Alloferon-1 Ameliorates Acute Inflammatory Responses In λ -Carrageenan-Induced Paw Edema In Mice

Xiangrui Zhang

Nanjing Tech University

Vladimir Retyunskiy

Nanjing Tech University

Shuai Qiao

Nanjing Tech University

Ye Zhao (✉ zhaoyev@163.com)

Nanjing Tech University

Chi-Meng Tzeng

Xiamen University

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Abstract

Alloferon-1 have been proposed as an effective peptide to enhance antitumoral immunity, antiviral defense and anti-inflammatory activity. This work aimed to assess anti-inflammatory effects of alloferon-1 against acute inflammation and histopathological deformations in λ -carrageenan-induced paw edema in mice. Systemic pretreatment with the alloferon-1 (22.0 mg/kg) injected mice showed a significant reduction in paw thickness and vascular permeability. Alloferon-1 prevented λ -carrageenan-evoked exudation and the neutrophil influx to the mouse pleura and the neutrophil migration into carrageenan-stimulated mouse air pouches based on the histopathological changes in the paw tissue. Administration of alloferon-1 also suppressed the expression of the inflammatory cytokines in the inflamed paw tissue such as tumor necrosis factor- α (TNF- α), monocyte chemoattractant protein 1 (MCP1), interleukin-5 (IL-5), etc. by Luminex liquid chip. Collectively, the present study provides evidences for the marked anti-inflammatory effects of alloferon-1 which might represent new therapeutic options for the treatment of acute inflammation inflammatory diseases.

Introduction

Inflammation refers to the defense response against allergens, toxic substances, infections or cell damaged, leading to the occurrence of redness, swelling, asthma, fever, pain and other symptoms in the affected site¹⁻³. Inflammation can be divided into acute inflammation and chronic inflammation. Acute inflammation is temporary and can be restored into normal tissue homeostasis by dispensing with the inciting stimulus. However, persistence acute inflammatory responses usually leads to rheumatoid arthritis, atherosclerosis, Alzheimer's disease and bowel disease⁴. Currently, prescribed anti-inflammatory drugs including steroidal anti-inflammatory drugs, non-steroidal anti-inflammatory drugs (NSAIDs) and antibacterial drugs are commonly applied for the treatment of inflammation. although often accompanied by mild or moderate adverse reactions, long-term use of large doses of these drugs have been correlated with serious health problems such as gastrointestinal bleeding and nephrotoxicity⁸. Therefore, it is important to understand the mechanisms of acute and chronic inflammation, and in particular to explore inhibitory intervention drugs to decline the immunological reactivity.

Antimicrobial peptides (AMPs), widely distributed in the biosphere, refer to a kind of conservative peptide molecules with antimicrobial activity. As a group of naturally occurring AMPs, alloferons have been proposed as effective peptide compounds to enhance antitumor immunity and antiviral defense. Alloferons are members of cytokine-like peptide family that were originally derived from a hemolymph of bacteria challenged maggots of the blow fly *Calliphora vicina* Diptera⁵. Two peptides of 13 and 12 amino acids, which stimulate in vitro natural cytotoxicity of mouse spleen lymphocytes and human blood mononuclear cells, were isolated and their amino acid sequences were identified as HIS-GLY-VAL-SER-GLY-HIS-GLY-GLN-HIS-GLY-VAL-HIS-GLY (alloferon-1) and GLY-VAL-SER-GLY-HIS-GLY-GLN-HIS-GLY-VAL-HIS-GLY (alloferon-2)⁵. Alloferon-1 reveals a capacity to stimulate activity of natural killer (NK) cells and synthesis of IFN in animal and human models, thereby exhibiting anti-tumoral and anti-viral capabilities⁶.

