

Therapeutic effect of an altered peptide ligand derived from heat-shock protein 60 by suppressing of inflammatory cytokines secretion in two animal models of rheumatoid arthritis

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Abstract

Rheumatoid arthritis is a systemic autoimmune disease mediated by T cells. Productive engagement of T cell receptors by major histocompatibility complex-peptide leads to proliferation, differentiation and the definition of effector functions. Altered peptide ligands (APL) generated by amino acid substitutions in the antigenic peptide have diverse effects on T cell response. We predicted a novel T cell epitope from human heat-shock protein 60, an autoantigen involved in the pathogenesis of rheumatoid arthritis. Three APLs were designed from this epitope and it was demonstrated that these peptides induce the activation of T cells through their ability to modify cell cycle phase's distribution of CD4 + T cells from RA patients. Also, IL-17, TNF- α and IL-10 levels were determined in PBMC from these patients. Unlike the wild-type peptide and the other two APLs, APL2 increased the IL-10 level and suppressed IL-17 secretion in these assays. Therapeutic effect of this APL in adjuvant arthritis (AA) and collagen-induced arthritis (CIA) models was also evaluated. Clinical score, histopathology, inflammatory and regulatory cytokine concentration were monitored in the animals. APL2 efficiently inhibited the progression of AA and CIA with a significant reduction of the clinical and histopathologic score. Therapeutic effect of APL2 on CIA was similar to that obtained with MTX; the standard treatment for RA. This effect was associated with a decrease of TNF- α and IL-17 levels. These results suggest that the therapeutic effect of APL2 is mediated in part by down-regulation of inflammatory cytokines and support the potential use of APL2 as a therapeutic drug in RA patients.

Keywords: *Rheumatoid Arthritis, AA, HSP60, CIA, APL, inflammatory cytokines*

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. Although the etiology of RA is not totally understood, many studies have shown that T lymphocytes, macrophages, and proliferating synovial cells play a major role in the pathogenesis of this disease [1,2].

In particular, cytokines produced by these cells appear to be critically involved in the initiation and

perpetuation of RA. Therefore, regulation of cytokine levels in patients with RA may also be a useful treatment for this disease [3]. Different studies have described successful treatment of refractory arthritis in patients with RA by intravenous infusion of antibodies against tumor necrosis factor α (TNF- α), indicating a key role of this cytokine in the pathogenesis of chronic arthritis [4,5].

On the other hand, excessive amounts of T helper 17 cells (TH17) are thought to play a key role in some experimental autoimmune models and human inflammatory conditions as RA [6]. These cells are a recently discovered subset of T helper cells producing

interleukin 17 (IL-17) that are considered developmentally distinct from TH1 and TH2 cells.

The heat-shock protein 60 (HSP60), a protein belonging to the HSP family is an autoantigen in RA. These proteins are highly immunogenic, with an exceptional degree of evolutionary conservation [7]. 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 [8]. Some authors have identified epitopes of the HSP60 involved in the regulation mechanisms in animal models [9,10,11].

Induction of peripheral tolerance by using epitopes derived from autoantigens involved in the autoimmune disease pathogenesis constitutes a novel therapeutic approach for treatment of these diseases. These epitopes can be modified to modulate their immunological properties. These modified peptides are named APL; which are similar to immunogenic peptides but with one or several substitutions in the essential contact positions with the TCR or the MHC, interfering the cascade of necessary events for the complete activation of T cells [12]. Some authors have suggested that APLs can affect T cell differentiation and the TH1/TH2 balance modifying disease outcome [13,14].

Different animal models for RA have been used to evaluate the therapeutic effect of APLs. 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. T cell reactivity against the mycobacterial HSP60 (Mt HSP60) is involved in the progression of AA. HSP65-specific T cells directed against the epitope from amino acid 180 to 188 cross-react with self-antigen present in cartilage and can adoptively transfer AA [12]. However, T cells that recognized a conserved sequence of Mt HSP, peptide M256-270, protect against subsequent arthritis induction in this model [15].

CIA has become the most widely used model for studying disease pathogenesis and validation of therapeutic targets. Arthritis is currently induced in mice or rats 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 [16].

Previously, we predicted a novel T cell epitope from human HSP60 (amino acids 90–109). This epitope was modified in one of the positions probably involved in the binding site to specific HLA class II molecules for increasing the affinity to these molecules. One of these APLs increased the proportions of Treg cells in

culture of PBMCs from RA patients, and efficiently inhibited the course of AA in rats, with significant reduction of the clinical and histopathologic scores. This effect was associated with an increase of the proportions of Treg cells and a decrease of TNF- α level in spleen [17].

In the present study, a novel T cell epitope was predicted by bioinformatics tools located in the N terminal region (amino acids 55–75) of human HSP60. This epitope, named E18-12, was used to design three APLs. E18-12, APL1 and APL2 induce the activation of CD4 + T cells, but only APL2 increases the IL-10 levels and suppress the IL-17 secretion in PBMCs from RA patients. Moreover, APL2 inhibits efficiently the course of arthritis in two animal models for RA. Therapeutic effect of this peptide was similar to MTX, the standard treatment for RA. These results suggest that APL2 is a potential therapeutic treatment for controlling RA.

