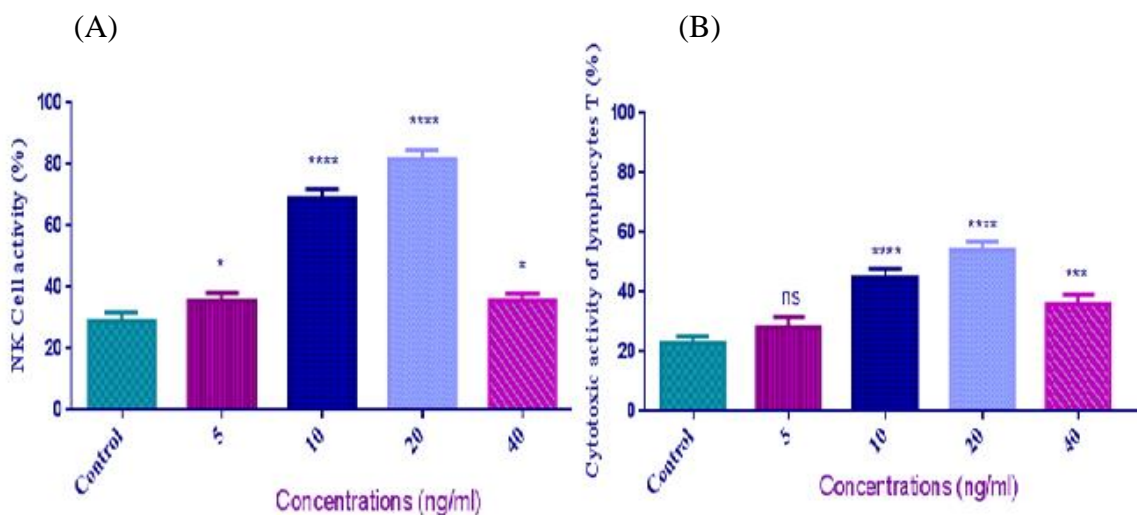


Fig. 1. *Ex-vivo* effect on Balb/c mice at different concentrations of Allostatine on (A) Natural Killer cells; (B) CTL After treating splenocytes for 24 h with increased doses of Allostatine , K562 and B16F10 target cells were added (at expected 100:1 effector-to-target ratio) respectively for NK and CTL activities. Data represent the mean \pm standard error of the mean of 3 independent experiments; by one-way ANOVA: *p < 0.05; **p < 0.01 and ***p < 0.001, ****P < 0.0001 compared to control cells treated with DMSO (1%),

Allostatine inhibits the cellular lysosomal enzyme activity and increases nitric oxide production

Peritoneal macrophages incubated with different concentrations of Allostatine ranging from 5 to 40 ng/ml for 48 h showed a cellular lysosomal enzyme activity inhibition in a dose-dependent manner. The most important inhibition was obtained at a 40 ng/ml concentration of Allostatine (Fig. 2A). In addition, the effects of Allostatine on nitric oxide (NO) production by macrophages in the presence of LPS revealed that Allostatin peptide dose-dependently induced NO production in macrophages, up to 49.3 μ M at the concentration of 40 ng/mL (Fig. 2B)

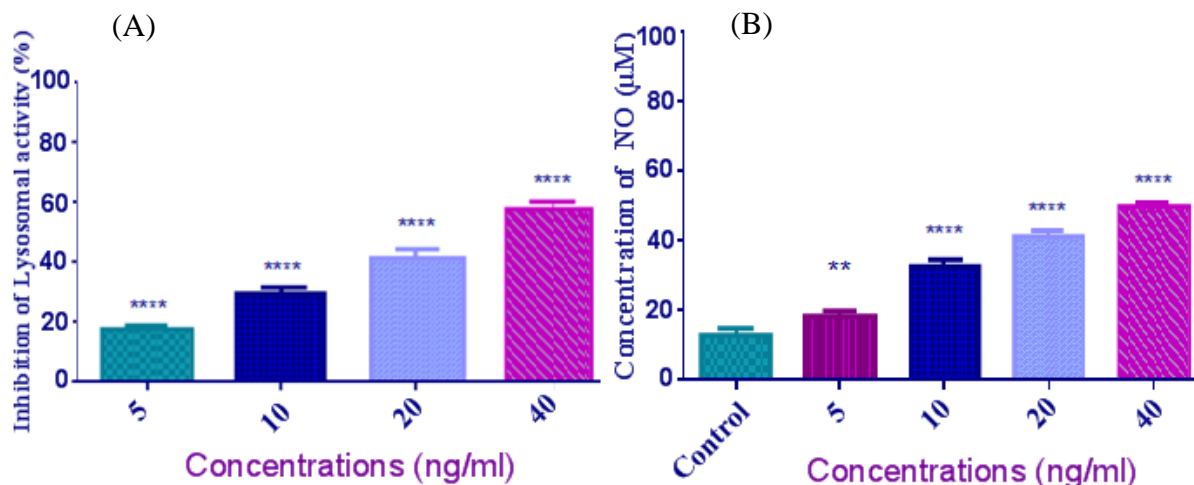


Fig. 2. Peritoneal Balb/c mice macrophage (A) Inhibition of lysosomal enzyme activity by Allostatine.(B) Production of the nitrite stimulated by lipopolysaccharide (LPS) (5 µg/mL) and Allostatine Data represent the mean \pm standard error of the mean of 3 independent experiments; * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$, **** $P < 0.0001$ compared to the control cells, cells treated with 1% DMSO. by one-way ANOVA.

NKp80 and CD244 expressions

NKs that have reacted with target cells decrease their expression of NKp80 and CD244 activation markers, thus turning into an inactive state. We analyzed how Allostatine affects the functional state of NK cells using a cytotoxic test performed with NK and K562 cells. Notably, in the presence of Allostatine, the number of inactive NK cells decreased 3-fold, suggesting that Allostatine uses the triggering of surface immunoreceptors CD244 and NKp80 to induce cytotoxicity (Fig. 3).

