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**APL-2, an altered peptide ligand derived from heat-shock protein 60, induces interleukin-10 in peripheral blood mononuclear cell derived from juvenile idiopathic arthritis patients and downregulates the inflammatory response in collagen-induced arthritis model**

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**TITLE: APL-1, an altered peptide ligand derived from heat shock protein, alone or combined with methotrexate attenuates murine collagen induced arthritis**

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**Abstract**
Induction of tolerance to autoantigens *in vivo* is a complex process that involves several mechanisms such as the induction of regulatory T cells and changes in the cytokine and chemokine profiles. This approach represents an attractive alternative for treatment of autoimmune diseases. APL-1 is an altered peptide ligand derived from a novel CD4+ T-cell epitope of human heat-shock protein of 60 kDa (HSP60), an autoantigen involved in the pathogenesis of Rheumatoid Arthritis (RA). We have shown previously that this peptide efficiently inhibited the course of adjuvant induced arthritis in Lewis rats and induced regulatory T cell (Treg) in *ex vivo* assay with PBMC isolated from RA patients.
This study was undertaken to evaluate the therapeutic effect of APL-1 and its combination with metotrexate (MTX) in collagen induced arthritis (CIA).
CIA was induced in male DBA/1 mice at eight weeks of age by immunization with chicken collagen. APL, MTX or both were administrated beginning from arthritis onset. Therapeutic effect was evaluated by arthritis and joint pathologic scores. In addition, TNFα and IL-10 in sera was measured by ELISA. Treg induction was assessed by FACS analysis
APL-1 inhibits efficiently the course of arthritis in CIA, similar to MTX. In addition, therapy with APL-1 plus MTX reduced CIA in mice, associated with an increase of Treg. These facts reinforce the therapeutic possibilities of APL-1 as a candidate drug for treatment of RA.

## Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by persistent inflammatory synovitis leading to various degrees of cartilage destruction, bone erosion with consequent joint deformity and loss of joint function (1). Disease prognosis can be significantly improved by early treatment with disease-modifying anti-rheumatic drugs (DMARDs) (2). During the nineties, methotrexate (MTX) became first-line therapy for RA, propelled by therapeutic successes when combined with other drugs and an acceptable toxicity profile at the dosages used for this indication (3). However, a complete remission is achieved in only a fraction of RA cases (4).

Biologic therapy is an alternative for patients not responding to MTX or other DMARDs, constitutes the best addition to the anti-rheumatic arsenal. Using biologicals to treat this disorder affords the possibility of targeting, in a more specific fashion, only those components playing an important role in the pathogenesis of RA (5). However, many patients have an inadequate response to such therapies (6,7). Besides, this therapy remains insufficient in 40-50 % of patients with RA (8).

In order to achieve additional significant gains in RA therapy, other approaches need to be evaluated intensely. One of which is the induction of peripheral tolerance by antigen-specific therapy. This approach is aimed at eliminating only T-cell clones that have escaped the control mechanisms of peripheral tolerance (9). The central role of T cells in the pathogenesis of RA is well established (10). In physiological condition, the induction of antigen-specific tolerance is indispensable for immune homeostasis and the control of autoreactive T cells responsible of the onset of autoimmune diseases. The function of regulatory T cells (Treg) populations in maintaining homeostasis is increasingly well understood (11). CD4+CD25$^{high}$ FoxP3+ Tregs are a critical subset of cells that play an essential role in controlling immune responses by suppressing the proliferation and effector functions of T cells (12).

Several studies have been conducted to evaluate the role of Treg cells in RA. There have shown a reduced suppressive function of Tregs in patients compared to healthy controls (13,14,15). However, in patients inflammation persists suggesting that these cells are unable to suppress ongoing disease (12). This phenomenon is possibly due to an inhibition of their functions by pro-inflammatory cytokines and/or because of the increased number of activated effector T cells [16,17,18].

The potentialities of the altered peptide ligands (APLs) as inductors of Treg have been broadly reported by several authors (19,20,21). The APLs are similar to original epitopes but with one or several substitutions in the essential contact positions with the TCR or with the MHC class II molecule, which interfering the cascade of necessary events for activation of T cells. These peptides can block the response of autoreactive T cells by different mechanisms in the control of autoimmune diseases (22,23,24).

Antigen specific approaches using APLs can manipulate in a more specific way the balance between Tregs and effector T cells. The selection of a specific autoantigen is an essential point in this approach. HSP60 has been successfully used in the induction of tolerance in autoimmune arthritis [25,26]. We previously predicted two novels CD4+ T cell epitopes (E18-3 and E18-12) from human HSP60 by the use of bioinformatics tools (27,28). In particular, the wild-type peptide E18-3 was modified in one aminoacid residue for increasing its affinity to HLA class II molecule related to RA. According to previous results, stimulation with this new peptide, called APL-1, increases the frequency of CD4+CD25$^{high}$FoxP3+ Tregs from peripheral blood mononuclear cells (PBMCs) of RA patients (27).

