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An altered peptide ligand corresponding to a novel epitope from heat-shock protein 60 induces regulatory T cells and suppresses pathogenic response in an animal model of adjuvant-induced arthritis

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Abstract

Induction of immune tolerance as therapeutic approach for autoimmune diseases constitutes a current research focal point. In this sense, we aimed to evaluate an altered peptide ligand (APL) for induction of peripheral tolerance in patients with rheumatoid arthritis (RA). A novel T-cell epitope from human heat-shock protein 60 (Hsp60), an autoantigen involved in the pathogenesis of RA, was identified by bioinformatics tools and an APL was design starting from this epitope. We investigated the ability of this APL for inducing regulatory T cells (Treg cells) in mice and evaluated the therapeutic effect of this peptide in an adjuvant-induced arthritis (AA) rat model. Clinical score, TNFα levels and histopathology were monitored, as well as the capacity of this APL for inducing Treg cells. Finally, the potentialities of the APL for inducing Treg cells were evaluated in *ex vivo* assays using mononuclear cells isolated from peripheral blood (PBMC). The APL induced an increase of the proportions of Treg cells in the draining lymph nodes of the injected site in mice. The APL efficiently inhibited the course of AA, with significant reduction of the clinical and histopathology score. This effect was associated with an increase of the proportions of Treg cells and a decrease of TNFα levels in spleen. Finally, stimulation of PBMCs from RA patients by the APL increases the proportions of the $CD4^{+}CD25^{high}FoxP3^{+}$ Treg cells. These results indicate a therapeutic potentiality of APL and support further investigation of this candidate drug for treatment of RA.

Keywords: *Rheumatoid arthritis, APL, HSP60, tolerance, regulatory T cells*

Introduction

Substantial progress in the understanding of the mechanisms of autoimmune diseases, including rheumatoid arthritis (RA), has taken place in recent years. The central role of T cells in the pathogenesis of RA is well established [1,2]. The function of Treg populations in maintaining homeostasis is increasingly well understood [3]. In physiological conditions, the induction of antigen-specific tolerance is indispensable for immune homeostasis and the control of autoreactive T cells responsible for the onset of autoimmune diseases.

Several recent studies have identified defects in the number or activity of Treg cells in patients with RA [4]. These defects have been attributed in part to the pathogenic role of TNFα in RA [5]. Biological therapeutic agents targeting this cytokine have been used successfully in the treatment of RA [6,7]. But, the anticytokine approaches remain vulnerable by the limitations associated eminently with generalized immunosuppression [8–10]. Also, this therapy remains insufficient in 40–50% of patients with RA [10]. However, it has been reported recently that this therapy restored the function of Treg cells in responder patients [11].

In this context, the modulation of peripheral pool of Treg cells that could suppress the pathogenic T cells specifically, without affecting other non-related T cells, constitutes the main challenge in the treatment of RA. This approach can induce tolerance mediated by activation of Treg cells, and in consequence

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avoiding the generalized immune suppression in patients as happen with anti-cytokine treatment.

The induction of peripheral tolerance using autoantigens involved in autoimmune disease pathogenesis constitutes an alternative to this purpose. The selection of a specific autoantigen is a crucial point in this approach. The HSP60, a protein that belongs to the HSP family is a candidate as autoantigen. Cohen and Young postulated that HSP could be part of the immunological homunculus, which includes a few dominant self-antigens encoded in a cell regulatory network comprising the immune system's picture of self [12]. The HSP60 could trigger the activation of Tregs [13–15]. Some authors had identified epitopes in the HSP60 involved in the regulation mechanisms in animal models [16,17].

Recently, Zanin-Zhorov et al. [18] found that $CD4^+CD25^+$ T reg cells are innately responsive to HSP60 *in vitro* assays, and when treated with HSP60 or its p277 peptide, these cells are significantly more effective than untreated Tregs for downregulating $CD4^+$CD25- or $CD8^+$ T cells.

On the other hand, other authors have selected epitopes of the human HSP with the objective of inducing tolerance in patients with autoimmune diseases [19–21]. The potentialities of the altered peptide ligands (APLs) as inductors of tolerance have been broadly reported by several authors [22–24]. 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 interfering with 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 [24–28].

Here, a novel T cell epitope located in the N terminal region of human HSP60 was predicted by bioinformatics tools and was used to design an APL. This APL induces an increment of T reg cells and inhibits efficiently the course of arthritis in an animal model for RA.

Materials and methods

Prediction of T cell epitope from hHsp60 and design of APL

A T cell epitope from human HSP60 was predicted using the program ProPred [29]. The peptide selected as a T cell epitope, was modified in one of the positions involved in the binding site specific to HLA class II molecules for increasing the affinity of the peptide for this molecule, using the prediction server ProPred [29]. The modification made in the peptide was in agreement with the HLA class II molecules frequently expressed by RA patients: DR1 (DRB1*01 and DRB*02) and DR4 (DRB*0401, DRB*0404, DRB*0405 and DRB*0408).

Antigens and adjuvants

Heat-killed Mycobacterium tuberculosis (Mt) (strain H37Ra) was obtained from Difco (Detroit, MI). Freund's incomplete adjuvant (IFA; Difco) was used as adjuvant. Peptides were manually synthesized by the Fmoc/tBu strategy in syringes using the Fmoc-AM-MBHA resin (0.54 mmol/g). After treatment with TFA, the peptides were lyophilized and analyzed by reverse phase HPLC and mass spectrometry.

Experimental animals

Female inbred *Lewis* rats, $RT1.B^L$ (5–8 weeks of age, weighing 101–120 g), were purchased from the National Center for the Production of Laboratory Animals (CENPALAB, Havana, Cuba). The animals were free from rat pathogens as tested in a health-monitoring program at the CENPALAB. Rats were kept in a 12-h light–dark cycle and housed in polystyrene cages (TECNIPLAC, Italy) containing aspen wood shavings, with full access to food and water during experiments. BALB/c and BALB/c Thy-1.1 mice were housed in filter-topped cages under specific pathogen-free conditions.