Recently, it was also reported that alloferon-1 effectively reduces the production of pro-inflammatory cytokines, such as interleukin 5 (IL-5), interleukin 6 (IL-6), and interleukin 17 (IL-17) in ovalbumin-induced asthma⁷. In UVB-induced skin inflammation, alloferon-1 effectively reduces the production of pro-inflammatory cytokines, such as IL-6, interleukin 8 (IL-8), and tumor necrosis factor- α (TNF- α)⁸. In addition, alloferon-1 attenuates dextran sulfate sodium-induced enteritis by downregulation of IL-6 and TNF- α ⁹. Based on the immune-modulating effects of alloferon-1, in the present study, we investigated the anti-inflammation effect of alloferon-1 in λ -carrageenan induced paw edema in mice.

Materials And Methods

Animals and Reagents

The study was approved by Ethics Committee of Nanjing Tech University. Mice husbandry and disposal were according to the compliance with Institutional Animal Care and Use Committee (IACUC) guidelines of Nanjing Tech University for laboratory animal use and all methods carried out were in compliance with the ARRIVE guidelines. Specific pathogen-free (SPF) male BALB/c mice (body weight 20-25 g) of 8-9 weeks of age were procured from the Nanjing Junke Bioengineering Corporation. All mice were kept under controlled environmental conditions ($25 \pm 3^{\circ}\text{C}$, 50-60% humidity and a 12-hour light-dark cycle) and maintained with ad libitum access to standard diet and water. Except for drinking water, all mice were deprived of food 12 hours before the start of the experiment. All the experimental animals were fed, and all the operations were performed by the same researcher.

Alloferon-1 (purity >98.62%) was supplied by Leon Biological Technology Co. Ltd. (Nanjing, China) and aspirin (purity >98%) was purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Evans Blue dye and λ -carrageenan were provided by Sigma (USA). PBS phosphate buffered saline was supplied by Leagene Biotech Co., Ltd. (Beijing, China). Formamide was supplied by Macleans Biochemical Technology Co., Ltd. (Shanghai, China). Fluorescence microscope was purchased from Scope Instruments Co., Ltd. (Nanjing, China) and Micro spectrophotometer was purchased from All for Life Science Co., Ltd. (Hangzhou, China). Other reagents used were of analytical grade.

λ -carrageenan-induced Acute Paw Edema In Mice

The mice were randomly divided into control, λ -carrageenan (CGN), Aspirin (AS) + λ -carrageenan (250mg/kg), and alloferon-1 + λ -carrageenan (22 mg/kg) groups, with 5 mice in each group. The control and λ -carrageenan groups were given 1 ml of 0.9% normal saline subplantar injection. Positive control groups were pretreated with (250 mg/kg) aspirin by intraperitoneal (IP), and 2 hours later 30 μL of 1% λ -carrageenan was injected into the left hind paw (along the midline of the mice's sole, insert the needle from the heel and gently inject it under the tendon). The alloferon-1 groups were treated with standard drugs for IP injection 6 hours in advance. The measurements were performed at 0, 1, 3, 5, and 7 hours

after λ -carrageenan injection with use of Vernier caliper and were compared with the controls. The inhibitory effect was determined by using the following formula:

$$\% \text{ Inhibition} = \frac{(C_t - C_o)_{CGN} - (C_t - C_o)_{treated}}{(C_t - C_o)_{CGN}} \times 100$$

Where $(C_t - C_o)_{CGN}$ is the difference in the size of paw at 7 hours in CGN mice, and $(C_t - C_o)_{treated}$ is the difference in the size of paw at 7 hours in mice treated either with the aspirin or alloferon-1.

Evaluation of paw histopathology by hematoxylin and eosin (HE) staining

Mice were sacrificed after injection of λ -carrageenan for 7 hours and the hind paw tissues were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (Sigma-Aldrich) and paraffin-embedded. The paw tissue was cut into five-micrometer thickness sections by microtome. Hematoxylin and eosin (H&E) staining (H-3404, Vector labs, USA) were performed according to the standard protocols. At least five sections from each experimental group were microphotographed by inverted microscope (Nikon ECLIPSE Ts2, Japan).