Diverse animal models for RA have been used to evaluate the therapeutic effect of APLs. We have shown previously that APL-1 efficiently inhibited the course of adjuvant induced arthritis (AA) in Lewis rats (27). AA is an experimental autoimmune disease with several features of RA. It can be induced in susceptible inbred strains, as Lewis rats, upon immunization with heat-killed *Mycobacterium tuberculosis* (Mt) in incomplete freund adjuvant (29). CIA has become the most widely used model for studying RA pathogenesis and validation of therapeutic targets. Arthritis is currently induced in mice by immunization with autologous or heterologous type II collagen in adjuvant. Susceptibility to collagen-induced arthritis is strongly associated with major histocompatibility complex class II genes, and the development of arthritis is accompanied by a robust T- and B-cell response to type II collagen (30).

Here, we aimed to evaluate the therapeutic effect of APL-1 and its combination with MTX in CIA. Both therapies inhibit efficiently the course of CIA in mice. This effect was associated with a decrease of TNFα levels. However, only therapy with APL-1 plus MTX increases the frequency of Tregs in mice.

## MATERIALS AND METHODS

### Antigens and adjuvants

Chicken type II collagen (CII) was obtained from Hooke Laboratories (USA). Incomplete Freund's adjuvant (IFA; Difco) and Complete Freund's adjuvant (CFA, Difco) were used as adjuvants. APL-1 was manually synthesized by the Fmoc/tBu strategy in syringes using the Fmoc-AM-MBHA resin (0.54 mmol/g). The peptide was purified to more than 95% by high performance liquid chromatography (HPLC), lyophilized and analyzed by reverse phase HPLC and mass spectrometry.

### Induction and clinical assessment of CIA

Each DBA/1 mouse was immunized intradermally with 50 µg/mL of chicken CII emulsified in Complete Freund Adjuvant (CFA), followed by a booster dose of chicken CII emulsified in IFA (Hooke, USA) on day 21. The severity of arthritis in each paw was determined according to an established scoring system as follows: 0, Normal paw; 1, one finger inflamed and swollen; 2, more than one fingers, but not entire paw, inflamed and swollen or mild swelling of entire paw; 3, entire paw inflamed and swollen; 4, very inflamed and swollen paw or ankylosed paw. Therefore, each mouse can receive a maximum score of 16 points.

### Peptide and MTX immunotherapy protocols

On day 26, CIA mice were randomly divided into four treatment groups (12 mice per group). Treatments were administered on days: 28, 31, 34, 37, 40, and 43 after disease induction. APL-1 (50µg) and PBS (50µL) were inoculated by subcutaneous route. MTX (60 µg) was inoculated by intraperitoneal route. The fifth group corresponds to healthy animals (12 mice).

Table 1 summarizes the therapy for each animals group

| Groups | Therapy |
|---|---|
| I | Placebo (PBS) |
| II | APL1 |
| III | MTX |
| IV | APL1 + MTX |
| V | Healthy |

**Histopathological analysis**

Ankle joints were harvested on day 46 after induction of CIA. Hind limbs were removed and fixed in 10% neutral buffered formalin (PANREAC, Spain) at room temperature during 5–7 days and were decalcified with formic acid (50% v/v) and sodium citrate (13% w/v). The tissues were dehydrated in alcohol gradient and embedded in paraffin. Tissue sections (2–3mm) were stained with haematoxylin and eosin. The histologic damage was defined according to the following system: Grade 0, normal; Grade 1, mild synovitis with hyperplastic membrane and no inflammatory reaction; Grade 2, moderate synovitis without pannus formation, bone and cartilage erosions limited to discrete foci; Grade 3, severe synovitis with pannus formation, extensive erosions of bone and cartilage, and disrupted joint architecture. All these histo-pathological procedures were performed totally blinded.

**Detection of TNFα and IL-10 levels**

Blood samples from four mice of each group were collected at day 46, TNFα and IL-10 were determined in serum. Cytokines were measured with commercially available ELISA kits (Quantikinew, R&D Systems) according to the manufacturer's instructions.