Materials and methods

Antigens and adjuvants

Heat-killed Mt (strain H37Ra) was obtained from Difco (Detroit, MI) and chicken type II collagen (CII) from Hooke Laboratories (USA). Incomplete Freund's adjuvant (IFA; Difco) and Complete Freund's adjuvant (CFA, Difco) were used as adjuvants. Peptides were manually synthesized by the Fmoc/tBu strategy in syringes using the Fmoc-AM-MBHA resin (0.54 mmol/g). The peptides were purified to more than 95% by high performance liquid chromatography (HPLC). The peptides were lyophilized and analyzed by reverse phase HPLC and mass spectrometry.

Patients

Eight patients with active RA as defined by the American College of Rheumatology criteria [18] were recruited from the National Institute of Rheumatology, Havana, Cuba (Table I). Peripheral blood sample collection and the investigation protocol were 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.

PBMC isolation and cytokine assays

Twenty mL of peripheral blood were extracted from each patient and diluted twice in PBS. Three mL of Ficoll-Paque (Amershan) were added to 5 mL of diluted blood and centrifuged in 15 ml tubes during 30 min at 1200 rpm. The ring corresponding to mononuclear cells was collected. Cells were washed twice with 15 mL of PBS 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 g/mL), HEPES 25 mM/L and L-glutamine 2 mM (all from Gibco BRL).

PBMC were cultured in triplicate (1 mL per well) in 24 well plates (Costar, Cambridge, MA) at 10^6 cells/well. Afterwards, 40 μ g/ml of APLs or wild-type peptide were added. The RPMI 1640 was used as control for cell growth. The mononuclear cells were incubated during 24 or 96 hours and cytokine concentration in supernatant was determined by specific kits (Quantikine®, R&D Systems) according to the recommendations of the manufactures.

Cell cycle phase's analysis

PBMC were plated (4×10^6) in RPMI 1640 10% FBS in 6-well plates and 40 μ g/ml of APLs or wild-type peptide were added. PBMC were stimulated with 5 μ g/ml soluble anti-CD3 (HIT3a; eBioscience) and 5 μ g/ml soluble anti-CD28 (CD28.2; eBioscience) as positive control. Seventy-two hours later, CD4 + T cells were purified by positive magnetic bead selection (MACS, Miltenyi Biotec) according to manufacturer's protocol.

The purity of the T cells was +95% as assessed by flow cytometry. Purified CD4 + T cells were then washed with PBS and fixed with ice-cold methanol/acetone (4:1). To analyze cell cycle and DNA fragmentation, cells were stained by incubation with a solution containing 100 μ g/mL propidium iodide (PI, Sigma) and 50 μ g/mL RNase (Sigma). All analyses were performed on a Becton Dickinson FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) by collecting a minimum of 20,000 events and analyzed using the WinMDI 2.8 and ModFit 3.0 software packages.

Experimental animals

Female inbred *Lewis* rats, RT1.B^L (5–8 weeks of age, weighting 101 to 120 g) were purchased from the National Center for the Production of Laboratory Animals (CENPALAB, Havana, Cuba). The animals were pathogen-free 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. DBA/1 mice were purchased from The Charles River Laboratory (Paris, France) and were housed in filter-topped cages under specific pathogen-free conditions. All animal procedures were performed in accordance with the guidelines approved by the Ethic Committees, the National Regulator Agencies and the European Union guidelines for animal experimentation.

Induction and clinical assessment of arthritis

AA in Lewis rats. Each rat was inoculated subcutaneously at the base of the tail with a freshly prepared emulsion (100 μ l) containing 1 mg of Mt in IFA. 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.

CIA in DBA/1 mice. Each mouse was immunized intradermally with 50 μ g/mL of chicken CII emulsified in 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.

Peptide immunotherapy protocols

AA in Lewis rats. On day 10, rats inoculated with Mt were randomly divided into 5 treatment groups (12 rats per group). Two groups were inoculated with the APL1 or APL2 by subcutaneous injection. The peptides were administered on days: 11, 14 and 18 after disease induction, each dose contained 200 μ g of peptide in PBS. Animal of two control groups were inoculated with wild-type peptide or PBS using the same procedure. The fifth group corresponds to healthy rats. The rats were anaesthetized using ketamine (50 mg/Kg, intramuscular) previous to the inoculation.

CIA in DBA/1 mice. On day 26, CIA mice were randomly divided into three treatment groups (12 mice per group). One group was inoculated by subcutaneous injection with APL2 and other group with MTX by intraperitoneal injection. The peptide or MTX were administered on days: 28, 31, 34, 37, 40, and 43 after disease induction, each dose contained 50 μ g or 60 μ g, respectively. PBS was administered as control using the same procedure. The fourth group corresponds to healthy mice.