All animal procedures were performed in accordance with the guidelines approved by the Ethical Committee and National Regulations for experiments with animals and by the European Union guidelines for animal experimentation.

Flow cytometry analysis of Treg cells recruitment after peptide administration in mice

BalB/c mice were inoculated with 50 μg/ml of wild-type peptide or APL by subcutaneous route and were boosted on day 5 in identical conditions. Four animals per group were sacrificed on day 4 or 9 after the first peptide inoculation. After mechanical dissociation, cells from spleen or peripheral LNs of mice were pre-incubated with 2.4G2 mAb (BD Biosciences) to block non-specific binding to Fc receptors and then stained in phosphate-buffered saline (PBS) 3% fetal calf serum with saturating amounts of combinations of the following mAbs from BD-Biosciences: APC-Cy7-conjugated anti-CD4 (clone RM4-5); APC-labeled anti-CD25 (PC61). Isotype-irrelevant mAbs (BD Biosciences) were used as controls. Intracellular labeling of transcription factor FoxP3 by anti-FoxP3 antibody conjugated to PE (FJK-16s; eBioscience) was performed according to the manufacturer's recommendations. Isotype-irrelevant mAbs were used as controls. Lymphocytes were gated according to their forward and side scatter characteristics and acquired on a LSR-II™ cytometer. Analyses were performed with FlowJo (Tree Star) software.

Induction and assessment of AA in Lewis rats

Each animal was inoculated subcutaneously at the base of the tail with a freshly prepared emulsion (100 μl) containing 1 mg of Mt in IFA. The rats were observed daily for signs of arthritis from day 0 until day 35 and then, every 3 days from day 36 until the end of the experiment at day 50. The severity of arthritis in each paw was determined according to an established scoring system as follows: 0, no disease; 1, slight swelling of the ankle or doll, or visible redness and inflammation of at least one finger, independently of the number of affected fingers; 2, moderate redness and swelling of the ankle and the doll; 3, severe redness and swelling of the whole paw including the fingers; 4, maximum swelling and deformity of the paw involving multiple joints. Therefore, each rat can receive a maximum score of 16 points.

Peptides immunotherapy protocols in Lewis rats

On day 10, rats inoculated with Mt were randomly divided into three treatment groups. Two groups were inoculated with the APL, one group by intradermal route and the other one by subcutaneous route. The third group was inoculated with the wild-type peptide by intradermal route.

The peptide was administered on days 11, 14, and 18 after disease induction; each dose contained 200 μg of peptide in PBS. The rats were anaesthetized using ketamine (50 mg/kg, intramuscular) previous to the inoculation.

Histopathological analysis of ankle joints from Lewis rats

Ankle joints were harvested on day 21 after induction of AA. 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 (2–3 μm) were stained with haematoxylin and eosin.

The histological damage, evaluated microscopically, was defined according to the following system: Grade 0, normal; Grade 1, mild synovitis with hyperplastic membrane, no inflammatory reaction; Grade 2, moderate synovitis without pannus formation, bone and cartilage erosions limited to discrete foci, and undisrupted joint architecture; Grade 3, severe synovitis with pannus formation, extensive erosions of bone and cartilage, and disrupted joint architecture. All these histopathological procedures were performed totally blinded.

Evaluation of Treg cells induced by peptides in Lewis rats

The spleen from three rats of each group were removed and homogenized on day 21 after induction of AA. Cells were stained using FITC anti-rat CD4 (clone OX35) and PE anti-rat CD25 (clone OX39) (eBioscience) according to the manufacturer's instructions. Cells (1 × 106) were resuspended in staining buffer (PBS containing 3% fetal bovine serum [FBS]) and stained with anti-CD4-FITC and anti-CD25-PE antibodies or isotype control for 30 min at 4°C. Stained cells were subsequently washed twice in staining buffer and resuspended in fixation/permeabilization buffer for intracellular staining of FoxP3 protein at 4°C for 45 min. The mononuclear cells were stained with anti-rat FoxP3 (clone FJK-16s) antibody or isotype control for 30 min at 4°C. FoxP3 was performed on $CD25^{high}$ $CD4^{+}$ gated T cells by a FACS Partec flow cytometer (Partec GmbH) using the Partec Flomax software. Results were expressed as the percentage of $CD4^{+}$ $CD25^{high}FoxP3^{+}$ T cells.

Determination of TNFα production

Splenocytes from three rats of each group were obtained as described here. Cells were washed once with PBS 1X and resuspended with RPMI 1640 supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 units/ml gentamycin, 25 mM/l HEPES (all from Gibco BRL, England). Cells were cultured by triplicate (1 ml per well) in 24-well plates (Costar, Cambridge, MA) at 10^{6} cells/well. Afterwards, 40 μg/ml of wild-type peptide or APL were added. RPMI 1640 was used as control for cell growth. Cells were incubated during 24 h and later on, the supernatant of each well was taken and TNFα concentration was determined by specific kits (Quantikine®, R&D Systems) according to the recommendations of the manufactures.

Evaluation of Treg cells induced by peptides in mononuclear cells from RA patients

Eleven patients with RA diagnosis were included in this study. The protocol was approved by the Ethics Review Committees of the Center for Genetic Engineering and Biotechnology and the National Institute of Rheumatology. Informed consent was obtained from all patients.

First, 10 ml of blood were extracted from each patient and diluted 1/2 in PBS 1X. Of Ficoll-Paque 3 ml (Amershan) were added to 5 ml of diluted blood and centrifuged in tubes of 15 ml during 30 min at 1200 rpm. The ring corresponding to mononuclear cells was collected. Cells were washed twice with 15 ml of PBS 1X and centrifuged at 900 rpm after each washing. Finally, the precipitate was resuspended in RPMI 1640 medium containing 10% of bovine fetal serum supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 25 HEPES mM/l and L-glutamine 2 mM (all from Gibco BRL).