**Evaluation of Treg Cells Induced by APL-1**

The spleen from four mice of each group were removed and homogenized on day 46 after induction of CIA. Cells were stained using anti-CD4-FITC (clone RMA-5) (eBioscience) according to the manufacturer's instructions. Cells (1x $10^6$) were resuspended in staining buffer (phosphate-buffered saline [PBS] containing 3% fetal bovine serum (FBS) and stained with anti-CD4-FITC or isotype control for 30 min at 4ºC. Stained cells were subsequently washed twice in staining buffer and resuspended in fixation/permeabilization buffer for intracellular staining of FoxP3 protein at 4ºC for 45 min. The mononuclear cells were stained with anti-mouse FoxP3 (clone FJK-16s) antibody or isotype control for 30 min at 4ºC. FoxP3 was performed on CD4+ gated T cells by a FACS Partec flow cytometer (Partec GmbH) using the Partec Flomax software. Results were expressed as the percentage of CD4+ FoxP3+ T cells.

**Statistical analysis**

Data analyses were performed using GraphPad Prism version 5.00 (GraphPad Sofware, San Diego California, USA). Samples were examined for normality and equal variance with Kolmogorov-Smirnov and Bartlett's tests, respectively. Results were expressed as mean ± standard deviation (SD) and differences between treatment groups were analyzed with ANOVA and Tukey's post-test. Kruskal Wallis and Dunn's post-test were also performed where appropriate. P-values less than 0.05 were considered statistically significant.

## Results

### APL-1 monotherapy and its combination with MTX reduced arthritis in mice

In order to confirm the therapeutic possibilities of APL-1 for RA, we evaluated healing effects of this peptide in the CIA model. In addition, APL-1 plus MTX was evaluated in this animal model. CIA was induced in male DBA/1 mice by twice subcutaneous immunization with CII. The mice were randomly divided into 4 treatment groups on day 26 after induction of arthritis: I) ill animals without treatment (inoculated with PBS as control), II) treatment with APL-1, III) treatment with MTX, IV) treatment with APL-1 plus MTX. The fifth group corresponds to healthy animals. Three independent experiments were performed, with 12 mice per group.

As it is shown in figure 1, the signs associated with the development of arthritis began gradually in all animals inoculated with CII. These signs were evident on day 17, characterized by a slight redness and inflammation of the posterior joints. It is also observed that the administration of APL-1 induced a significant reduction of the clinical signs of arthritis in mice. However, in animals of group I (placebo), the arthritis signs were expanding to the rest of the joints until became severe in all mice.

**Figure 1**

Treatment with APL-1 reduced the severity of CIA compared to PBS treated mice from day 37 after the first immunization until the end of protocol (Fig.1). Similar results were observed in mice treated with MTX or treated with APL-1 plus MTX. These mice showed significantly lower arthritis score compared to PBS treated mice ($P<0.05$) but not with APL-1 (Fig.1).

Clinical improvement of CIA induced during therapies was compared with decrease of the joint destruction by the arthritic process. Four animals were sacrificed per group and ankle joints were collected on day 46 after the induction of arthritis and scored for severity of inflammation in the synovium, pannus formation, and cartilage and bone erosion.

A considerable correspondence between the data obtained by the evaluation of the clinical signs and the histopathological report was found. The monotherapy with APL-1 led to significant improvement of the histological score of the joints (Table 2). The mice inoculated with APL-1 presented very slight damages. Similar results were finding in mice inoculated with MTX or APL-1 plus MTX. In contrast, the animals inoculated with PBS showed severe erosion of

cartilage and bone as well as massive inflammatory cell infiltration and pannus formation in the joins (Table 2 and Figure 2).

**Figure 2, Table 2**

**APL-1 monotherapy and its combination with MTX reduced TNFα in CIA mice**

The production of TNFα and IL-10 was investigated in the serum from four sacrificed mice at day 46 after disease induction. As shown in Fig. 3, the treatment with APL-1 significantly reduced TNFα secretion compared to PBS inoculated mice ($P<0.05$).

**Figure 3**

In addition, therapy with APL-1 plus MTX or MTX alone significantly reduced TNFα secretion compared to mice inoculated with PBS and mice treated with APL-1 ($P<0.05$). But, levels of TNFα in these two groups were similar to those obtained in healthy animals.

No difference was observed in the levels of serum IL-10 among the four groups (results not shown).

These results evidence that APL-1 monotherapy or its combination with MTX inhibits production of TNFα. But, these treatments do not have influence on the level of IL-10.

**APL-1 plus MTX induced Treg in CIA mice**

Four mice of each group were sacrificed on days 46 (after CIA induction), and variations of Treg in spleen were evaluated by Flow Cytometric Analysis. Samples were screened for frequency of FoxP3 positive cells among CD4+ T cells. Figure 4 A and B show the analysis corresponding to a mouse treated with APL-1 alone and a mouse treated APL-1 plus MTX, respectively.