Histopathological analysis

Ankle joints were harvested on day 21 or day 46 after induction of AA or CIA, respectively. 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 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 cytokine levels by ELISA

Spleen from 4 rats of each group of AA model were removed and homogenized 21 days after immunization. The splenocytes were washed once with PBS, erythrocytes were lysed with 0.83% NH_4Cl , washed 3 times and resuspended with RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS), L-glutamine 2 mM, gentamycin 100 units/mL, HEPES 25 mM/L (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 $\mu\text{g}/\text{ml}$ of wild-type peptide or APLs were added. RPMI 1640 was used as control for cell growth. Cells were incubated during 24 hours. In CIA model, blood samples from 4 mice of each group were collected at day 46 and cytokines were determined in serum. $\text{TNF-}\alpha$, IL-10 and IL-17 concentrations were measured with commercially available ELISA kits (Quantikine®, R&D Systems) according to the manufacturer's instructions.

Statistical analysis

Data analyses were performed using GraphPad Prism version 5.00 (GraphPad Software, San Diego California, USA). Results were expressed as mean \pm standard deviation; the differences between groups were analyzed with the ANOVA, Tukey's test or Students *t*-test. Kruskal-Wallis and Dunn's test were also performed where appropriate. P-values less than 0.05 were considered statistically significant.

Results

Prediction of a novel T cell epitope from human HSP60 and design of APLs

A new T cell epitope in the N-terminal region of human HSP60 (amino acids 55 to 75) was predicted using the program ProPred [19] and the corresponding peptide was named E18-12. According to the

prediction, this peptide represents an epitope directly involved in the interaction with HLA class II molecules frequently expressed by RA patients as: DRB1*0101, DRB*0102, DRB*0401, DRB*0404, DRB*0405 and DRB*0408. This epitope presents a 100% similarity with rat and mouse HSP60 proteins. Its sequence is shown in Figure 1.

The ability of E18-12 to modify proinflammatory cytokine levels was evaluated in *ex vivo* assays using PBMC from RA patients. A significant increment of $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ levels was observed after stimulation with E18-12 ($P \leq 0.05$) (data not shown), suggesting that this peptide induces a TH1 phenotype in $\text{CD4} + \text{T}$ cells present in PBMC isolated from RA patients.

Amino acid residues potentially involved in the interaction with the HLA class II molecules were substituted in the E18-12 peptide with the purpose to modify the T cell response induced by this peptide to a TH2 or a regulatory phenotype. These new peptides were named APL1, APL2 and APL3 and their sequences are shown in Figure 1.

Effect of E18-12 and APLs on cell cycle progression of $\text{CD4} + \text{T}$ cells from RA patients

We first characterized the effect of peptides on activation of $\text{CD4} + \text{T}$ cells from RA patients through cell cycle analysis by PI staining. Cells stimulated with anti-CD3 and anti-CD28 Abs (CD3/CD28) were used as positive control in these experiments, because it represents the most physiological condition of antigenic activation of T-cell effector functions.

Cell cycle analysis showed that E18-12, APL1 and APL2 peptides reduced the number of T cells in sub-G1 phase (Table II). Furthermore, these peptides increased the number of T cells in S + G2/M phase, similarly to that observed after CD3/CD28 stimulation.

APL3 did not affect the cell cycle phase's distribution of $\text{CD4} + \text{T}$ cells.

These results suggest that E18-12, APL1 and APL2 constitute a survival stimulus for $\text{CD4} + \text{T}$ cells inducing exit from G0/G1 and entry into S + G2/M phase of the cell cycle.

Evaluation of IL-17, $\text{TNF-}\alpha$ and IL-10 levels induced by APLs in PBMC from RA patients

Once we demonstrated that peptides induced proliferation of $\text{CD4} + \text{T}$ cells, we proceed to evaluate the

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Wild Type(E18-12): MGPKGRTVIIIEQSWGSPKVTK
APL1: MGPKGRTVIILQSWGSPKVTK
APL2: MGPKGRTVIIIEQSLGSVPKVTK
APL3: MGPKLRTVIIIEQSWGSPKVTK

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

Table I. Characteristics of the RA patients.

RA patient	Age (years)	Sex	Disease Duration (years)	DQ Allele	DR allele	Treatment
P1	63	F	6	0301	0411	NSAID
P2	44	F	8	0602	1515	NSAID
P3	33	F	3	0301	0311	NSAID, MTX
P4	49	F	22	0501	1314	NSAID
P5	43	F	10	0205	0103	NSAID
P6	60	M	4	0301	1113	NSAID, MTX
P7	69	F	2	0302	0404	NSAID
P8	58	M	1	0301	1113	NSAID

NSAID, nonsteroidal anti-inflammatory drug; MTX, methotrexate.

effect of these peptides on cytokine secretion. PBMC without peptide stimulation were used as negative control (C -).

All peptides suppressed the IL-17 levels *in vitro* by more than 50% (Figure 2A). However, only E18-12 and APL1 induced an increment of TNF- α secretion (Figure 2B). Remarkably, APL2 increased IL-10 levels more than 3 times compared to negative controls in most patients (Figure 2C). Although APL1 induced secretion of IL-10 in some patients, it was not significant. On the contrary; E18-12 did not induce any variation on IL-10 concentration.

APL3 did not affect the concentration of any cytokine. We selected APL2 for further evaluation in experimental animal models of arthritis because APL2 induced a significant increment of IL-10 levels and strongly reduced IL-17 secretion in PBMCs from RA patients.