In the case of mononuclear cells isolated from synovial fluid (SFMC), fluid was transfer to a sterile 50 ml conical tube and incubated with 10 μg of

hyaluronidase (Sigma, USA) per ml of fluid during 1 h at room temperature. Then, the tubes were centrifuged at 12,000 rpm for 15 min at room temperature. Supernatants were taken and diluted 1/2 in PBS 1X. Then, 3 ml of Ficoll-Paque (Amershan) and treated as in the case of mononuclear cells isolated from peripheral blood (PBMC).

PBMC or SFMC were cultured in triplicate (1 ml per well) in 24-well plates (Costar) at 10^6 cells/well. Afterwards, 40 μg/ml of APL or wild-type peptide was added. The RPMI 1640 was used as control for cell growth. The mononuclear cells were incubated during 5 days and stained using Human Regulatory T cell Staining Kit (w/APC FoxP3, FITC CD4, PE CD25) (eBioscience) according to the manufacturer's instructions.

Cells (1×10^6) were resuspended in staining buffer (PBS containing 3% FBS) and stained with anti-CD4-FITC (clone RPA-T4)/anti-CD25-PE (clone BC96) antibody cocktail for 30 min at 4°C. Stained cells were subsequently washed twice in staining buffer and resuspended in fixation/permeabilization buffer for intracellular staining of FoxP3 protein at 4°C for 45 min. After washing, cells were blocked with normal rat serum for 15 min at room temperature. The mononuclear cells were stained with anti-human FoxP3 antibody (clone PCH101) or isotype control for 30 min at 4°C. FoxP3 was performed on $CD25^{high}CD4^+$ gated T cells by a FACS Partec flow cytometer (Partec GmbH) using the Partec Flomax software. Results were expressed as the percentage of $CD4^+CD25^{high}FoxP3^+$ T cells.

Statistical analysis

Statistical analysis was performed with Sigma Stat software (version No. 2, GraphPad Software, Inc.). Data were analyzed using the Mann–Whitney rank sum test and the Student–Newman–Keuls test. Bilateral Student's *t*-test was used in experiments with mice. Statistical significance was established at $p < 0.05$.

Results

APL design

In contrast, with previous studies, we focused on the N-terminal region of human Hsp60. In this region, the computer algorithm Propred predicted a new epitope, corresponding to amino acid (aa) 90–109, directly involved in the interaction with HLA class II molecule (Figure 1). This peptide was called E18 and the sequence is shown in Figure 1. E18 presents a 100% similarity between human, rat and mice but 50% similarity with *Mycobacterium tuberculosis*.

SIDLKDKKYKNIGAKLVQDVANNTNEEA (E18-3)

SIDLKDKKYKNIGAKLVQ**L**VANNTNEEA (E18-3 APL1)

Figure 1. Amino acid sequence of the wild-type peptide and the derived APL. The amino acid modified in the APL has been represented in bold.

An amino acid residue involved in the interaction with the HLA class II molecule was changed for increasing the affinity to this molecule. The HLA class II molecules frequently expressed by RA patients were considered. This new peptide was called APL-1 and its sequence is shown in Figure 1.

Induction of Treg cells by APL-1 in mice

The hypothesis that APL-1 could induce an increase of Treg cells was evaluated *in vivo*. We evaluated Treg proportion in draining and non-draining lymph nodes (dLN) of mice inoculated subcutaneously with 50 μg/ml of wild-type peptide or APL-1 on days 0 and 5. A group of animals was inoculated with PBS as control. Four animals of each group were sacrificed on days 4 and 9, and variations of T cells in spleen and peripheral LNs were evaluated by flow cytometric analysis.

APL-1 induced an increase of the proportions of $CD4^+FoxP3^+$ Treg cells in the dLN at the two time points of the analysis, and in the spleen at day 9. This increase was statistically significant at day 9 as shown in Figure 2. In contrast, the mice treated with the wild-type peptide presented a statistically significant increase of the proportions of $CD4^+FoxP3^-$ helper T cells in the spleen at day 4 compared to control mice and a significant increase of the proportions of T helper cells in the dLN at day 9, which is statistically significant compared to non-draining lymph node (ndLN), but not to the control mice.

Reduced arthritis in rats treated with APL-1

Female Lewis rats were immunized with 100 μg MT to induce AA. The rats were randomly divided into 3 treatment groups and 2 control groups on day 10 after induction of arthritis: (I) treatment with APL by intradermal route (II) treatment with APL by subcutaneous route, (III) treatment with wild-type peptide by intradermal route, (IV) no treatment, (V) healthy animals. Three independent experiments were performed, with 12 rats per group. The rats from groups I to III were inoculated with 200 μg/ml of specific peptide by intradermal or subcutaneous route, at days 11, 14 and 18 after induction of arthritis.

As is shown in Figure 3, in the non-treated animals, the signs associated with the development of arthritis began gradually and were evident on day 10, characterized by a slight redness and inflammation of the posterior joints. At later time points, the arthritis signs were expanding to the rest of the joints until became severe in all rats. The mean arthritis score on day 21 (the day of maximum arthritis severity) was the main parameter used in this study to measure the clinical outcomes and evaluation of the effects of the peptides.