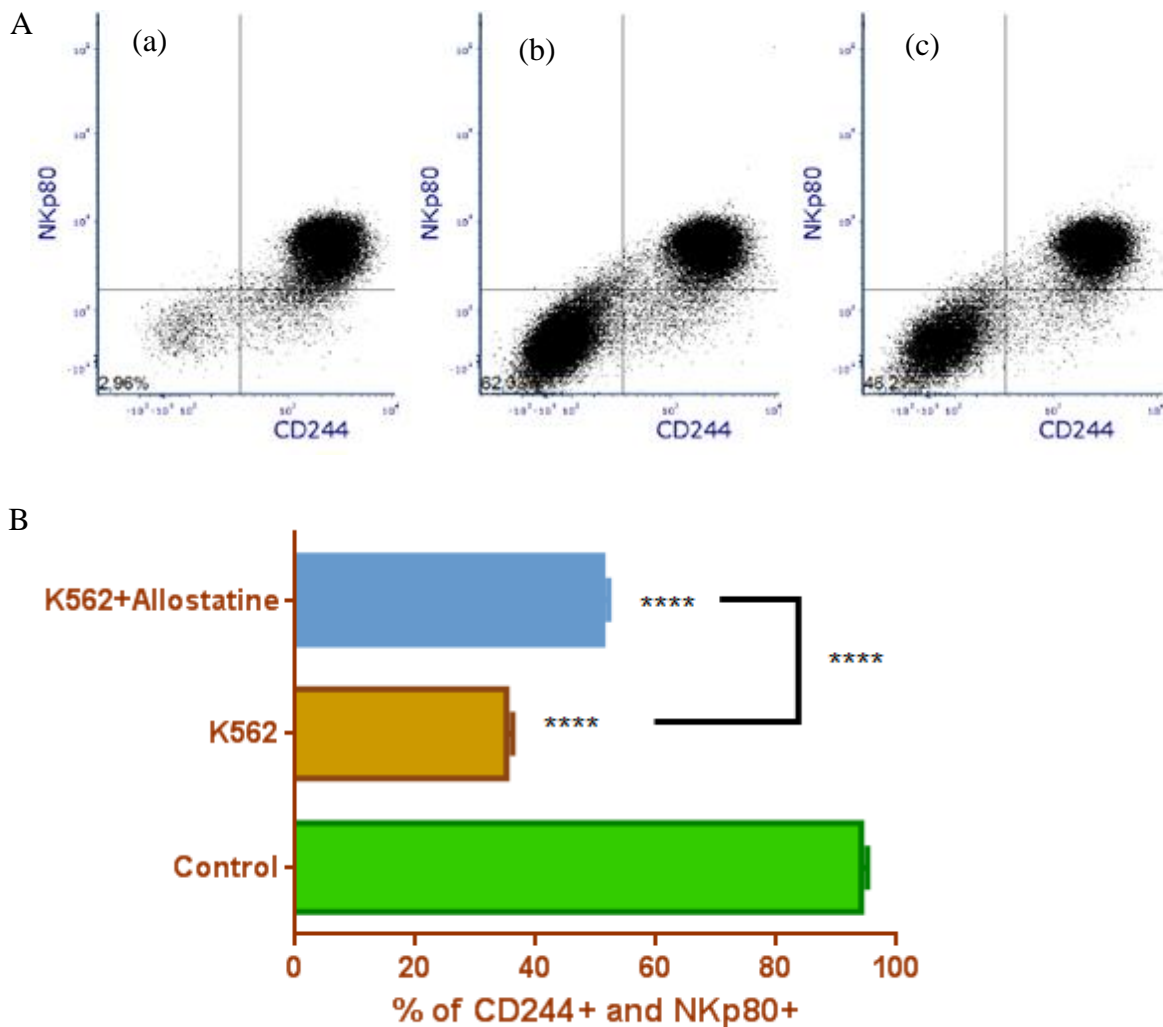


Figure 3. Changes in the expression of CD244 and NKp80 receptors by NK cells in cytotoxic test with tumor cells (K562) after pre-incubation with Allostatine (A) Representative flow cytometry dot plots of NK cells stained with NKp80 and CD244 antibodies (a) shows the initial expression level of activation markers in the absence of K562 cell target, (b) expression level of activation markers with target K562 cells, (c) expression level of activation markers with target K562 cells co-incubated with 10 μ g/ml of Allostatine.

The expression on activation markers CD244 and CD226

Another activating marker which maintains its expression after Allostatine treatment is CD226 (Fig. 4). Therefore, a possible Allostatine mode of action is by supporting the cytotoxic ability of NK cells. We evaluated the mechanism of action of Allostatine on the functional state of NK

cells. It was shown that the treatment with Allostatine increases the number of activated NKs carrying CD244 and CD226 activation markers when reacting to K562.