Therapeutic evaluation of APL2 on AA model

Therapeutic effects of APL2 on arthritis was evaluated and compared with E18-12 and APL1 in the AA model. As shown in Figure 3, the signs associated with the development of arthritis began gradually in all animals inoculated with Mt. These signs were evident on day 10, characterized by a slight redness and swelling of the posterior joints.

On day 11, the rats were randomly divided into 5 groups ($n = 12$ per group) and treated with peptides. PBS was injected as negative control and the fifth group corresponds to healthy rats.

The mean arthritis score on day 21 (the day of maximum arthritis severity) was the main parameter used in this study to measure clinical outcomes and evaluation of the effects of the peptide. A significant reduction of AA mean arthritis score on day 21 ($p < 0.001$) was observed with APL2 (4.6 ± 3.6) compared to rats treated with PBS (12 ± 5.32), E18-12 (8.1 ± 2.4) and APL1 (9.45 ± 3.57) (Figure 3). The improvement induced by APL2 was comparable to healthy animals. This trial was repeated three times obtaining similar results.

Clinical improvement of AA with the APL2 is corresponded with a decrease of joint destruction by the arthritic process. Four animals were sacrificed per group and ankle joints were collected on day 21 after induction of AA and scored for severity.

The histologic examination of the joints showed severe erosion of cartilage and bone as well as massive inflammatory cell infiltration and obvious pannus formation in all rats inoculated with PBS. In contrast, subcutaneous administration of APL2 resulted in a suppression of these histologic signs characteristic of AA. Histologic damage in the joints was not observed. However, in all rats treated with APL1 or wild-type peptide presented a histologic score of 2, with massive cellular infiltration, synovitis and moderate erosions of bone and cartilage (Table III and Figure 4). These results indicate that APL2 suppressed AA efficiently.

Effect of APL2 on cytokine secretion in AA model

In addition, the production of TNF- α , TGF- β and IL-10 was investigated in the spleen from sacrificed

Table II. Effects of E18-12 and APLs on cell cycle phase's distribution of T CD4 + cells from RA patients.

Stimulation	% Cells in G0/G1	% Cells in S + /G2/M	% Cells in SubG1
Unstimulated	44.68	17.41	32.43
E18-12	47.35	24.32	22.87
APL-1	52.56	25.11	17.96
APL-2	47.45	25.30	21.71
APL-3	44.21	16.98	29.40
CD3/CD28	61.27	29.36	4.54

Representative results obtained by PI staining of CD4 + T cells from a patient are shown.

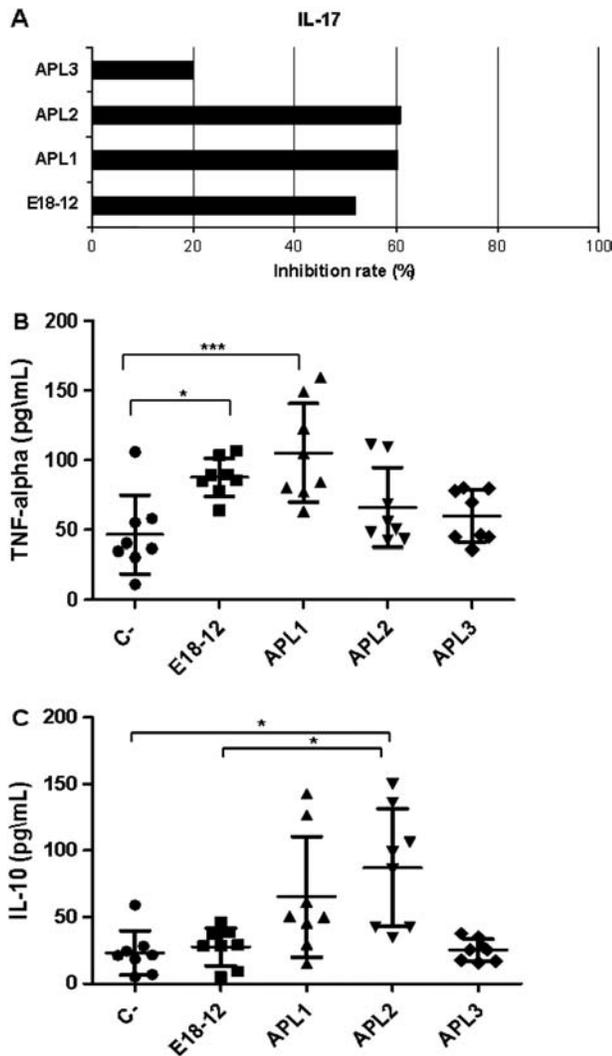


Figure 2. APL-2 increases IL-10 levels and suppresses IL-17 secretion in PBMC from RA patients. PBMC were stimulated with 40 microgram/ml of each peptide during 24 or 96 hours. Concentrations of IL-17, TNF- α and IL-10 were measured in the culture supernatants by specific ELISA. PBMC without peptide stimulation were used as negative control (C -). Inhibition rate of IL-17 is expressed as percentage inhibition against the PBMC without peptide stimulation (A). Concentrations of TNF- α (B) and IL-10 (C) are expressed as mean \pm standard deviation and were analyzed using the ANOVA and Tukey post-test (*, $P \leq 0.05$, *** $P \leq 0.001$). These results are representative of three similar experiments.

rats at day 21 after disease induction. As shown in Figure 5, administration of APL2 significantly reduced TNF- α levels ($P < 0.05$), but it has not any effect on IL-10 or TGF- β levels. In contrast, treatment with wild-type peptide significantly increased TNF- α level ($P \leq 0.05$) compared to rat treated with APLs and PBS. Besides IL-10 and TGF- β levels in E18-12 treated rats were significantly lower ($P \leq 0.05$) compared to the rats treated with APL2 and PBS (Figure 5). No difference was observed in the levels of these cytokines in APL1 treated rats compared to PBS (Figure 5).