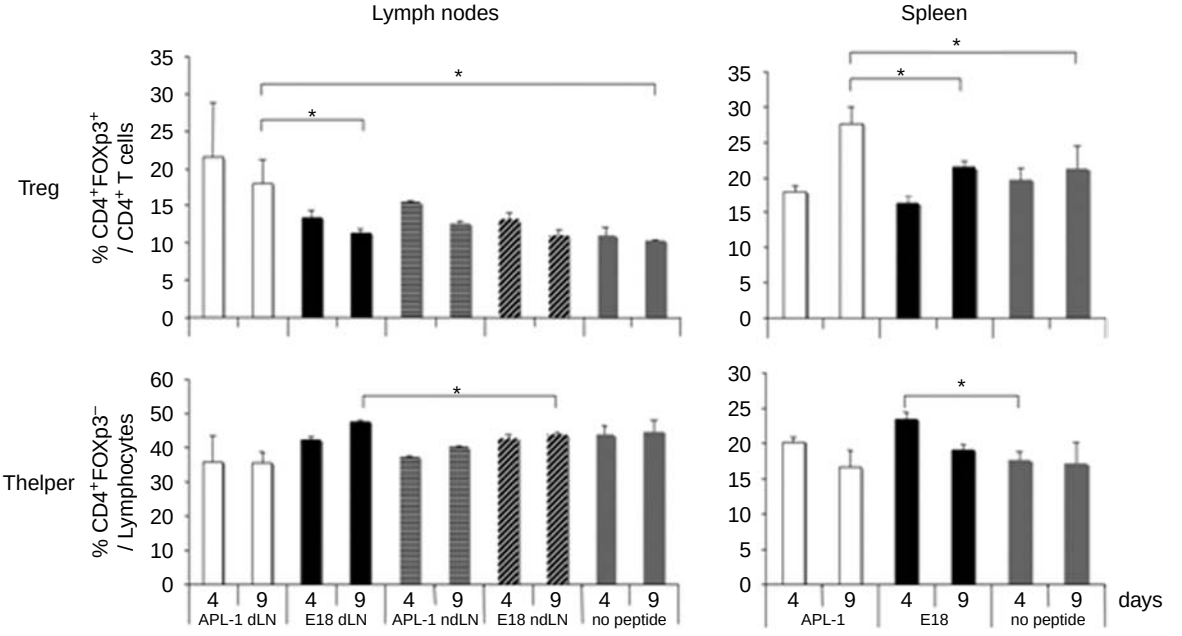

Figure 2. Treatment with APL-1 induced an increase of the proportions of $CD4^+FoxP3^+$ Treg cells in mice. Four animals of each group were sacrificed on days 4 and 9 after subcutaneous immunization with wild-type peptide or APL1. Treg proportion was evaluated in dLN, ndLN and spleen by flow cytometric analysis. (*) mean statistically significant differences $p < 0.05$.

Treatment with the wild-type peptide showed a trend towards reduction of arthritis, but statistically significant differences were not observed versus non-treated animals (Figure 3).

A significant reduction of AA mean arthritis score on day 21 ($p = 0.001$) was achieved with the APL compared to non-treated animals and rats inoculated with the wild-type peptide. Similar results were obtained in three independent experiments.

The histological signs of arthritis were also evaluated: four animals were sacrificed per group and ankle joints were collected on day 21 after induction of arthritis and scored for severity of inflammation in the synovium, pannus formation, and cartilage and bone erosion. A close correlation between the clinical signs and the histopathological findings was found. All rats without treatment presented a histological score of 3. The rats from group 1, inoculated with APL-1 by intradermal route did not present any histological damage in the joints, as shown in Figure 4. Although the group 2 corresponding to rats inoculated with APL-1 by subcutaneous route, present very slight damage, the rats treated with the wild-type peptide did not present differences of the histological score with respect to the ill group.

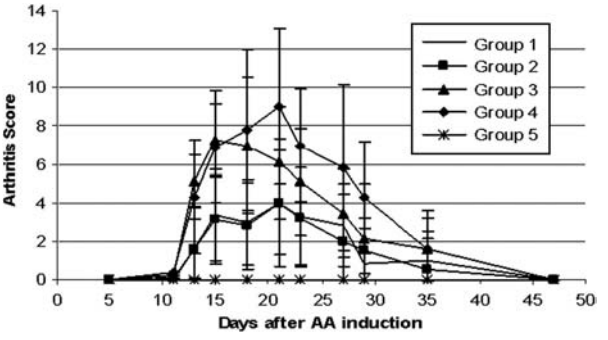

Figure 3. Treatment with APL-1 caused significant reduction of adjuvant arthritis (AA) in ill rats. Arthritis was induced on day 0 by immunization with MT in Incomplete Freund Adjuvant. On day 10, rats were randomly divided into five groups: Group I: rats inoculated with wild-type peptide by intradermal route, Group II: rats inoculated with APL-1 by intradermal route, Group III: rats inoculated with APL-1 by subcutaneous route, Group IV: non-treated rats. Group V: healthy rats. Arthritis scores were assessed every other day from day 5 onward. $N = 12$ rats per group. *** mean statistical differences, $p = 0.001$.

The capacity of APL-1 to induce regulatory T cells during the therapy with this peptide in rats was confirmed by a significant increase in the percentages of T cells with phenotype $CD4^+CD25^{high}FoxP3^+$in rats treated with APL-1 by both routes, compared with rats without treatment or treated with wild-type peptide as shown in Figure 5.

In addition, we monitored the quantitative changes in TNFα level upon APL-1 treatment, since TNFα is the major cytokine responsible for RA pathogenesis. At day 21 after disease induction, the TNFα levels were reduced significantly in rats treated with APL by both routes (Figure 6).

Evaluation of Treg induced by peptides in PBMC from RA patients

The induction of regulatory T cells by the APL-1 peptide in *ex vivo* assays using PBMC or SFMC from RA patients was analyzed. In total, 13 patients were included in this study. Information about the patients is provided in Table I. PBMC were stimulated with