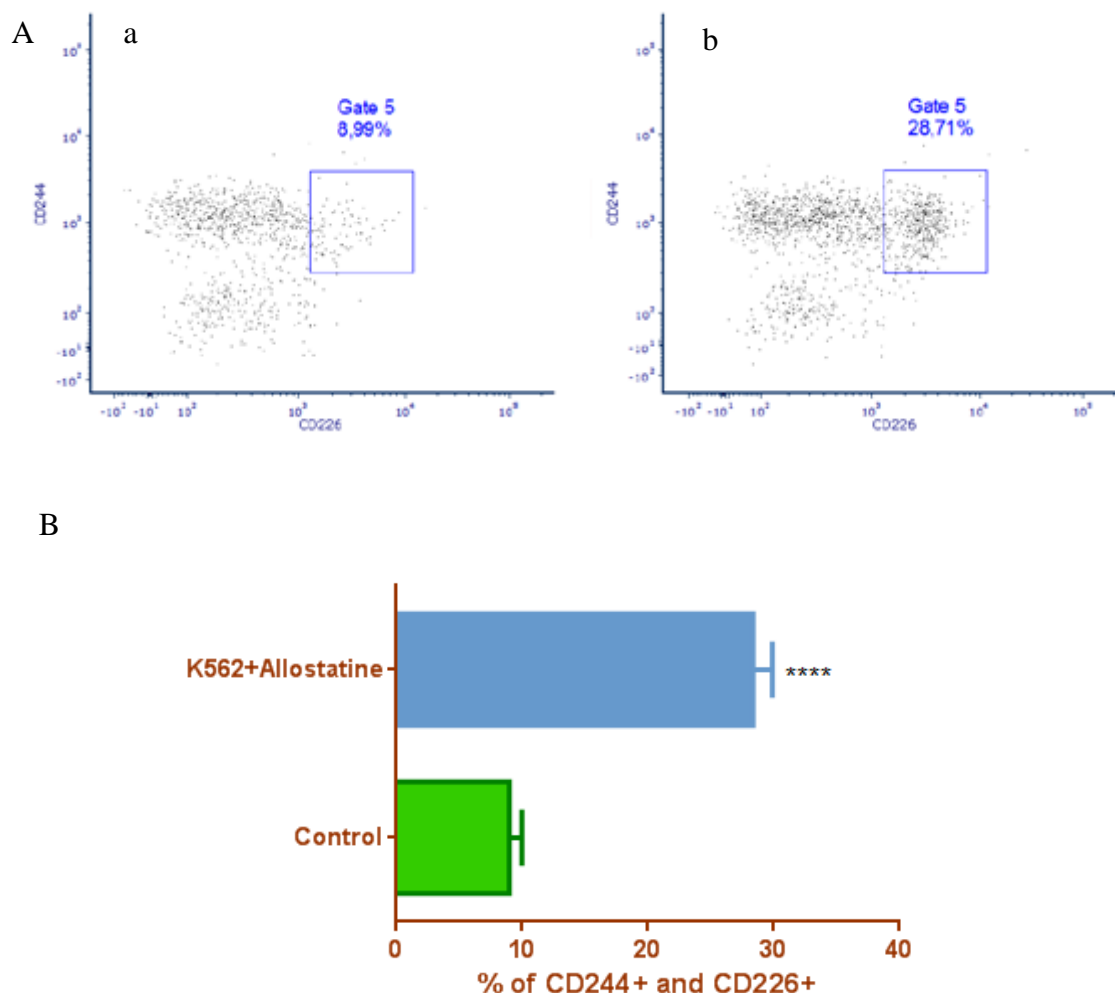


Figure 4. The effect of Allostatine on expression of activation markers CD244 and CD226 by NK cells during the cytotoxic reaction with K562 cells; (A) Representative flow cytometry dot plots of NK cells stained with CD244 and CD226 (a): control NK cells cultured with only K562 target cells, (c): NK cells with K562 target cells co-incubated with Allostatine. (B) Percentages of live CD244+ and CD226+ (n = 3 biological replicates) ,*p < 0.05; **p < 0.01 and ***p < 0.001, ****P < 0.0001 compared to the control cells by one-way ANOVA.

Induction of CD25 on Allostatine preactivated Human NK Cells

Assays for CD25 Expression

Beyond phenotype markers the interaction of NKs with Allostatine influences the expression of functional markers (Fig. 5). In particular, NK cells express higher level of CD25 marker (IL-2 receptor) which is crucially important for activation and survival of NK cells.

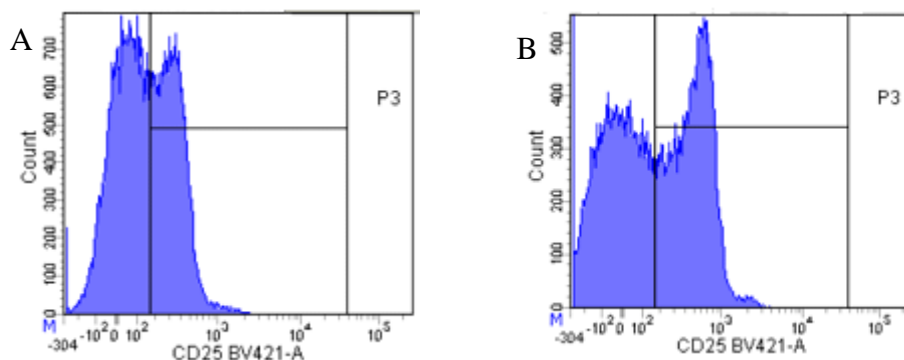


Figure 5. Representative flow histograms of Allostatine effect on the activation marker CD25 level on NK cells during the cytotoxic reaction with K562 cells. A: Control ; B: Treated with Allostatine (3 independent experiments).

Interaction of Allostatine with human leucocytes

In order to identify possible Allostatine-sensitive target cells, a fluorochrome-labeled peptide was added to whole blood, after which the distribution of the label in leukocytes was studied. One of the questions that should be clarified is where the peptide localizes and whether the peptide enters the nucleus. The analysis showed that human leukocytes selectively bind the labeled Allostatine (Fig. 6). In this case, the label is localized in the cytoplasm and does not penetrate the nucleus.

