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REPORT

A modified variant of human Interleukin-15 as a novel antigen for active immunotherapy in rheumatoid arthritis

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ABSTRACT

Interleukin (IL)-15 is a pro-inflammatory cytokine that plays a crucial role in the pathogenesis of rheumatoid arthritis (RA), a chronic inflammatory disease for which there is no effective therapy. In this report, we describe a novel vaccine based on active immunization with modified human IL-15 for the treatment of RA and others diseases related with IL-15 overexpression. The IL-15 obtained in *E. coli* exhibits a conformation of the disulfide bridges different to the one described for the native cytokine, which may favor the development of an immune response against this antigen. The results show that immunization with modified human IL-15 generates specific polyclonal antibodies against the cytokine in non-human primates, which suggests a rupture of B cells tolerance as consequence of immunization. These antibodies inhibited the biological activity of native IL-15 without affecting the human IL-2-induced proliferation of CTLL-2 cells, demonstrating the specificity of the antibodies by autologous IL-15. Additionally, we show that vaccination induces a regulated response of antibodies that neutralize the biological activity of simian IL-15, when aluminum hydroxide was used as adjuvant. The present work also provides the first safety elements of the anti-IL-15 vaccine in *Macaca fascicularis* monkeys, an animal model in which IL-15 shares a 97 % homology to the human molecule. This work received the Annual Award of the Cuban Academy of Sciences for the year 2017.

Keywords: IL-15, immunization, rheumatoid arthritis, neutralizing antibodies, non-human primates

RESUMEN

Una variante modificada de la interleucina-15 humana como antígeno novedoso para la inmunoterapia activa en la artritis reumatoide. La Interleucina-15 (IL-15) es una citocina pro-inflamatoria que desempeña un papel crucial en la patogénesis de la artritis reumatoide (AR), enfermedad inflamatoria crónica para la cual no existe una terapia efectiva. En este reporte, describimos una nueva vacuna basada en la inmunización activa con la IL-15 humana modificada para el tratamiento de la AR y otras enfermedades relacionadas con la sobreexpresión de la IL-15. La IL-15 obtenida en *E. coli* exhibe una conformación de los puentes disulfuro diferente a la descrita para la citocina nativa, lo que puede favorecer el desarrollo de una respuesta inmune contra este antígeno. Los resultados muestran que la inmunización con la IL-15 humana modificada genera anticuerpos policlonales específicos contra la citocina en primates no humanos, lo que sugiere una ruptura de la tolerancia a las células B como consecuencia de la inmunización. Estos anticuerpos inhiben la actividad biológica de la IL-15 nativa, sin afectar la proliferación de células CTLL-2 inducida por la IL-2 humana, demostrando la especificidad de los anticuerpos por la IL-15 autóloga. Además, demostramos que la vacunación induce una respuesta regulada de anticuerpos que neutralizan la actividad biológica de la IL-15 de simio, cuando se emplea el hidróxido de aluminio como adyuvante. El trabajo aporta, además, los primeros elementos de seguridad de la vacuna anti-IL-15 en monos *Macaca fascicularis*, un modelo animal en el que la IL-15 comparte una homología del 97 % con la molécula humana. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba en el año 2017.

Palabras clave: IL-15, inmunización, artritis reumatoide, anticuerpos neutralizantes, primates no humanos

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects about 1 % of the world population [1]. This disorder presents an imbalance in the production of anti-inflammatory and pro-inflammatory cytokines, the latter being found at high levels in the synovial fluid and the serum of patients. Experimental

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evidence has shown that many of these cytokines are involved in the pathogenesis and development of the disease, including TNF-α; a cytokine validated in clinical practice [2]. Although, the use of TNF-α antagonists has resulted in a great clinical benefit, it is described that 50 % of patients do not respond to treatment [3]; hence still requiring to develop new therapeutic strategies.

In this scenario, interleukin (IL)-15 a pro-inflammatory cytokine that plays a crucial role in the pathogenesis of RA, could provide a therapeutic target to at least ameliorate the disease. Previous studies have described elevated levels of IL-15 in the synovial fluid and in the serum of patients with RA. It is also known that this cytokine participates in the development of an inflammatory response through the induction of TNF-α, promoting the attraction of autoreactive T cells to synovial fluid [4]. On the basis of these antecedents, our group has directed its investigations towards the development of new therapeutic strategies to inhibit the inflammatory activity of IL-15.