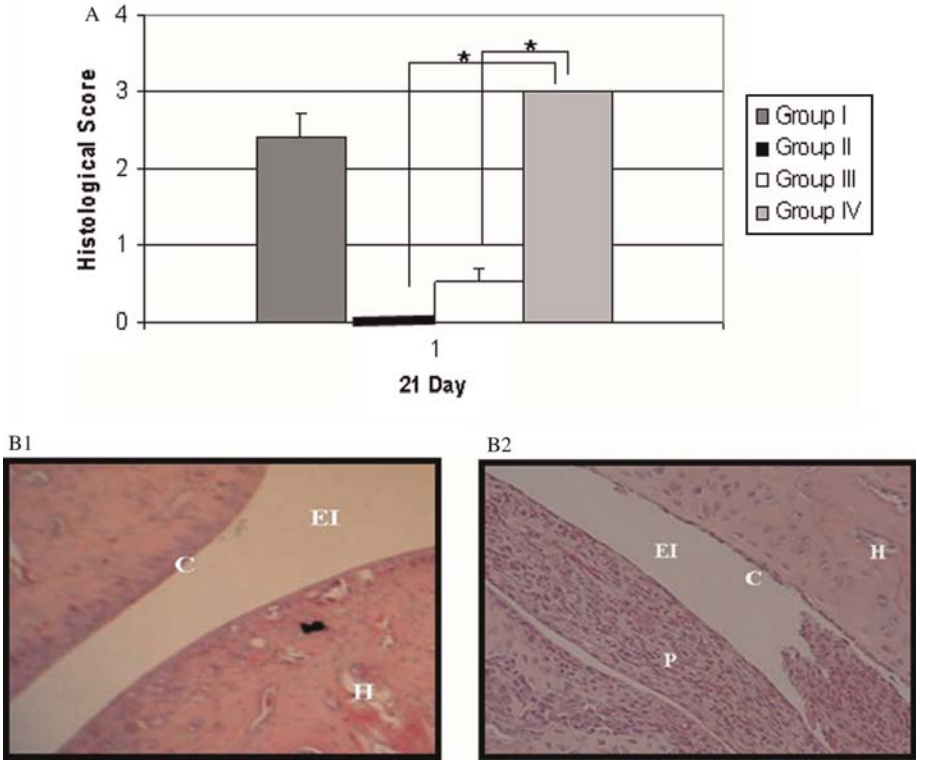

Figure 4. (A) Treatment with APL-1 peptide caused significant reduction of histological damage in ankle joints from rats. Joints were collected on day 21 after induction of arthritis. The histological damage was defined as: Grade 0, normal; Grade 1, mild synovitis with hyperplastic membrane, no inflammatory reaction; Grade 2, moderate synovitis without pannus formation, bone and cartilage erosions limited to discrete foci, and undisrupted joint architecture; Grade 3, severe synovitis with pannus formation, extensive erosions of bone and cartilage, and disrupted joint architecture. $N = 4$ rats per group. Group I: rats inoculated with wild-type peptide by intradermal route, Group II: rats inoculated with APL-1 by intradermal route, Group III: rats inoculated with APL-1 by subcutaneous route, group IV: non-treated rats. Error bars indicate the SE of the mean of 4 rats per group (*): mean statistical differences. $p = 0.029$. (B) Representative Hematoxilin and Eosin stained joint tissue sections from rats of group 1(B1) and group 4 (B2) on day 21 after AA induction. Intra-articular cavity (EI), bone (H), cartilage (C), pannus (P). Amplification 40 ×.

40 μg/ml of APL-1 or wild-type peptide for 5 days. The samples were screened for frequency of FoxP3 positive cells among $CD25^{high}CD4^+$ T cells by flow cytometry.

For this purpose, $CD4^+$ lymphocytes were first gated, and further gates applied on $CD4^+$ lymphocytes to select those coexpressing low or high levels of CD25. FoxP3 was performed on $CD25^{high}CD4^+$ gated T cells. Figure 7 shows the analysis corresponding to patient 2. The frequency of $FoxP3^+CD25^{high}CD4^+$ T cells in PBMC was higher when these were stimulated with APL-1. To confirm these results, the relative increase of $FoxP3^+CD25^{high}CD4^+$ T cells induced by APL-1 in PBMCs was calculated in comparison with PBMC stimulated with wild-type peptide and without stimulation. An increment of Treg cells frequencies of more than 1.5-fold in the case of PBMC stimulated with the APL-1 was found for 12 patients (Figure 8 and Table I).

Discussion

The approach presented here is based on the induction of peripheral tolerance using a peptide derived from Hsp60. Conceptually, this therapeutic intervention is based on modulation of T cell function and therefore higher specificity and lower toxicity is expected [30–33].

In this study, the Hsp60 was selected due to several characteristics of this protein: first this protein belongs to the HSP family, which are conserved proteins, however they are extremely immunogenic [34]. Second, Hsp60 can be used as an inductor of RA in animal models, but immunization with conserved HSP60 epitopes increases resistance to arthritis induction in rats [35,36]. Also, patients with chronic inflammation have antibodies and T cell response against this antigen [37].

In addition, we are in contact with this protein along of our life, during infections and immunizations or in foods and these previous contacts could induce regulatory response [15,38]. These indicated that this protein may have a function in the immune regulation.

On the other hand, several authors have reported that peptides derived from Hsp60 may play a role in amplification of autoimmune processes [39–41]. These peptides are identified as a "danger" signals and cause an inflammatory physiological response for clearing a possible pathogen invasion, but also they induce T cells with regulatory function [15,42].