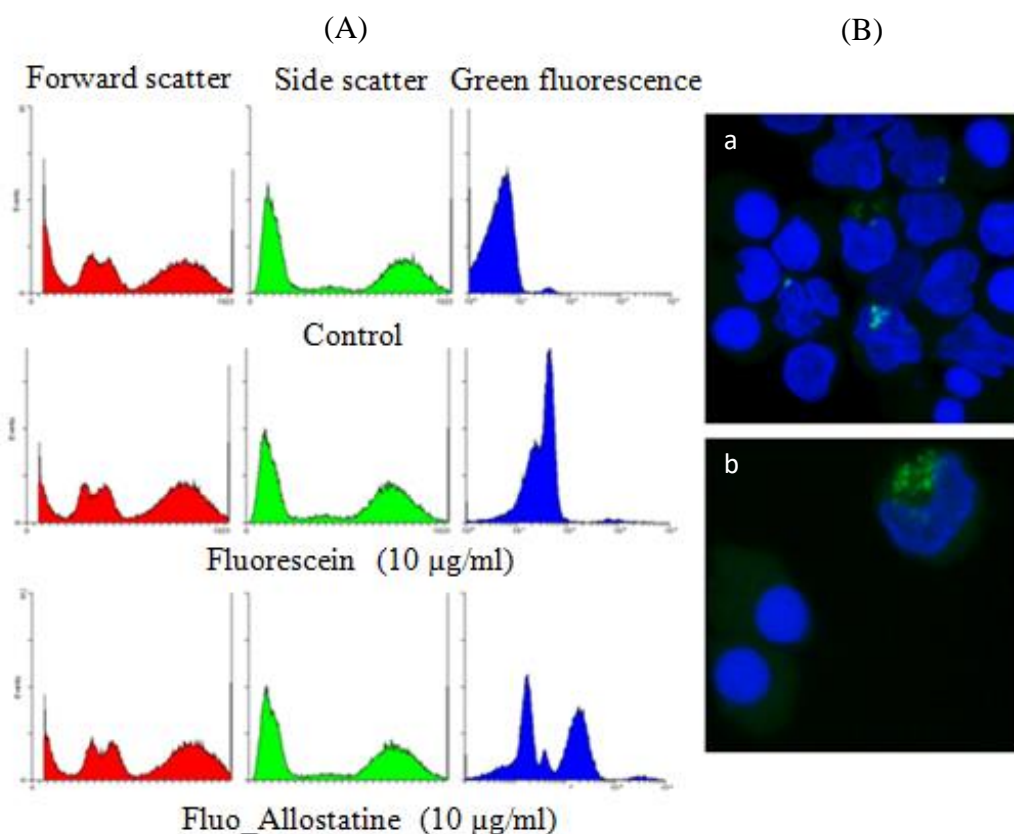


Fig. 6 The specificity of the interaction of Allostatine with human leukocytes (for explanations see the text); Panel B shows human leukocytes and localization of Allostatine within the cytoplasm. The green fluorescence is labeled Allostatine, the blue fluorescence is DAPI. Allostatine interacts with different types of cells: granulocytes (a) and monocytes (b).

Analysis for IFN- γ or IL-2 cytokine expression

To assess the effect of Allostatine on the production of cytokines by activated T cells cells, part of the cells were preincubated for 12 hours in a medium containing the peptide at a concentration of 10 $\mu\text{g} / \text{ml}$. When comparing the obtained results, it turned out that the preincubation of T-lymphocytes with Allostatine increased the number of activated cells producing IFN- γ and IL-2 by approximately two fold (Figure 7).

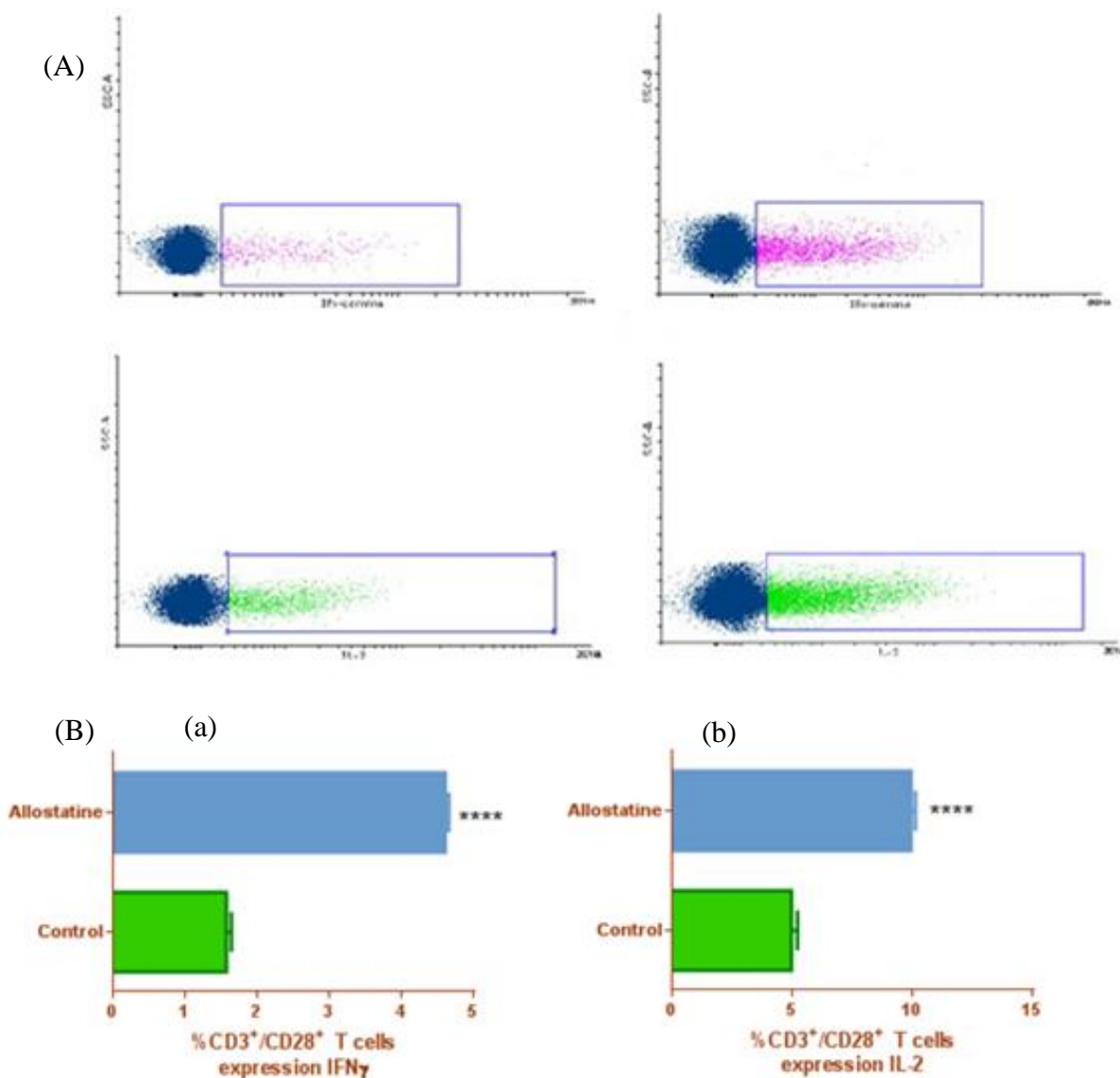


Figure 7. Effect of Allostatine on cytokine production by activated T-lymphocytes

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Preliminary clinical studies

The dynamics of the expression of activation markers is presented in Table 1. The analysis showed that in all volunteers, after injection of Allostatine at a dose of 1 mg, a population of cells (peak) with the CD3 + / CD25 + phenotype appears in the peripheral blood. The timing and magnitude of this reaction varied from volunteer to volunteer (Figure 8).