Evans Blue Dye Extravasations In λ -carrageenan-induced Paw Edema

The permeability of Evans Blue dye in the blood vessels of mice is mainly affected by two factors. The internal factors mainly depend on the weight, strain and age of mice, and the external factors are mostly affected by the temperature, humidity and the stress handling of mice¹⁰. Before tail vein injection, the prepared 0.5% Evans blue PBS sterile solution was put through filter again to remove incompletely dissolved particles. 200 μ l of 0.5% Evans blue dye was injected into tail veins of all mice after 7 hours of λ -carrageenan injection to measure extravasations of dye in hind paw tissues of mice as a marker of inflammation, and then after 30 minutes the photographs of mice paws were taken. Meanwhile, the mouse paws were sustained in a 65°C oven for 48 hours, and to eliminate the difference in moisture. After weighed and crashed, paw tissues were soaked with 500 μ l formamide and further heated in a water bath at 55°C for 24-48 hours to extract Evans blue. Afterwards, the formamide/Evans blue mixture was centrifuged at 4000 $\times$ g for 10 minutes. The Evans blue level in the supernatants was determined by its absorption at the wavelength of 630 nm by using a microplate reader (Molecular Devices).

Luminex liquid suspension chip detection

The serum was collected from retro orbital plexus of mice 7 hours after the 0.1% λ -carrageenan was injected. Luminex liquid suspension chip for inflammatory factors detection was performed by Wayen Biotechnologies (Shanghai, China). The Bio-Plex Pro Human Chemokine Panel 23-plex kit was applied accordance with the manufacturer's instructions. Each sample was assessed in triplicate.

Statistical analysis

Statistical analysis was carried out by two-tailed unpaired t-tests using inStat version 5.0 for Windows (GraphPad Software, San Diego, California, US). The mice samples were randomly collected in all experiments and measurement differences were considered statistically significant at $P < 0.05$. All data are presented as mean values $\pm$ standard deviation (SD).

Results

Effect of alloferon-1 on λ -carrageenan-induced paw edema

Before experiments, alloferon-1 was synthesized by chemical routes, detected by high pressure liquid chromatography (HPLC) and analyzed by mass spectrometry (MS) (Fig. 1A). In the λ -carrageenan-induced paw edema model, the subplantar injection of λ -carrageenan led to a time-dependent increase in the paw thickness and degree of swelling, as shown in Fig. 1B and C, which reached a maximum at 5 hours and sustained elevated thereafter for 24 hours. Alloferon-1 was pretreated for 6 hours at the concentration of 22 mg/kg of body weight based on the preliminary experiments. Aspirin, as a positive control, was pretreated for 2 hours at the concentration of 250 mg/kg of body weight as reported¹¹. Both drugs showed negligible effect to paw edema at 0 time. At 1 hour after λ -carrageenan-induction, aspirin caused significant reduction of paw edema. Meanwhile, alloferon-1 still showed negligible effect at 1 hours after λ -carrageenan treatment. However, after 3 hours of λ -carrageenan induction, alloferon-1 significantly reduced the paw edema with a thinner paw and less swelling ($P \leq 0.05$). As time proceeded, alloferon-1 produced a time-dependent inhibitory effect. At 7 hours, alloferon-1 reached maximum and almost the same reduction effect as aspirin in paw edema. The changes in paw swelling of mice were measured by digital vernier caliper. As shown in Fig. 1C, alloferon-1 and aspirin exhibited 23.03% and 48.82% maximum inhibition rates respectively in comparison to the mice treated with normal saline.

Histopathological examination of paw

The effects of alloferon-1 on the histopathological changes in the paw edema were evaluated by HE staining. As shown in Fig. 2A, in the acute inflammatory response induced by λ -carrageenan, the mice paw exhibited an intense edema, characterized by epithelial and conjunctive tissue blisters with substantial numbers of infiltrated inflammatory cells, most of which are neutrophils. The λ -carrageenan groups pretreated with alloferon-1 and aspirin showed a significant reduction in the extent of edema as well as decrease in the infiltration of inflammatory cells. The neutrophils were circled with the red dotted line and the average number of neutrophils was quantified in Fig. 2B. It was showed that the inflammation response with infiltration of neutrophils was significantly decreased by alloferon-1 and by aspirin compared to the λ -carrageenan induced mice. Additionally, it was observed that both alloferon-1 and aspirin prevented λ -carrageenan-evoked exudation and the neutrophil influx to the mouse pleura and the neutrophil migration into carrageenan-stimulated mouse air pouches in the paw tissue.