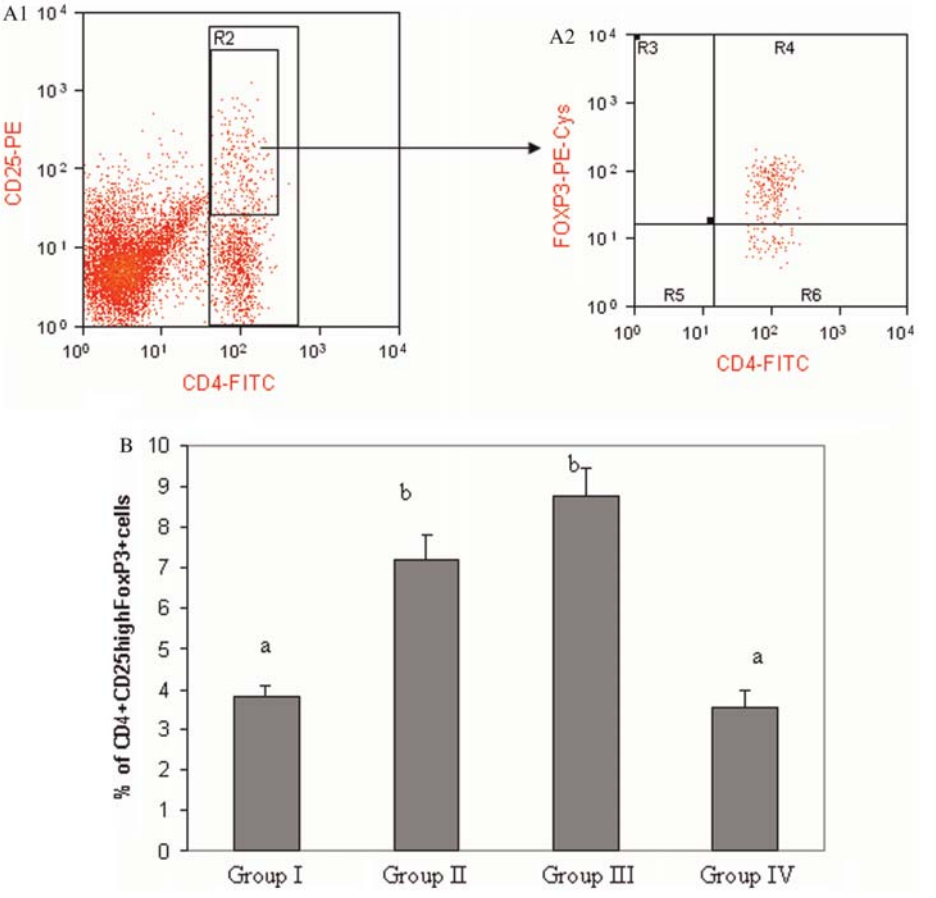

Figure 5. Treatment with APL-1 caused a significant increase in the percentages of T cells with phenotype $CD4^{+}CD25^{high}FoxP3^{+}$ in rats. (A) Representative results obtained by staining splenocytes from a rat with mAbs specific for CD4, CD25 and FoxP3 are shown. $CD4^{+}$ lymphocytes were gating according to low or high degree of CD25 expression (A1). FoxP3 was performed on $CD25^{high}CD4^{+}$ gated T cells (A2). (B) Percentage of $CD4^{+}CD25^{high}$Foxp3 T reg cells in spleens derived from rats sacrificed at day 21 after AA induction. Group I: rats inoculated with E18 peptide by intradermal route, Group II: rats inoculated with APL-1 by intradermal route, Group III: rats inoculated with APL-1 by subcutaneous route, Group IV: non-treated rats. Different letters mean statistically significant differences $p < 0.05$.

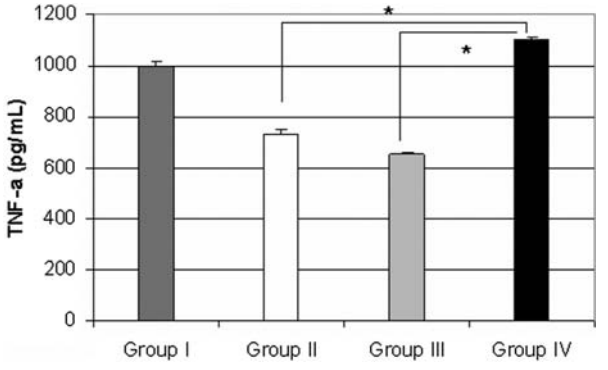

Figure 6. Treatment with APL-1 led to significant reduction of TNFα. The mononuclear cells were isolated from spleen and were stimulated *in vitro* with 40 μg/ml of APL for 24 h. The TNFα in supernatants were determined by commercial ELISA kit. Group I: rats inoculated with wild-type peptide by intradermal route, Group II: rats inoculated with APL-1 by intradermal route, Group III: rats inoculated with APL-1 by subcutaneous route, group IV: non-treated rats. Data are representative for three independent experiments (2mu*) mean statistically significant differences.

In this study, a novel T cell epitope from human Hsp60 was predicted. According to our prediction this peptide represents a strong epitope directly involved in the interaction with HLA class II molecules, particularly those related to RA. In a previous study, we evaluated the potentiality of this wild-type peptide to modify proinflammatory and immunoregulatory cytokine levels in *ex vivo* assays using PBMC from patients with RA (results not shown). In theses assays, an increment of TNFα and INFγ levels was confirmed.

An APL was designed from this wild-type peptide sharing with the wild-type peptide the binding characteristics to HLA class II molecules, but containing one modification in an essential contact position with HLA class II molecules. The purpose of this modification was to transform the T cell response induced by the wild-type peptide in order to induce tolerance in patients with RA.

First, the ability of the APL-1 for inducing Treg cells *in vivo* was tested in BALB/c mice, by inoculation of the APL and its wild-type peptide as control. These results corroborated that APL-1 induced an increase

Table I. Characteristics of patients with RA included in the study and induction of T reg by APL1-1 and E18.