APL-1 plus MTX induced an increase of the proportions of CD4+FoxP3+ Treg cells in the spleen at day 46, after CIA induction as shown in figure 4C. In contrast, APL-1 monotherapy does not induce this effect.

**Figure 4**

**Discussion**

APL-1 is considered as a therapeutic candidate for RA and other autoimmune diseases. One of its possible modes of action is the induction of Tregs (27). Conceptually, such a peptide mediated therapeutic intervention is based on modulation of antigen specific T cells and therefore lower toxicity is expected (31), compared to biological therapeutic agents that target broadly active inflammatory cytokines (32).

We have reported that APL-1 increases the frequency of CD4+CD25$^{high}$FoxP3+ Treg in *ex vivo* assays using PBMC or SFMC from RA patients. Also, APL-1 efficiently inhibited the course of adjuvant-induced arthritis (AA) in Lewis rats. This therapeutic effect was associated with an increase of the proportions of Tregs (27). In AA, the disease is induced by immunization with *Mycobacterium tuberculosis*; however at the same time it protects against subsequent arthritis induction and this protection is mediated by T cells that recognized a conserved sequence of Mt HSP60, peptide M256-270. Also, this effect was associated with the production of regulatory cytokines (33,34).

Here, therapeutic effect of APL-1 was evaluated in CIA model, where arthritis is induced by other autoantigen. CIA has also been the model of choice in terms of testing new potential therapeutic agents for treatment of human RA. Previous animal experiments have shown that APLs derived from CII administered subcutaneously or intravenously can inhibit the progression of CIA (35).

The susceptibility to CIA is determined by the I-Aq (MHC class II molecule). APL-1 contains an epitope that could be presented by mouse MHC class II molecules, according to the program RANKPED (36).

In the present case, APL-1 monotherapy induced excellent clinical control of CIA. This effect was correlated with improvement of the histological score of the joints induced by the peptide and it was comparable to mice treated with APL-1 plus MTX. These results were associated with a decrease of TNFα levels. TNFα is known to be involved in stimulating inflammatory cytokines (including itself) production, enhancing the expression of adhesion molecules and neutrophil activation, and it is also a costimulatory of T-cell activation and antibody production by B cells (37). The pivotal role of TNFα in the induction and progression of rheumatoid synovitis is well established (38,39). Additionally, pannus formation and joint damage was not observed. Such facts suggest that infiltration of macrophages and neutrophils did not occur in the synovium of

mice inoculates with APL-1 alone or combined with MTX. Similar results were obtained in mice treated with MTX, which is the current standard treatment for RA. This result is in agreement with the work by Neurath et al (40), who demonstrated that intraperitoneal administration of MTX reduced TNFα and INF-γ levels in sera of CIA mice.

APL-1 increases Treg and a decrease TNFα levels in spleen of AA rats, during the treatment (27). Here, APL-1 did not induce Treg in CIA mice. However, the combination of APL-1 plus MTX increases the proportions of the Treg in the spleen of CIA mice. In this sense, this result could be associated with molecular action of MTX in inflammatory diseases. This drug produces a decrease of neutrophils and macrophages, which secrete high levels of TNFα and INF-γ (40). In our experiments, the reduction of TNFα induced by MTX alone or combined with APL-1 was superior to induce by APL-1. This fact could contribute to increase of Treg in the spleen of CIA mice treatment with APL-1 plus MTX. Although the molecular mechanism of MTX has been studied exhaustively, this does not associate with an increase of Treg (41). These facts suggest that MTX and APL-1 could have a complementary molecular effect in CIA model. Also, it is possible that Treg induced by APL-1 was not detected on day 46. Taking in consideration this possibility, Treg induced by APL-1 previous on day 46 should be quantified in further study.

Nevertheless, we think that the mechanism of action of APL-1 in both animal models is very similar. Probably, the potent therapeutic effect of the APL-1 in the reduction of AA and CIA is due to processing and presentation of the peptide by the APC to the autoreactive T lymphocytes in periphery. The recognition of this altered ligand may induce the expansion of Treg. These cells migrate to the inflammation site and could attenuate autoreactive T cells responsible of the arthritis pathogenesis, inhibiting the TNFα expression. Consequently, the demonstration that APL-1 inhibited the expression of TNFα in two animal model of arthritis represents a beneficial effect for the control of inflammatory process.

These results indicate a therapeutic potentiality of APL-1 and support further investigation of this candidate alone or combined with MTX for treatment of RA. Intervention on T cell function in a specific manner as shown here would give the possibility of focusing on one or more antigens involved in the RA pathogenesis, thus avoiding the patients generalized immune suppression as happen with anti-TNF treatment. This study contributes to the knowledge of mechanisms and tools needed for induction of tolerance in humans using autoantigens or theirs variants.

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