Extravasation of Evans blue dye in paw tissues of mice

Acute inflammation caused by λ -carrageenan can increase the vascular permeability, which can be adjudged by the Evans blue extravasation from the tissues. As shown in Fig. 3, the λ -carrageenan induced paw edema tissues shown a strikingly increasing of Evans blue measure at the absorption wavelength of 630 nm. Meanwhile, the treatment of both alloferon-1 and aspirin suppressed extravasation of Evans blue dye and exhibited 19.63% and 43.63% inhibition, respectively in comparison to the λ -carrageenan induced paw edema groups.

The effect of alloferon-1 on the expression of inflammatory factors

To further explore the potential mechanism of alloferon-1 in λ -carrageenan-induced inflammation, levels of cytokines in serum were measured by Luminex Liquid Cytokine Chip technology. The expression heat map in Fig. 4A and B showed that IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-12(P40) was remarkably decreased by alloferon-1 compared to λ -carrageenan-induced paw edema model. Meanwhile, the levels of IL-9 and IL-10 were also slightly decreased by alloferon-1. Additionally, the expression of macrophage inflammatory protein 1 α (MIP-1 α) and MIP-1 β , monocyte chemoattractant protein-1 (MCP-1) and granulocyte macrophage-colony stimulating factor (GM-CSF) was downregulated in λ -carrageenan-induced model treated by alloferon-1 compared to λ -carrageenan-induced group. On the contrary, the changes of IL-1 α , IL-17A, granulocyte-colony stimulating factor (G-CSF), keratinocyte (KC) and eotaxin did not show apparent changes after the treatment of alloferon-1. IFN- γ and TNF- α showed a slight upregulation by alloferon-1 but not significant. Moreover, the expression of IL-6 showed slightly upregulation by alloferon-1. Interestingly, the aspirin showed opposite regulatory effects with alloferon-1 in MIP-1 α , MCP-1, GM-CSF expression, which were increased after aspirin treatment, suggesting that the anti-inflammation mechanism of aspirin and alloferon-1 was different. Collectively, these evidences indicated that alloferon-1 might have an inhibitory effect on the in the λ -carrageenan-induced paw edema by regulation with variety of cytokines.

Discussion

According to previous studies, alloferons are polypeptides with biological activities including anti-virus, anti-tumor, anti-inflammation, immune regulating, etc.^{13, 14} As an immunomodulatory peptide, alloferon-1 has been supported clinically and has been proven to be efficient in patients suffering with herpes simplex virus (HSV) and human papilloma virus (HPV) infections, as well as several Coxsackie viruses^{12,13, 14, 15}. However, its anti-inflammation potential needs further preclinical assessment. With the aim of defining the anti-inflammatory properties of alloferon-1, in this study, λ -carrageenan-induced acute paw edema mice model has been chosen. λ -carrageenan-induced paw edema in mice is a well-researched model which has been fully characterized previously^{16,17}. According to relevant data, the λ -carrageenan induced inflammation can be divided into three stages, and the inflammation-mediated effects in the first and third stages are mainly E series prostaglandins, histamine and serotonin¹⁸. The main signs of inflammation appear immediately after subcutaneous injection-edema, hyperalgesia and erythema, which are caused by the action of pro-inflammatory agents-bradykinin, histamine, tachykinin,

complement and reactive oxygen species as well as nitrogen substances¹⁹. Peripheral edema is known to be mainly caused by venous obstruction and increased vascular permeability²⁰. Therefore, in this investigation, the changes of paw edema thickness after the injection of λ -carrageenan had been measured to evaluate the anti-inflammation effect. Results showed that alloferon-1 alleviated the paw edema thickness significantly, which proved apparent evidences for the anti-inflammation effect of alloferon-1 in λ -carrageenan paw edema.