Patients	Sex	Age	Duration	% Foxp 3′ T cells of PB CD4′ CD25^{high} Median (Range)	% Foxp 3′ T cells of PB CD4′ CD25^{high} stimulated with APL Median (Range)	% Foxp 3′ T cells of PB CD4′ CD25^{high} stimulated with wild peptide Median (Range)
1	F	49	5m	2.85 (2.7–3.02)	4.71 (4.52–4.9)	2.98 (2.97–3.0)
2	F	40	2y	1.85 (1.80–1.90)	3.0 (2.97–3.02)	1.90 (1.63–2.17)
3	F	54	5y	1.65 (1.56–1.75)	3.1 (2.9–3.3)	na
4	F	18	8y	1.69 (1.63–1.75)	3.67 (3.13–4.2)	1.94 (1.86–2.02)
5	F	56	4y	1.27 (1.22–1.32)	2.25 (2.15–2.35)	1.88 (1.63–2.12)
6	M	54	1y	0.83 (0.81–0.85)	3.04 (2.64–3.44)	1.57 (1.48–1.66)
7	F	30	1y	0.73 (0.7–0.75)	0.93 (0.90–0.96)	0.95 (0.8–1.07)
8	F	45	2y	1.9 (1.85–2.02)	3.2 (2.99–3.36)	2.1 (1.9–2.25)
9	M	68	7y	3.1 (3.05–3.08)	4.30 (4.1–4.43)	3.7 (3.51–3.95)
10	F	58	12y	2.32 (2.25–2.4)	4.30 (4.1–4.5)	2.20 (2.1–2.3)
11	F	51	15y	1.28 (1.22–1.34)	4.34 (4.18–4.50)	na
12	F	46	3y	0.82 (0.74–0.90)	4.78 (4.55–4.92)	na
13	F	39	1y	0.57 (0.44–0.69)	1.18 (1.06–1.26)	na

Note: na, not analyzed; m, month; y, years.

of Treg cells with a CD4$^+$FoxP3$^+$ phenotype in the dLN and spleen. Contrary to humans, the expression of Foxp3 correlates with suppressor activity of T reg in mice, irrespective to CD25 [43].

On the other hand, wild-type peptide seems to recruit more conventional effector T cells, indicating that the modification of the wild-type peptide was efficient for inducing Treg cells and reinforce the therapeutic possibilities of this peptide for treatment of RA patients, because the Treg cells are capable of reducing the inflammatory response by suppressive mechanisms [44,45]. These therapeutic potentialities could be extensive to other autoimmune diseases where Hsp60 is an autoantigen as diabetes type I or Crohn's disease.

To explore the therapeutic effect of the APL-1, AA was chosen, a T cell, Hsp-dependent model of RA [46]. In this model, the disease is induced by immunization with Mt; 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 protection was associated with the production of regulatory cytokines [47,48]. In the present case, the treatment with the APL-1 induced excellent clinical control of AA. This effect was correlated with improvement of the histological score of the joints induced by the peptide, and it was comparable to healthy animals.

In contrast, the wild-type peptide did not induce any clinical or histological improvement in the rats. According to theses results, we can confirm that the modification carried out in the wild-type peptide was very effective for attenuation of the pathogenic inflammation in this animal model. The clinical efficacy achieved by the treatment with APL-1 administrated by subcutaneous or intradermal route was associated with an increment of Treg cells in the spleen. The Treg cells induced by APL-1 in periphery apparently could migrate to the joints and induce suppression of the local self-reactive response.

In addition, we found that the therapy with the APL-1 reduces significantly the TNFα level in spleen. Given these facts, we think that probably the potent therapeutic effect of the APL-1 in the reduction of AA is due to the 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 T cells with immunoregulatory phenotype like CD4$^+$CD25^{high}FoxP3$^+$ Treg cells. The activated cells migrate to the inflammation site and they could cross-recognize the native epitope from the HSP60, where it is highly expressed due to the inflammation process.

This new contact with the autoantigen may induce potent immunoregulatory effect, attenuating the autoreactive T cells responsible for arthritis pathogenesis and inhibiting the TNFα expression. The pivotal role of TNFα in the induction and progression of rheumatoid synovitis is well established [49]. Consequently, the demonstration that APL-1 inhibited the expression of TNFα in an animal model of arthritis represents a beneficial effect for the control of inflammatory process.

Recent studies have reported defects in the number and/or activity of Treg cell in humans with RA that are similar to those observed in the mouse models of arthritis [50,51]. Failures in the function of the Treg cells can therefore be responsible for the development of autoimmune diseases, and enhancing their functions may represent a treatment strategy.

We suggested that the modification carry out in APL-1 could induce T reg cells in patients that may attenuate the pathogenic T cells with specificity, without affecting other non-related T cells. To test this hypothesis, the induction of Treg cells by APL-1 was analyzed in *ex vivo* assays using PBMC or SFMC from RA patients, and it was confirmed that APL-1 induced