Table 1 The percentages of activated leucocytes in volunteers' blood after Allostatine injection

	CD3 ⁺ /HLA-DR ⁺			CD3 ⁺ /HLA-DR ⁺			CD3 ⁺ /CD25 ⁺		
	0h	12h	5 days	0h	12h	5 days	0h	12h	5 days
Volunteer 1	8.18	6.20	8.33	13.95	12.05	9.14	15.58	56.04	13.94
Volunteer 2	6.05	6.56	7.70	5.49	8.21	3.35	20.39	17.19	81.52

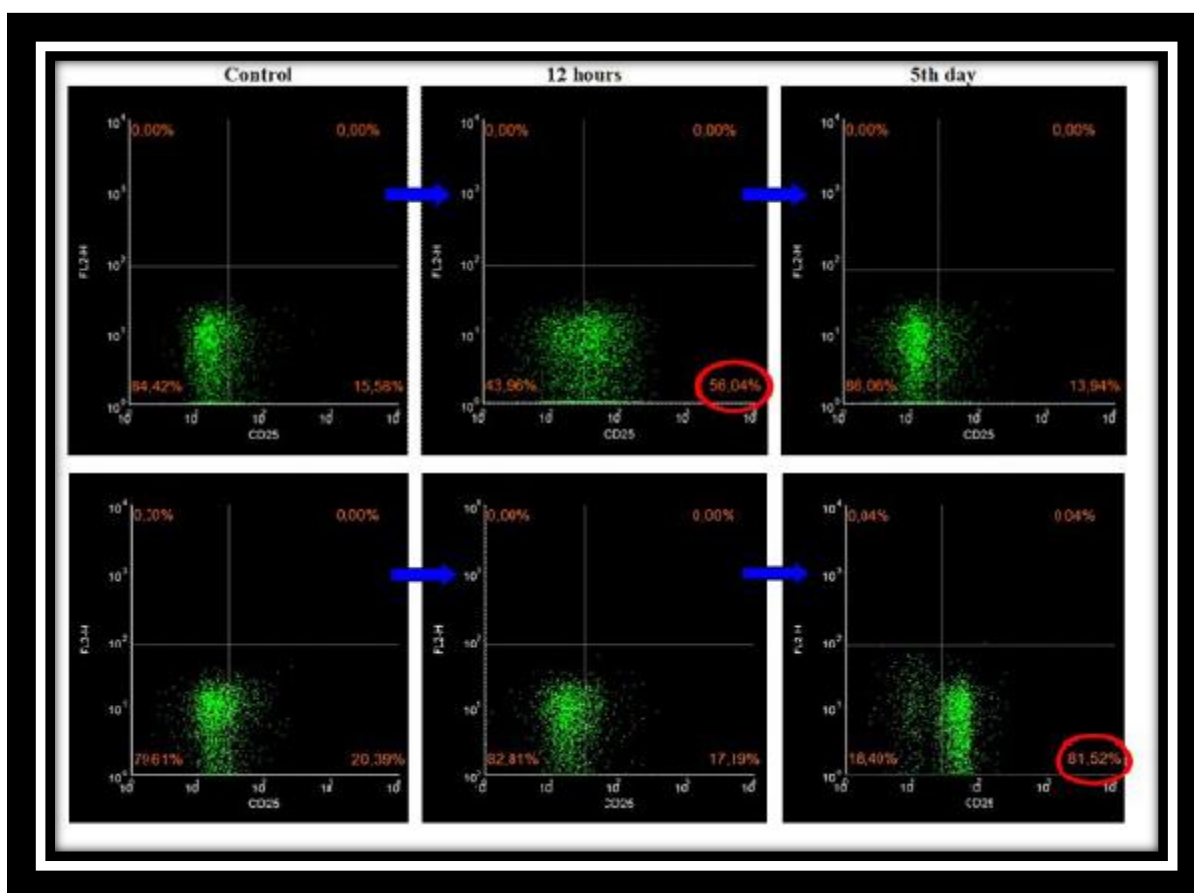


Fig. 8: Dynamics of T-lymphocyte activation after injection of 1 mg Allostatine
Please describe the panels briefly otherwise it is not clear.

HSV infections in patients experiencing relapsing herpes lesions for more than 4 years.

The preliminary clinical data characterizing Allomedin's therapeutic efficacy in patients infected with *Herpes simplex* virus involved results from 104 patients (81 females, 23 males) experiencing relapsing herpes of different localizations for more than 4 years before the study. The age of the patients varied from 18 to 60 years. Allomedin was applied onto the surface of herpetic lesions 2 to 3 times a day during 3 to 5 days. Results of the studies are summarized in Table 2.

Table 2. Allomedin clinical efficacy in the therapy of herpes lesion relapses with reference to the lesions localization (summary of a multicenter clinical study).

Localization	Therapeutic efficacy evaluation		
	Patients number	Positive responses	
		N	%
Skin	27	26	96,3
Vagina or urethra mucous tunic	44	41	93,1
Mouth mucous tunic	33	20	66,6

Data of Table 2 demonstrate that the majority of patients with skin herpes and genital mucous tunic herpes (labial and genital herpes, correspondingly) can be effectively treated by Allomedin. Clinical studies of Allomedin efficacy in labial and genital herpes demonstrated also an extraordinary fast relief of herpes symptoms, including reduced burning duration and reduction in size of oedema zone, when compared to Acyclovir which was the comparative standard antiviral. Tables 3 and 4 illustrate this fact. Allomedin rapidly eliminated inflammation symptoms like itch, burning, oedema such that the duration of the symptoms decreases about 10-fold when compared to acyclovir treatment.