In 2012, our group received the Annual Award of the Cuban National Academy of Sciences for the identification of a synthetic peptide that inhibits the biological effects of IL-15, by blocking the interaction of this cytokine with its specific receptor. This research strategy focused on a novel therapeutic candidate based on a modified variant of the IL-15 protein, as an antigen for active immunotherapy. In this work, we further expanded the concept of a modified human IL-15 as therapeutic molecule for RA, by inducing a neutralizing antibodies response against native IL-15. A modified variant of human IL-15 was obtained, which exhibits a different localization of the intramolecular disulfide bridges with respect to the native cytokine. We demonstrated that the immunization with this modified human IL-15 molecule induces neutralizing antibodies in non-human primates; suggesting that the vaccination is capable to disrupt B-cells tolerance. This work received the Annual Award of the Cuban Academy of Sciences for the year 2017.

Results and discussion

Obtention and characterization of modified human IL-15

hIL-15 was expressed in *Escherichia coli* and purified to 95 % of purity [5]. The molecule was characterized by mass spectrometry, showing that the disulfide bridges in the purified protein was formed between the contiguous cysteines (Cys^{35}-Cys^{42} and Cys^{85}-Cys^{88}), which differ from those described for the native protein (Cys^{35}-Cys^{85} and Cys^{42}-Cys^{88}) [6]. These results confirmed that the purified hIL-15, previously described by Santos *et al*. [5], was structurally modified with respect to the native protein. Taking into account this characteristic, the purified hIL-15 was denominated as modified human IL-15 (mhIL-15).

This study was the first report on the obtention of a modified variant of hIL-15 with a disulfide bridges pattern different from those described for the native cytokine. This feature favors the exposure of subdominant or cryptic epitopes, making possible to generate an effective antibody response against the autologous protein, and thereby, providing a strategy to try to break immune tolerance.

Immunization with mhIL-15 induces a neutralizing antibodies response in non-human primates

The immunogenic capacity of mhIL-15 was evaluated in *Macaca fascicularis* monkeys using aluminum hydroxide (alum), Montanide ISA-51 or Freund's Incomplete Adjuvant (FIA) [7]. Anti-IL-15 antibody titers were greater than 1/20 000 in all immunized groups (Figure 1). The highest titers were obtained in the group immunized with mhIL-15 formulated in FIA. These results demonstrated, for the first time, the break of immune tolerance to a self-cytokine molecule and the generation of a specific antibody response against IL-15. This was relevant, considering the high level of homology between human and macaque IL-15, sharing up to 97 % of its amino acid sequence.

The neutralizing capacity of sera was evaluated using a CTLL-2 cell proliferation assay [8]. We demonstrated that sera from immunized animals inhibited the biological activity of native IL-15 by decreasing the IL-15-induced proliferation of CTLL-2 cells. The highest neutralizing effect was obtained in the animals immunized with the mhIL-15 protein formulated in Alum (Figure 2). This also supported the selection of this adjuvant for active immunotherapy with mhIL-15. These results demonstrated, for the first time, the use of the IL-15 as antigen for a therapeutic vaccine to generate polyclonal neutralizing antibodies against autologous hIL-15 [7].

Characterization of the anti-IL-15 antibody response induced by mhIL-15 formulated in alum

The selected formulation of the mhIL-15 adjuvanted with Alum was further used to establish the timespan of the antibody response. Results showed that neutralizing antibody titers generated by immunization had a half-life of approximately 3 months. Noteworthy, the antibody response recovered to a similar extent of that achieved in previous immunizations before the administration of a boosting dose

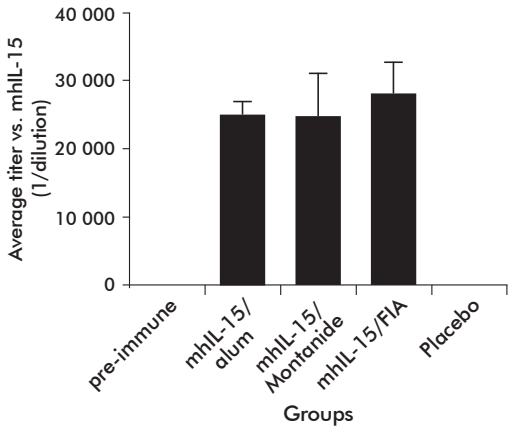

Figure 1. Antibody titers against modified human IL-15 (mhIL-15) in sera of immunized *Macaca fascicularis* monkeys. Sera was extracted 15 days after the third immunization with each antigen. Titers were expressed as 1/serum dilution, and values are shown as means (error bars stand for SD). FIA: Freund's Incomplete Adjuvant.