The occurrence of edema causes the increase of vascular permeability, thereby enhancing the efficacy of fluid transport across capillaries and allowing protein-rich liquid to enter the tissue²¹. Inflammatory mediators such as histamine and serotonin cause increase of vascular leakage²⁵. Moreover, the extravasation of the liquid including plasma proteins will cause the leukocytes aggregation and adhesion, which are important features of acute inflammation^{20,22,23}. Jane Jeon et al. found that combined application of alloferon-1 and prednisolone to treat asthma significantly reduced the number of eosinophils, macrophages, and neutrophils in bronchoalveolar lavage fluid (BALF) of asthmatic mice induced by ovalbumin (OVA)²⁴. In this study, injections of Evans blue dye into the tail veins of mice were used to evaluate the degree of vascular permeability after the inflammation response caused by λ -carrageenan²⁵⁻²⁷. It was observed that both alloferon-1 and aspirin suppressed extravasation of Evans blue dye in comparison with the λ -carrageenan induced paw edema, suggested that alloferon-1 administration inhibited the vascular permeability in acute inflammation site. According to the studies, combined application of alloferon-1 and prednisolone to treat asthma significantly reduced the number of eosinophils, macrophages, and neutrophils in bronchoalveolar lavage fluid (BALF) of mice with ovalbumin-induced asthma²⁸. Additionally, at 7 hours after λ -carrageenan-induction, alloferon-1 significantly inhibited the aggregation and infiltration of neutrophils at the edema location, which further confirmed the consistent anti-inflammatory effect of alloferon-1 in λ -carrageenan-induced neutrophils aggregation.

As known, NK cells participate in the development of adaptive immune response through the ability to produce cytokines. When NK cells are activated, NK cells release interferon- γ (IFN- γ) and TNF- α to reverse the suppression of immune system. Interferon- γ (IFN- γ) and TNF- α are biological factors which kill a large amount of tumor cells and activate lymphocytes (such as T and B lymphocytes)^{8,29-34}. NK cells were identified as the peptide pharmacological target responding to alloferon-1 with immediate growth of cytotoxic activity³⁵. Alloferon-1 increased the IFN- γ , TNF- α and granular exocytosis produced by NK cells, thus antagonizing cancer cells^{5,24,35-39}. In addition, previous research has also demonstrated that NF- κ B activation is involved in IFN synthesis¹⁴, and alloferon-1 inhibited the degradation and phosphorylation of inhibitor of κ B (I κ B) induced by TNF- α in Colo205 colon cancer cells⁴⁰, which provide clues to the basis of alloferon's capability to stimulate IFN synthesis. In this study, the expression of IFN- γ and TNF- α were upregulated after λ -carrageenan-induction, which may because that both factors were involved in inflammation reaction. However, after alloferon-1 treatment, IFN- γ and TNF- α were detected to be decreased, which implied a different mechanism of alloferon-1 on IFN- γ and TNF- α regulation. The

cytokines IL-2, -12, -15, -18 and -21 are commonly upregulated to increase cellular cytotoxicity of NK cells^{41 42}. In this study, IL-1 β , IL-2, IL-3, IL-4, IL-12(P40) was remarkably downregulated by alloferon-1 treatment, which suggested that NK cells mediated acute inflammatory reaction was inhibited by alloferon-1. Further investigation had demonstrated that alloferon-1 prevented inflammatory cell infiltration by down-regulating IL-5 and IL-17 and reduced IgG1 and IgE by inhibiting type 2 T helper auxiliary immune response²⁸. IL-5 induces the chemotaxis and activation of integrin CD11b at the molecular level during inflammation and prolongs eosinophil survival by inhibiting apoptosis⁴³. In this study, IL-5 was decreased by alloferon-1, which further validate this conclusion in λ -carrageenan-induced inflammations.