Table 3. Allomedin and acyclovir comparative efficacy in the treatment of cold sores (labial herpes) symptoms.

Index	The index rate (duration in hours)	
	Acyclovir	Allomedin
Itch and burning duration	60 – 84	4-8
50% decrease in the size of oedema zone	84 -108	8-12

Total relapse duration	168 – 192	72 – 96
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Table 4. Allomedin and acyclovir comparative efficacy in the elimination of genital herpes symptoms

Indices and stages	Symptoms duration, hours	
	Acyclovir	Allomedin
Itch	84,3 ± 9,4	13,6 ± 3,7
Burning	93,2 ± 12,8	14,0 ± 2,2
Vesicular-erosive stage	96,2 ± 12,8	54,6 ± 10,2
Scab stage	114,1 ± 20,8	98,0 ± 12,8

Treatment of HSV infections in patients with chronic recurrent herpes simplex.

In this second study 156 patients, 80 men and 76 women with a clinical diagnosis of chronic recurrent herpes simplex were included for the treatment. Genital herpes was diagnosed in 53 patients (33.97%), orofacial herpes in 98 (62.82%), herpes in other sites in 5 (3.21%) (belly - 1, brush - 1, nates - 3 patients). All patients participating in the study had exacerbation of herpes infection (no later than 3 days from the onset of relapse).

For the division of patients into 3 groups, depending on the drug used, the method of adaptive randomization was used, while gender characteristics and localization of the herpetic process were not taken into account (genital and labial herpes were taken as equivalent pathology). Patients of group 1 received for external treatment for 10 days gel “Allomedin” (every 8 hours - 3 times a day for the lesions), patients of group 2 were given - Zovirax cream (every 4 hours - 5 times a day), the third group was treated with "Fenistil Pentsivir" –cream (every 2 hours - 8 times a day). The patient received the drug in the form of monotherapy. If the lesions regressed earlier, the use of the drug was interrupted. For “Allomedin” treatment in group 1, itching, pain and burning sensation regressed towards the 2nd visit (on the 3rd day). Also, all complaints for patient in group 1 treated with Allomedin regressed almost simultaneously: itching - for 3.8 ± 0.4 days, pain and burning - for 3.7 ± 0.4 days. Pain regressed in 3.3 ± 0.3 and 4.3 ± 0.5 in groups 2 and 3 respectively. Burning sensation was registered over 3.9 ± 0.5 and 4.3 ± 0.7 days, in groups 2 and 3 respectively. Herpetic lesions and itching were regressed somewhat slower compared with group 1 (4.3 ± 0.5 , $p > 0.2$ and 5.3 ± 0.7 , $p > 0.05$ in group 2 and 3 respectively).

Objective symptoms of genital infection (GI) in patients of group 1 regressed in an average of 8.76 ± 0.4 days (for 210.4 ± 10.2 hours), for the group 2 the regression was registered 9.7 ± 0.3 days (for 232.9 ± 7.1 hours, $p > 0.05$), while patients in group 3 present regression after 9.5 ± 0.3 days (for 227.3 ± 7.7 hours, $p > 0.1$). These data can be described as the average duration of relapse.

All the researchers involved noted a potential epithelial effect of Allomedin compared with other drugs. When compared with the control drugs treatment 58.8% of patients in group 1 presented eruptions in the exposed vesicular eruptions site. However, 88.2% * ($p < 0.05$) and 94.1% * patients ($p < 0.01$) presented eruptions in group 2 and 3, respectively, when compared with group 1. Furthermore, we registered a significantly faster epithelialization in 5.0 ± 0.6 days in group 1, when compared with other groups. The process of epithelialization in group 2 took 7.1 ± 0.6 * days ($p < 0.02$ compared with group 1), for the third group this indicator was 6.6 ± 0.4 * days ($p < 0.05$ compared with group 1). Consequently, the formation and drop of crusts on the sites of the former eruptions also occurred faster in the Allomedin group at 8.4 ± 0.4 days (in group 2 at 9.5 ± 0.3 * days, $p < 0.05$; in group 3 at 9.2 ± 0.5 days, $p > 0.2$, when compared with group 1).

A decrease in the level of herpetic eruptions was noted by a factor of 2 (in group 1, it was 4.8 ± 0.4 days (114.4 ± 10.8 hours). In the 2nd group, 6.0 ± 0.4 days (in 144.0 ± 10.2 hours, $p < 0.05$ compared with group 1), in the 3rd group ~~day~~ - by 5.7 ± 0.5 days (for 136.9 ± 11.0 hours, $p > 0$, 1 compared to group 1). We registered for 1 patient of the 3rd group with genital herpes at the second visit, the appearance of 2 small fresh elements along the periphery of the main nidus (against the background of regular use of Fenistil Pentsivir gel).

By the end of the observation period, a complete regression of lesions was noted in 47 patients (90.4%) in the 1st group; in 36 patients (69.23%) patients in the second group, and in 35 patients (67.3%) of group 3 (Figure 9). We registered slight edema and hyperemia remaining in the lesion focus, crusts at the at location of lesions in 5 patients (9.6%) in group 1. For the group 2, 16 patients (30.8%) present slight hyperemia and poorly defined crusts remained. While 17 patient in group 3 (32.69%) experienced a crusts in lesions location.