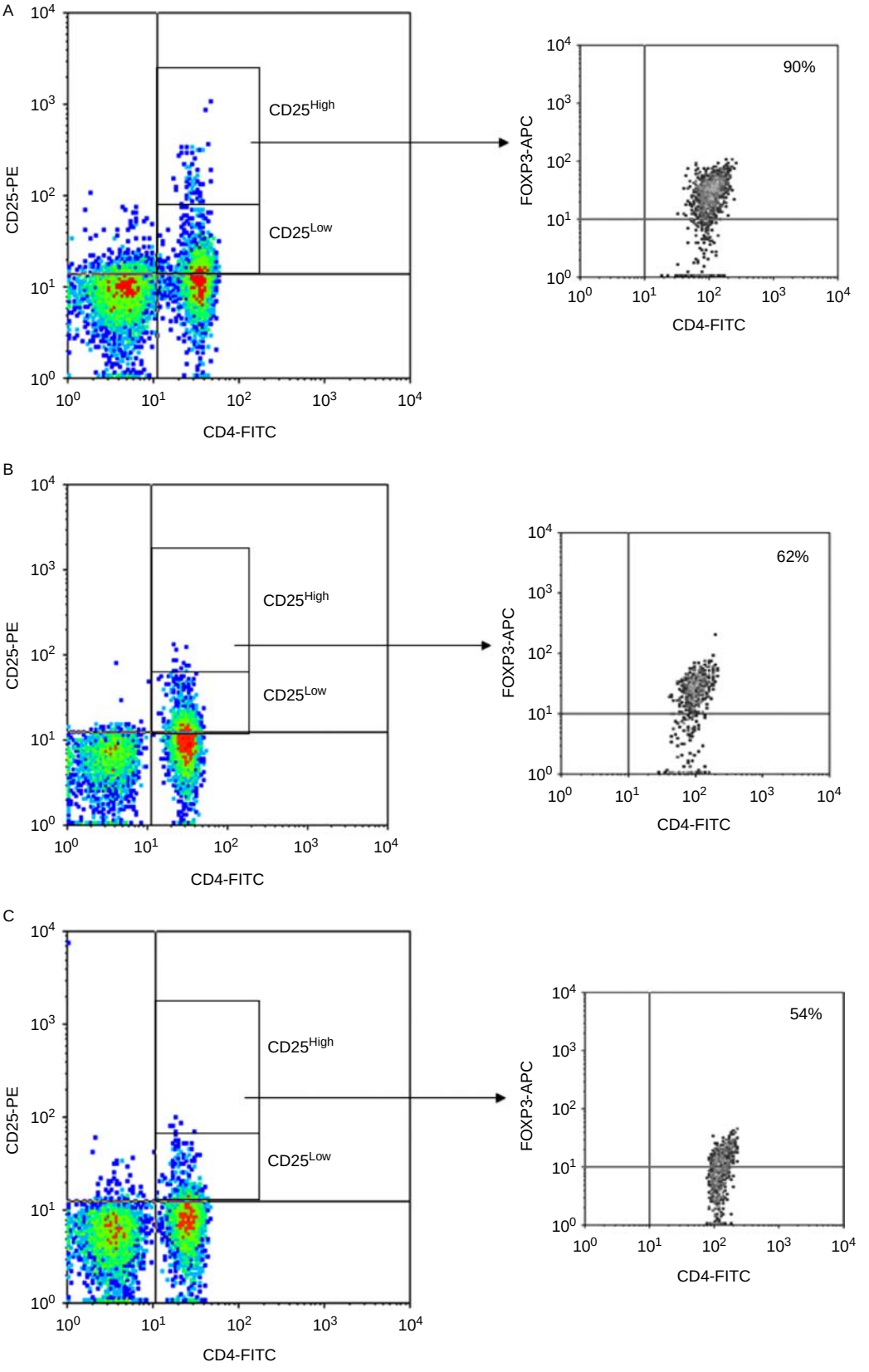

Figure 7. APL-1 induces regulatory T cells in *ex vivo* assays with PBMC from rheumatic patients. Representative results obtained by staining PBMC from a patient with mAbs specific for CD4, CD25 and FoxP3 are shown. PBMC isolated from patient were stimulated with 40 μg/ml of APL-1 (A) or E18 (B) during 5 days. PBMC in absence of peptide were used as control of basal levels in each patient (C). Then, cells were stained using Human Regulatory T cell Staining Kit and analyzed by flow cytometry. $CD4^+$ lymphocytes were gating according to low or high degree of CD25 expression. FoxP3 was performed on $CD25^{high}CD4^+$ gated T cells. Numbers indicate the percentage of positive cells. Eleven samples were analyzed.

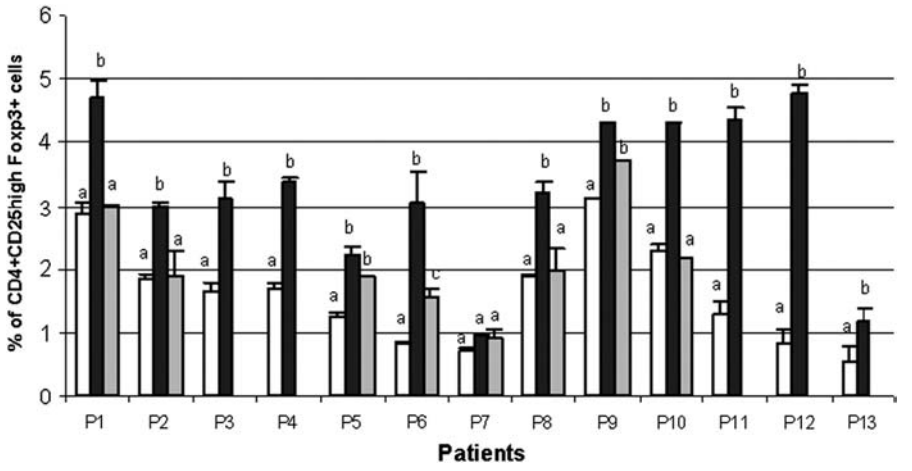

Figure 8. APL-1 increases frequency of $CD4^+CD25^{high}Foxp3$ Treg cells in *ex vivo* assays with PBMC from rheumatic patients. PBMC are stimulated with APL-1 or E18 peptide for 5 days. PBMC in absence of peptide were used as control of basal levels in each patient. Samples were screened for the frequency of $CD4^+CD25^{high}Foxp3^+$ T cells by flow cytometry. White bar: PBMC without stimulus. Blank bars: PBMC stimulated with APL. Gray bars: PBMC stimulated with wild-type peptide. Different letters mean statistically significant differences $p < 0.05$.

an increment of Treg cells with a $CD4^+CD25^{high}$ $FoxP3^+$ phenotype in all the analyzed patients, except in Patient 7 (Figure 8). This patient was pregnant at that moment. She did not present any sign or symptom of RA. Several authors have analyzed the influence of the pregnancy in RA, reaffirming the clinical improvement during this condition [52,53]. On the contrary, the wild-type peptide did not induce any increment of Treg cells.

In addition, we had assessed the induction of regulatory T cells by the APL-1 in *ex vivo* assays using PBMC from patients with Crohn's disease and Juvenile Idiopathic Arthritis. In both cases, we also found that APL-1 induced an increase of Treg cells with a $CD4 + CD25^{high}FoxP3 +$ phenotype in patients (results not shown).

In summary, we designed a novel HSP60-derived APL that inhibited, by induction of Treg cells, the development of experimental arthritis. This effect was found also in healthy mice inoculated with this peptide and *in vitro* assays using PBMC isolated from RA patients. Interference of the pathogenic T cell function in a specific manner using an APL derived from an autoantigen that can induce tolerance mediated by activation of Tregs as shown here, represents an attractive therapeutic approach for autoimmune diseases.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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