Alloferon-1 has been reported to play a positive role in the treatment of UVB-induced skin inflammation and ovalbumin-induced asthma^{6,44}. The application of alloferon-1 on the UVB-exposed skin of hairless mice showed significantly lowered the increase of skin epithelial thickness of mice, exposed to chronic UVB irradiation³⁸. Pro-inflammatory cytokines including IL-1 α , IL-1 β , IL-6 and IL-18 induced by UVB were reduced by alloferon-1⁸. Recently, it has been reported that alloferon-1 can improve colitis model in mice induced by dextran sulfate sodium (DSS)⁴⁰. Compared with the control groups, the symptoms of edema, epithelial erosion, and immune cell infiltration in the experimental group with the treatment of alloferon-1 were alleviated, and the level of IL-6 in the mouse plasma decreased⁷. In this study, the expression of IL-1 β , IL-9 and IL-10 has been detected in λ -carrageenan-induced model treated by alloferon-1 and results also showed the downregulation of these cytokines. Additionally, we provided experimental evidences that the expression of MIP-1 α and MIP-1 β , MCP-1 and GM-CSF were downregulated in λ -carrageenan-induced model treated by alloferon-1, compared to λ -carrageenan-induced group. MIP-1 α , MIP-1 β and MCP-1 are inducible chemokines in response to various proinflammatory stimuli and exhibits a variety of activities including leukocyte chemotaxis⁴⁵. GM-CSF was identified to promote MCP-1-mediated inflammation⁴⁶. Inhibition of MIP-1 α , MIP-1 β and MCP-1 by alloferon-1 may inhibit the recruitment of monocytes. In this study, we demonstrate for the first time that alloferon-1 downregulated MIP-1 α , MCP-1, GM-CSF in immune response. Further studies are still needed to expand the generalizability of these findings.

Declarations

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Author contributions

YZ, CT conceived and designed the experiments; XZ, SQ and YZ performed all experiments; XZ, YZ and CT analyzed data; XZ, VR and YZ wrote and reviewed the manuscript.

Conflict of Interest statement

The authors declare no competing interests.

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Figures

Figure 1

The effect of alloferon-1 on the paw edema in carrageenan-induced mice. Mice were pretreated by intravenous injection with alloferon-1 for 6 hours (22mg/ body weight) or aspirin for 2 hours (250mg/body weight) before subplantar injection of λ -carrageenan. Ctrl: saline solution; CGN: λ -carrageenan; AF: alloferon-1; ASA: aspirin. (A) a, Structure of alloferon-1; b, High pressure liquid chromatography (HPLC) detection of synthesized alloferon-1; c. mass spectrometry (MS) analysis of synthesized alloferon-1. (B) Appearances of paw edema in each group. (C) Measurements of the thickness of paw edema in mice using digital vernier caliper. Data are presented as the mean $\pm$ SEM (n = 5). *P < 0.05 were considered as significant difference compared to the control group.

Figure 2

Histopathological analysis of λ -carrageenan (CGN), aspirin + λ -carrageenan (CGN+ASA) and alloferon-1 + λ -carrageenan (CGN+AF) treated groups. The neutrophils in site were circled with the red dotted line. (A) Histopathological microphotography under microscope with the magnification of 40 $\times$ and 400 $\times$. (B) The average number of neutrophils was quantified by image J. Data are presented as the mean $\pm$ SD (n = 5). *P < 0.05 were considered as significant difference compared to the control group.

Figure 3

Extravasation of Evans blue in tissues. (A) Evans blue injection and observation in paw edema. (B) The Evans blue level in the paw edema tissue was collected and the level was determined by its absorption at the wavelength of 630 nm by using a microplate reader.

Figure 4

Effect of alloferon-1 on the expression of inflammatory cytokines. (A) Heat map analysis of inflammatory cytokines in the serum detected by Luminex liquid cytokine chips (n=3). (B) Expression level of cytokines in serum. Values are expressed as mean $\pm$ SEM. *P<0.05 for drug treatment groups vs. λ -carrageenan induced groups.

Abbreviations: pg/ml, picograms/milliliter; IL, Interleukin; GM-CSF, Granulocyte- macrophage colony stimulating factor; TNF- α , Tumor necrosis factor- α ; IFN-interferon- γ ; MCP-1, monocyte chemoattractant

protein-1; MIP-1, Macrophage inflammatory protein-1; G-CSF, Granulocyte colony stimulating factor.