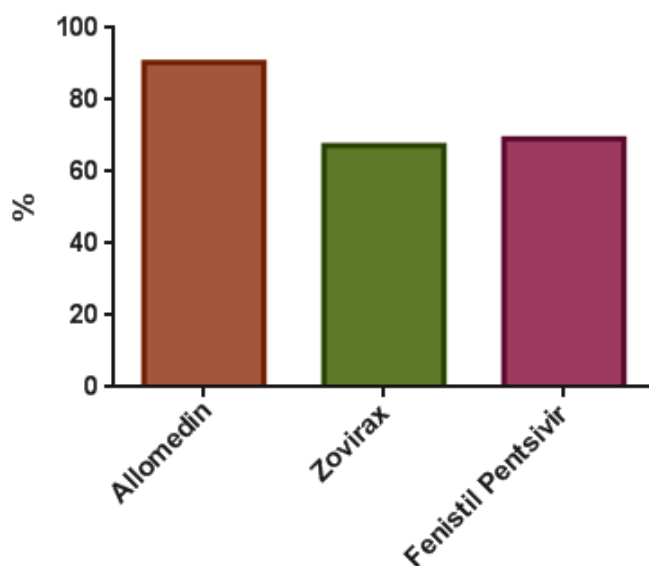


Figure 9. Complete regression of herpes clinical manifestations after Allomedin, Fenistil Pentsivir and Zovirax treatments

Based on the total summation of all indicators (regress of complaints, clinical symptoms, tolerance of the prescribed drug), patients and Medical Researchers evaluated the effectiveness of the treatment of Herpes Infection exacerbation. A good effect after treatments was indicated by 92.3% of patients in group 1, 75% in group 2 and 69.23% of patients in group 3. We noted as a “satisfactory” treatment result in 7.7% of the patients for group 1, and 25% of the patients in group 2, and 28.85% of the patients in group 3. The ‘unsatisfactory’ effect of the treatment was noted by 1 patient of the 3 groups. The effect of the treatment in this case was regarded by the doctor as satisfactory.

The subjective assessment of the treatment performed by the patient and the doctor in most cases coincided in all groups. In group 1, the opinions of the patient and the doctor coincided in 98.08% of cases, only in 1 case the patient assessed the result of treatment as satisfactory, and the doctor as good. In group 2 in 5 patients (9.61%) the opinions of the doctor and the patient differed in the assessment of treatment: in 3 cases the patient rated the result as satisfactory, and the doctor as good, in 2 cases the opposite. In group 3, the discrepancy was noted for 4 patients - 7.69%: in 1 case the patient rated the treatment effect as good, and the doctor as satisfactory, in 2 cases - on the contrary, in the 4th case the patient considered the effect of treatment unsatisfactory and the doctor is satisfactory (table).

The safety of the use of the gel "Allomedin" is proven by the absence of adverse events associated with the use of the drug, as well as the absence of local and common adverse and allergic reactions. In this regard, the drug can be recommended as a choice for widespread use in clinical practice. Based on the total summation of all indicators (regression of complaints, clinical symptoms, tolerance of the prescribed drug), the patients and medical researchers made an evaluation of the effectiveness of the treatment of herpes virus infection exacerbation (Table 5). 92.3% of patients in group 1, 75% of patients in group 2 and 69.23% of patients in group 3 indicated a positive effect from the treatment. As a "satisfactory" result of treatment, 7.7% of patients in group 1 and 25% of patients in group 2 and 28.85% of patients in group 3 were evaluated. An unsatisfactory effect of the treatment was noted by 1 patient of the 3 groups (the effect of the treatment in this case was regarded by the doctor as satisfactory). The subjective assessment of the treatment carried out by the patient and the doctor in most cases coincided in all groups. In group 1, the opinions of the patient and the doctor coincided in 98.08% of cases, only in 1 case the patient assessed the result of treatment as satisfactory, and the doctor as good. In group 2 in 5 patients (9.61%) the opinions of the doctor and the patient differed in the assessment of treatment: in 3 cases the patient rated the result as satisfactory, and the doctor as good, in 2 cases the opposite. In group 3, the discrepancy was noted for 4 patients - 7.69%: in 1 case the patient rated the treatment effect as good, and the doctor as satisfactory, in 2 cases - on the contrary, in the 4th case the patient considered the effect of treatment unsatisfactory and the doctor considered it satisfactory.

Table 5: Evaluation of the effectiveness of treatment

Effect	Patient's Opinion			Researcher's Opinion			Difference of Opinions
	Good	Acceptable	Non-acceptable	Good	Acceptable	Non-acceptable	
Group 1 (52 patients)	48 (92,3%)	4 (7,7%)	-	49 (94,23%)	3 (5,77%)	-	1 (1,92%)
Group 2 (52 patients)	39 (75%)	13 (25%)	-	40 (76,92%)	12 (23,08%)	-	5 (9,61%)
Group 3 (52 patients)	36 (69,23%)	15 (28,85%)	1 (1,92%)	37 (71,15%)	15 (28,85%)	-	4 (7,69%)

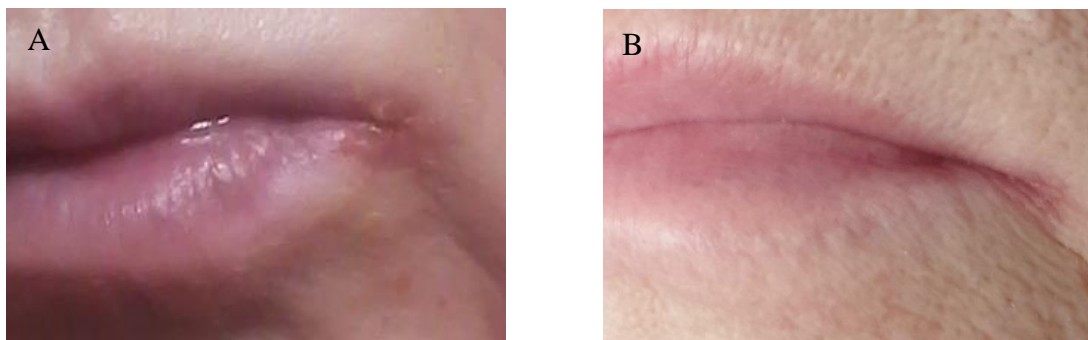


Figure 9. Labial herpes relapse.; Panel A: Before treatment, Panel B: After Allomedin treatment

Discussion

In the present study we demonstrate that Allostatine induces different immune reactions which are in line with a favorable antiviral immune response. When formulated in a gel (named Allomedin®) Allostatin exerts beneficial therapeutic efficacy for skin lesions in patients infected with both *Herpes simplex* virus type 1 and 2.