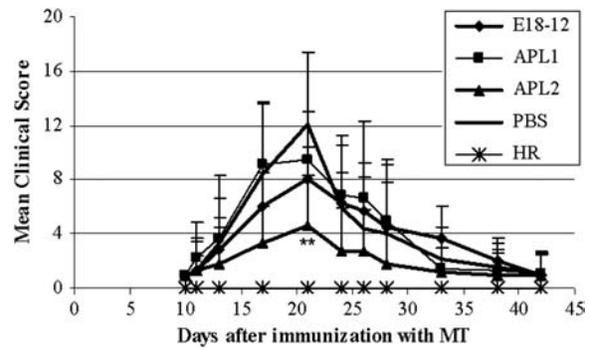


Figure 3. Treatment with APL-2 significantly reduced of AA in ill rats. Arthritis was induced on day 0 by immunization with Mt in IFA. On day 10, rats were randomly divided into five groups ($N = 12$). E18-12, APL1 or APL2 were administered s.c. on days 11, 14 and 18 after disease induction. PBS was injected as negative control. The clinical score of arthritis is expressed as mean \pm standard deviation. Data were analyzed using the ANOVA and Tukey post-test (** $P \leq 0.01$). HR: healthy rat.

Therapeutic evaluation of APL2 on CIA model

Next, therapeutic effect of APL2 was evaluated and compared to MTX, in another animal model where arthritis is induced by a different autoantigen. CIA was elicited in DBA/1 mice by twice subcutaneous immunization with CII. Thirty-two out of 36 mice (88%) developed arthritis between days 23 and 26 showing evidences of clinical inflammation in one or two hind paws. CIA mice were randomly allocated into 4 groups ($n = 12$ per group), and there was not a difference in arthritis score among them. Then, the CIA mice were treated with APL2 or MTX. PBS was injected as negative control, and the fourth group corresponded to healthy animals.

Table III. Histopathologic analysis of AA rats per treatment group.

Treatments groups	# Animal	Score histopathological
E18-12	1	2
	2	2
	3	1
	4	2
APL1	1	2
	2	2
	3	2
	4	2
APL2	1	0
	2	0
	3	0
	4	0
PBS	1	3
	2	3
	3	3
	4	3
Healthy mice	1	0
	2	0
	3	0
	4	0

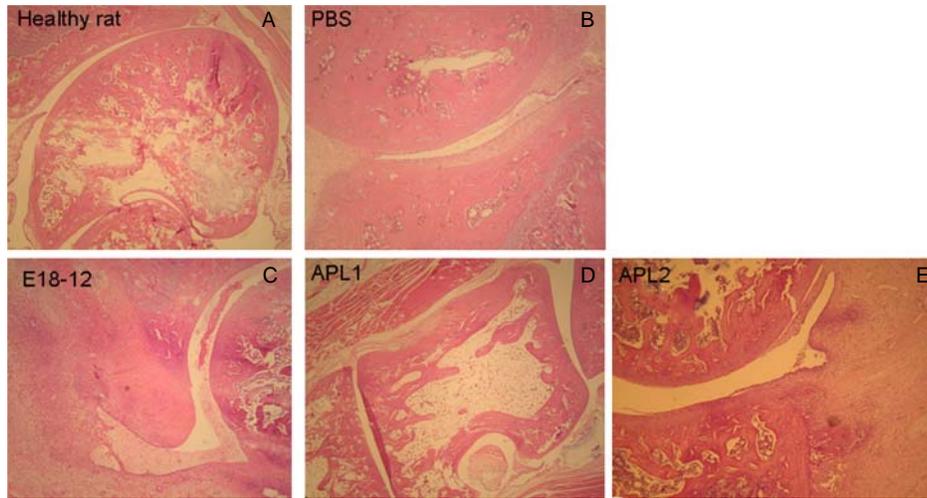


Figure 4. Treatment with APL2 peptide caused reduction of histological damage in ankle joints from Lewis rats. Joints were harvested on day 21 after induction of arthritis and were stained with haematoxylin and eosin and represent original magnification 10X ($N = 4$ rats per group). Paw sections were examined by two independent observers who were blinded to the experimental group. The histopathological damage score in ankle joints was assigned using values between 0–3. (A) Normal: joint with a conserved synovial space; (B) PBS: control mice with pannus infiltration, diminished synovial space and cartilage damage; (C–D) E18-12 and APL1: massive cellular infiltration and moderate synovitis; (E) APL2: joint with a conserved synovial space.