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[7]. These results indicated that regulation of the antibody response is controlled by vaccination and also suggests that the generation of memory B cells, which are activated in response to a new immunization. This is a very relevant concept since this could imply that vaccination with this immunogen would allow the manipulation of the immune response, by generating a transient therapeutic effect mediated by the antibody response against the endogenous hIL-15, without other undesired side effects due to uncontrolled autoimmune responses. This is of paramount importance in diseases such as RA which are characterized by periods of uncontrolled immune activation which lead to relapses and remission [9].

In order to know the specificity of the antibody neutralizing activity, the effect of sera from immunized animals on the IL-2-induced proliferation of CTLL-2 cells was evaluated. These cells proliferate in response to IL-2 or IL-15, two cytokines that share the β and γ subunits of their trimeric receptors [10, 11]. It was shown that sera from animals immunized with mhIL-15 did not affect the proliferation induced by IL-2 [7], demonstrating that the neutralizing effect of sera is specific for IL-15.

Effect of vaccination on IL-15 dependent cells and simian IL-15 biological activity

In order to elucidate the possible safety issues of vaccination with mhIL-15 formulated in alum, IL-15-dependent immune cell populations were studied. In this case, the number of $CD56^+$ NK cells and $CD8^+$ T cells was determined by flow cytometry in samples corresponding to 10 months after the fourth immunization and 15 days past the fifth inoculation [7]. Additionally, there was no variation detected on body weight, corporal temperature or cardiac and respiratory rates immunized vs. non-immunized monkeys (placebo group). There were also no changes in the main blood biochemical parameters. All these results support the use of the mhIL-15 formulated in Alum to generate a regulated, specific and safe anti-IL-15 antibody response in *M. fascicularis* monkeys.

Additionally, the simian IL-15 (siIL-15) was obtained [12], in order to evaluate the effect of immune sera on the activity of siIL-15 in IL-15-dependent cell lines. It was demonstrated that, despite the high sequence homology in the amino acid sequence between hIL-15 and siIL-15, sera from monkeys immunized with mhIL-15 were able to neutralize the biological activity of siIL-15 in the CTLL-2 and Kit225 cells [12].

These results suggest that the antibodies generated by immunization with mhIL-15 effectively recognize epitopes that are conserved within the sequence of IL-15 of both species, and proves the usefulness of *M. fascicularis* as robust species for the proof of concept of anti-IL-15 vaccines. This could foster the elucidation of the clinical potential of this cytokine in humans, due to the high homology of the protein between both species. Other species, such as rodents, are not adequate for the evaluation of this therapeutic strategy due to protein sequence divergence. This statement is supported by results published by our group where we demonstrated that sera from mice immunized with mhIL-15 do not neutralize murine

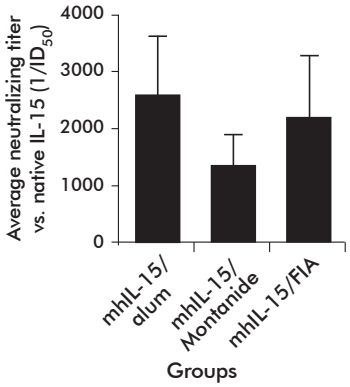

Figure 2. Neutralizing antibody titers against native IL-15 in sera of immunized *Macaca fascicularis* monkeys. Titers were calculated from data obtained in the CTLL-2 cell proliferation assay. Titers were expressed as the inverse of the serum dilution required to inhibit cell proliferation by at least 50 % (ID_{50}). Values are shown as means (error bars stand for SD). FIA: Freund's Incomplete Adjuvant.

IL-15 [8], a cytokine that shares only 73 % of amino acid identity with the human IL-15. Therefore, the demonstration of the usefulness of this monkey species for the pre-clinical characterization of the anti-IL-15 vaccine is another novel aspect of this work.

Relevance of the study

This work provided the first report on the obtention of a structurally modified hIL-15, as antigen for a therapeutic vaccine, which was able to induce a regulated response of specific and neutralizing antibodies against autologous IL-15 in *M. fascicularis* monkeys. This strategy has advantages over current therapeutic strategies using similar molecules (monoclonal antibodies) in that it requires a lower frequency of injections, a lower dose, a transient regulated response and the absence of anti-drug response, and significantly, is able to break the immune tolerance against the endogenous molecule. Taking into account the inflammatory activity of IL-15 in some autoimmune diseases, the strategy presented in this work could be applied to the treatment of patients with RA, psoriasis, multiple sclerosis and inflammatory bowel disease.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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