Allostatine (primary structure: His-Gly-Val-Ser-Gly-Trp-Gly-Gln-His-Gly-Thr-His-Gly) belongs to Alloferons, a group of natural antiviral peptides isolated from *Calliphora vicina* insects [2] that are able to induce synthesis of interferon and modify cytokine production. It is considered as a novel structural analog to Alloferon-1 (His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly), which is known to regulate the transcription factor NF-kB and to modulate the response of NKs and T-cells [1].

NK and T cells play an important role in the host response against viral infections. Activated NK and T cells are able to directly kill virus infected cells by the production of different directly cytolytic cytokines and cytokines which stimulate antiviral adaptive immune response, particularly IFN- γ [3].

NK-cell effector mechanisms of action respond upon engagement of different cellular receptors. The present study demonstrated the potential of Allostatine to elicit a response by triggering such receptors while interacting with NKs; therefore Allostatine can be considered as an antiviral peptide. Allostatine co-incubated with K562 target cells increases the expression of surface molecules CD244 (2B4), NKp80 (Killer cell lectin-like receptor subfamily F member

1 - KLRF1) and CD226. CD244 signaling pathway involves the interaction with the small intracellular SH2 domain-containing protein 1, SH2D1A and with the Src homology 2 (SH2)-domain of the Src tyrosine-family member Fyn, a non-receptor tyrosine-protein kinase expressed in NK, T and B-cells (Engel et al., 2003) [5]. NKp80 is known to behave as an activator of C-type lectin-like immunoreceptor (CTLR) in human NK cells [6]. It has also been shown that Allostatine may activate CD226 (also known as DNAM-1 or DNAX accessory molecule-1), a 65 kDa glycoprotein expressed on the surface of NKs, platelets, monocytes and a subset of T cells [7].

Interestingly, CD244, Nkp80 and CD226 have been previously described as inducers of IFN γ production [8]. CD244 is a member of SLAM (signaling lymphocytic activation molecule) protein family with the ability to induce IFN γ secretion and cytotoxicity. This stimulatory effect is due to the presence in the intracytoplasmic tail of the extra tyrosine motif (Watzl et al., 2000) [9]. Cytokine production and NK cytotoxicity can be explained as a consequence of NKp80 up-regulation and its binding to the cognate ligand ‘activation-induced C-type lectin’ (AICL) [10]. Furthermore, CD226 induces the stimulation of NK cell-mediated cytotoxicity and enhance secretion of IFN- γ [11].

After incubation of Allostatine with T cells, activated by CD3 and CD28, we observed an increased secretion of both IL2 and IFN γ .

These findings are in line with data that have been demonstrated previously, indicating that peptides of the Alloferons family induce NK cells cytotoxicity against cancer cells by up-regulating the NK-activating receptors CD244 and with the production of IFN γ , TNF- α and the promotion of granule exocytosis [13].

In this study, we highlighted the potential generation of immune responses against viral infections following Allostatine treatment by demonstrating activation of the CD25 marker of NK cells *in vitro*. Also, we evaluated the increase of CD3 and CD25 expression by T lymphocytes in Allostatine-treated patients. CD25 is the α -chain receptor of interleukin 2 (IL-2R α) (Waldmann, 1989) [14]. Our data demonstrated that Allostatine stimulates IL-2R α receptor on human NK and activated T cells, followed by cytokine pre-activation.

Many studies demonstrated that the initial stages of an HSV infection are influenced by the activity of IFNs, NK cells and T cells which serve to limit the spread of virus to the nervous system [5, 6, 15]. Other experiments report that macrophages are considered as potent early inhibitors of HSV infection [16]. Activated macrophages inhibit pathogen replication by releasing nitric oxide [17].

The observed allostatine-related immune response data are coherent with the results of the clinical study performed with allostatine as a the treatment of recurrent herpes simplex lesions. Our data proved the effectiveness and safety of Allostatine against both HSV1 and HSV2. Its high efficacy and good tolerability as a topical treatment, even as a monotherapy for these diseases.

The allostatine-containing gel "Allomedin" quickly suppressed subjective sensations and objective manifestations of exacerbation of herpes infection, ~~especially with early treatment~~ (please explain). Allomedin treatment showed a superior and significantly different clinical benefit when compared to Zovirax and Fenistil Petsivir cream treatments. The clinical data obtained also showed high degree of compliance of the treatment. In addition, Allomedin evoked a more pronounced epithelial effect, reducing the period of formation and falling away of the crusts. The application of the drug 2-3 times a day showed excellent cosmetic properties suggesting the use of Allomedin gel to stop exacerbations of labial or genital herpes of any severity. This was also confirmed by a subjective assessment of the treatment, given by the patients and the doctor. These finding support Allomedin as a means of choice for widespread use in clinical practice.

The tolerability of all the drugs for external use was rated as Good, with no registered adverse local neither general reactions, nor allergic. Notably, patients of the group treated with Allomedin noted the convenience of using Allomedin gel only 2-3 times a day. Allomedin is reported also for its good organoleptic property such us the transparency and no-shining was reported after Allomedin application.

Conclusion

According to our clinical data, Allostatine and it gel formulation Allomedin, demonstrate excellent therapeutic efficacy and safety as a therapy for skin and mucous tunic HSV1 and HSV2 infections. In fact, the clinical data from the present study confirm that 90 to 95% of colds sores and genital herpes cases can be effectively controlled by Allomedin treatment. To the best of our knowledge, there is no other drug or cosmeceutical that is currently as effective as Allomedin for this indication. Nowadays about 90% of the human population is infected with HSV. HSV causes lifelong infections manifesting in different forms varying from mild disorders (cold sores) to severe chronic forms such as relapsing genital herpes. According to European STD guidelines approved by the WHO [Patel et al., 2017], a group of drugs is

recommended for systemic administration (*per os* or *iv*) since their topical formulations have very limited efficacy. We demonstrated that Allomedin eliminates most of the unpalatable symptoms of HSV infections such as itch, burning, oedema, much faster than acyclovir. Worthy to note is that long term treatment with acyclovir give rise to acyclovir resistant strains that, obviously, worsen herpes therapy perspectives. Since the Allostatine mode of action is completely different from that of acyclovir, Allomedin can be effectively used against acyclovir resistant HSV as well.

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