Treatment with APL2 reduced the severity of CIA compared to PBS treated mice (3.7 ± 3.0) from day 21 after the first immunization until the end of protocol (Figure 6). Similar results were observed in MTX treated animals. These mice showed significantly lower arthritis score (3.8 ± 2.8) compared to PBS treated mice (8.5 ± 1.95) ($P \leq 0.05$) but not with APL2 (Figure 6).

On day 46, 4 mice per group were sacrificed for histologic examination. All PBS treated mice presented a histologic score of 3 (Table IV). Severe synovitis with pannus formation and severe erosions of bone and cartilage were evident (Figure 7B). Two

animals of the APL2 group did not show any histologic damage and other two mice had only moderate synoviocyte hyperplasia (Table IV and Figure 7D). No inflammatory reaction was observed in animals treated with MTX, only synovial hypertrophy and a discrete angiogenesis (Table IV and Figures 7C).

Effect of APL2 on cytokine secretion in CIA model

To investigate the mechanism of action of APL2 in suppressing CIA, the levels of TNF- α , IL-17 and IL-10 were measured in serum of mice sacrificed on day 46. As shown in Figure 8, the treatment with

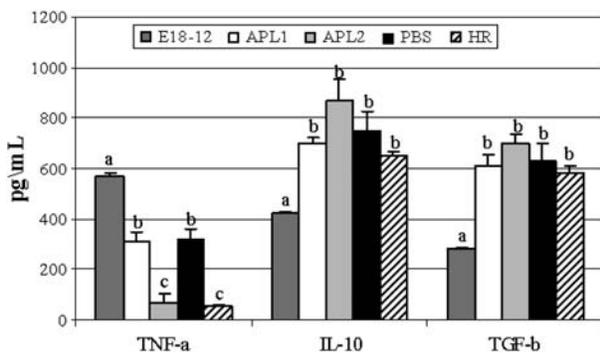


Figure 5. Treatment with APL2 led to significant reduction of TNF- α . On day 21 after arthritis induction 4 rats per group were sacrificed. The splenocytes were isolated and stimulated *in vitro* with 40 μ g/ml of corresponding peptide for 24 hours. The TNF- α , IL-10 and TGF- β in culture supernatants were measured by ELISA. HR: healthy rat. Data are mean + standard deviation and are representative for three similar experiments. The results were analyzed using Kruskal-Wallis and Dunn post-test. Different letters indicate statistically significant differences ($P \leq 0.05$).

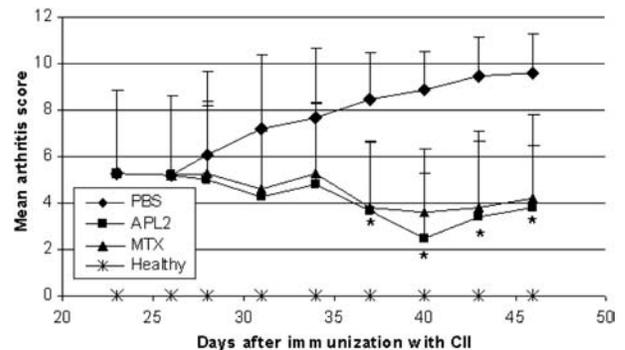


Figure 6. Treatment with APL2 caused significant reduction of CIA in ill mice. Arthritis was induced in DBA/1 mice with chicken CII. APL2 or MTX were administered s.c and i.p, respectively, on days 28, 31, 34, 37, 40, and 43 after immunization of CII. PBS was injected s.c as control in similar conditions. The clinical score of arthritis is expressed as mean for 12 mice per group + standard deviation. Data were analyzed using ANOVA and Tukey post-test ($*$, $P \leq 0.05$).

Table IV. Histopathologic analysis of CIA mice per treatment group.

Treatments groups	# Animal	Score histopathological
PBS	1	3
	2	3
	3	3
	4	3
APL2	1	2
	2	2
	3	0
	4	0
MTX	1	1
	2	2
	3	0
	4	0
Healthy mice	1	0
	2	0
	3	0
	4	0

APL2 significantly reduced TNF- α and IL-17 secretion compared to PBS inoculated mice ($P \leq 0.05$). Levels of both cytokines in this group were similar to those obtained in healthy animals. The administration of MTX significantly reduced TNF- α secretion ($P \leq 0.05$), but not modify IL-17.

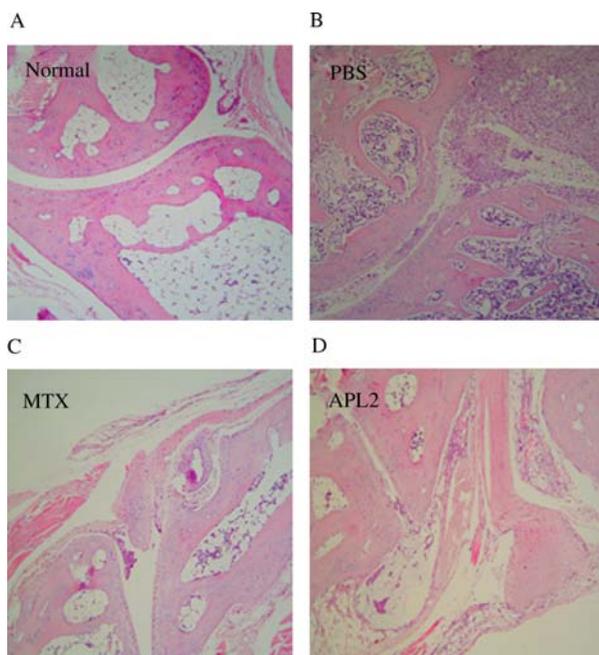


Figure 7. Treatment with APL2 significantly prevented of histological damage in ankle joints from CIA mice. DBA/1 mice were immunized and boosted with chicken CII Joints were harvested on day 46 and were stained with haematoxylin and eosin and represent original magnification 10X ($N = 4$ rats per group). The histopathological damage score in ankle joints was assigned using values between 0–3. All these histopathological procedures were performed totally blinded. $N = 4$ mice per group. (A) Normal: joint with a conserved synovial space; (B) PBS: severe synovitis with pannus formation, extensive erosions of bone and cartilage; (C) MTX: mild synovitis with hyperplastic membrane, no inflammatory reaction; (D) APL2: moderate synovitis without pannus formation.

No difference was observed in the levels of serum IL-10 among the 4 groups (Figure 8). These results demonstrate that the APL2 inhibit production of two inflammatory cytokines, TNF- α and IL-17, but have not influence on the level of IL-10, an anti-inflammatory cytokine, in these experiments.

Discussion

The use of APLs as therapeutic approach for T mediated autoimmune diseases has been studied extensively in recent years and the *in vitro* effects of some APLs suggest their *in vivo* functions [12]. It has been shown that APLs were extremely effective in the treatment of several animal models of human autoimmune diseases [13,14,17]. The aim of the approach presented here is the induction of peripheral tolerance using an APL derived from human HSP60. APLs mediate a number of different functional outcomes, including the T cell receptor antagonism [20], induction of T cell anergy [21], or partial activation (partial agonist) states [22] and the deviation to a regulatory/TH2 cytokine profile [23]. Theoretically, this therapeutic intervention is based on modulation of T cell function; therefore higher specificity and safe is expected [24].

HSP60 influences T-cell responses by at least two different mechanisms: as a ligand for innate immune receptors and as an antigen recognized by adaptive immune receptors. HSP60 can control T cells by TCR-dependent mechanisms. For example in type 1 diabetes mellitus, vaccination with HSP60 epitopes activates HSP60-specific regulatory T cells that affect the T cell response to disease-associated antigens by inducing a shift from secretion of INF- γ to IL-10 [25].

HSP60 vaccination induces a similar cytokine shift in the response to mycobacterial antigens triggering

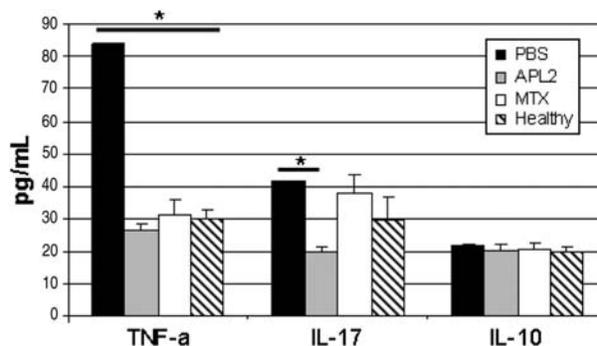


Figure 8. In mice treated with APL2 the serum IL-17 and TNF- α were lower than that of PBS treated mice. DBA/1 mice were immunized and boosted with CII. APL, MTX were administered s.c. and i.p., respectively on days 28, 31, 34, 37, 40, and 43 after immunization of CII. IL-17, IL-10 and TNF- α concentration from serum in mice sacrificed on day 46 were measured by ELISA. Data are mean + standard deviation and were analyses using Kruskal-Wallis and Tukey post-test ($*P < 0.05$).

AA [26]. Also a new mechanism by which HSP60 down-regulates inflammation has been reported [27]. The activated effector T cells up-regulate HSP60 and present the HSP60 epitopes to anti-ergotypic regulatory T cells. Thus, by functioning as an ergotope, HSP60 can control both the activated T cells and the regulatory HSP60-specific T cells.

Here, a novel T cell epitope in the N-terminal region of human HSP60 was predicted. *In vitro* experiments corroborated the prediction results, showing that E18-12 was able to induce proliferation of CD4 + T cells from RA patients and significantly increased TNF- α and IFN- γ levels. These findings suggest that this peptide induces a TH1 phenotype in CD4 + T cells from these patients. It has been reported, INF- γ produced by TH1 cells inhibited TH17 cells [28]. In agreement with this, E18-12 peptide suppresses IL-17 secretion in these cultures.

Three APLs were designed from E18-12 with the purpose to modify the T cell response induced by this peptide to a regulatory phenotype. APL1 and APL2 maintained the ability of wild-type peptide to stimulate CD4 + T cells. Our data suggest that these peptides provide a survival signal that induced the exit of G0/G1 phase and the progress through the G1 restriction point to the S + G2/M phases of the cell cycle.

However, APLs had different effect on cytokines profile in PBMCs from RA patients suggesting these peptides could activate different CD4 + T cell subsets. We evaluated the levels of the most important pro-inflammatory cytokines involved in the joint destruction of RA patients: TNF- α and IL-17. Also, IL-10 levels were measured because this cytokine has an essential role for controlling the magnitude of the immune response [29].

APL2 preferentially increased IL-10 and decreased IL-17 but has not any effect on TNF- α levels, suggesting a deviation from inflammatory to regulatory cytokine profile. This peptide increased IL-10 levels in all patients irrespective of their HLA background; therefore APL2 may bind to several allelic variants of the human HLA class II (DQ and DR). Different authors have reported pan-DR-binding HSP60 T cell epitopes that induce a disease-specific anti-inflammatory T cell response in PBMCs from Juvenile Idiopathic Arthritis and RA patients, underlining the potential of these epitopes as candidates for antigen-specific immunotherapy [30,31].

Conversely, APL1 increased TNF- α and decreased IL-17 levels as wild-type peptide do. However, this peptide increased IL-10 levels in some patients but it was not significant. So, this peptide has a different response pattern to E18-12 or APL2.

APL3 has not any effect on the secretion of these cytokines or in the proliferation of CD4 + T cells. These results suggest that APL3 could become a null

ligand [32]. All these findings confirm that minor variations of the peptide sequence may affect its binding affinity with MHC class II molecules, modifying the strength of the interaction of peptide-MHC complex with the TCR, modulating the immune response.

We have not *in vitro* evidence showing that E18-12, APL1 and APL2 bind to rat MHC class II molecules (RT1.B^L), but these peptides contain an epitope that could be presented by RT1.B^L, according to the program MHC2PRED [33]. APL2 was effective for down-regulating the inflammatory response in AA model with a reduction of TNF- α level, but does not affect the IL-10 or TGF- β levels. However, wild-type peptide increases the TNF- α secretion and decreases the IL-10 and TGF- β secretion in AA rats showing that this peptide enhances the inflammatory response in this model.

AA is mediated by cross-reactive CD4 + T cells recognizing Mt HSP60 as well as rat HSP60, most likely due to the high degree of similarity of these proteins. However, Alberta et al. showed that protection induced by preimmunization with Mt HSP60 in this model is mediated by T cells recognizing a mycobacterial peptide and this protection was associated with the production of regulatory cytokines [15].

The therapeutic effect of APL2 was also evaluated in the CIA model, where arthritis is induced by a different autoantigen, to verify this effect. 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 [34,35].

The susceptibility to CIA is determined by the I-A^q (MHC class II molecule). We have not *in vitro* evidence showing that APL2 can bind to I-A^q molecules, but this peptide contains an epitope that could be presented by mouse MHC class II molecules, according to the program RANKPED [36].

Studies to characterize and modulate cytokine production within the joint during CIA have identified critical roles of TH1-associated pro-inflammatory cytokines: TNF- α and IL-1, in joint inflammation and tissue destruction [16]. Primarily, secreted by mononuclear phagocytes following activation, TNF- α contributes to inflammatory cell infiltration, while IL-1 promotes neutrophilia and pannus formation. APL2 reduced the TNF- α level in sera of these animals to concentrations comparable to healthy animals.

Additionally, pannus formation and joint damage was not observed. These results suggest that infiltration of macrophages and neutrophils did not occur in the synovium. Similar results were obtained in MTX treated mice, which is the current standard treatment

for RA. This is in agreement with the work by Neurath et al. [37], who demonstrated that intraperitoneal administration of MTX reduced TNF- α and INF- γ levels in sera of CIA mice.

Many evidences support the role of IL-17 in the pathogenesis of human RA and its animal models such as CIA [38,39,40]. The decrease of IL-17 and TNF- α secretion induced by APL2 could indicate that the therapeutic effect of this peptide is mediated by down-regulation of inflammatory cytokines rather than induction of regulatory T cells. Consistent with this, APL2 treatment does not induce regulatory T cells with a Foxp3 + CD4 + phenotype at day 46 in spleen of CIA mice (results not shown).

However, MTX had no effect on IL-17 production. The arthritis suppressive effect of low dose MTX treatment used currently has not been clarified, although cytokine patterns are disturbed, and it is unclear whether the disease promoting mechanism also involves immune priming or only effector functions of the inflammatory attack [41].

Differences in cytokine profile found in *ex vivo* experiments with PBMCs from RA patients and two animal models suggest that APL2 works through different molecular mechanisms in these experiments. APL2 is derived from human HSP60; therefore, its binding affinity to MHC class II molecule could be different in human, rat and mouse affecting also the interaction of the TCR with the APL2-MHC complex and thus altering the functional outcome of the immune response.

In conclusion, we designed an HSP60-derived APL that increased the IL-10 levels and suppressed IL-17 secretion in PBMCs from RA patients. APL2 effectively reduced the joint inflammation and also decreased joint injuries in AA and CIA by inhibition of inflammatory response. These results demonstrate the potentiality of APL2 as a therapeutic agent in RA patients.

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