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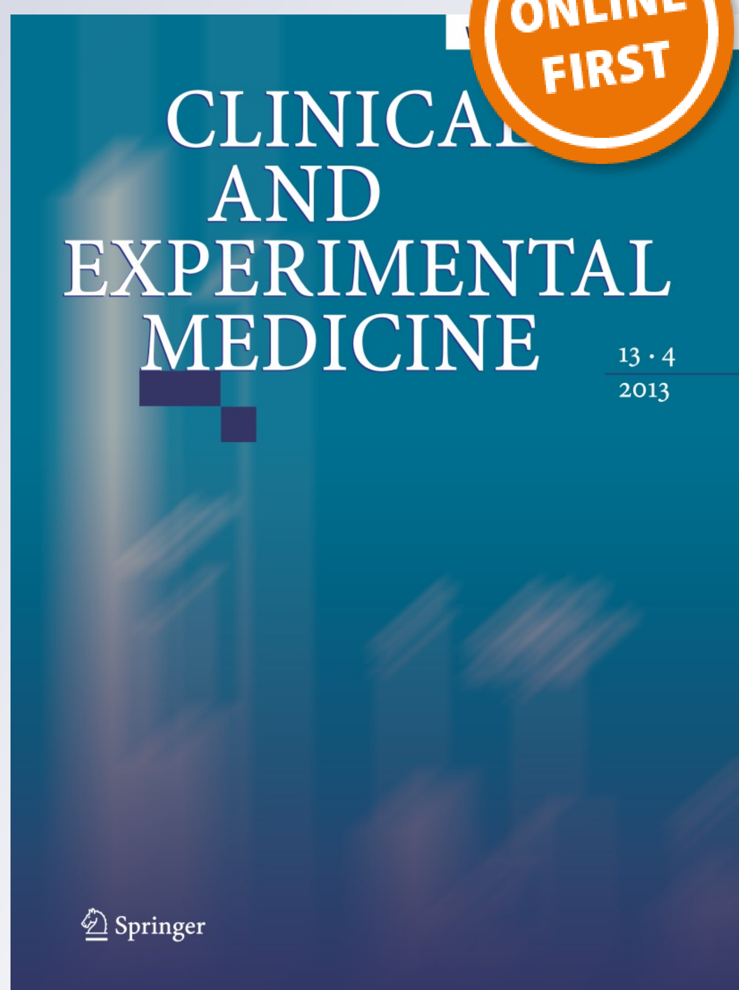

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ORIGINAL ARTICLE

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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Abstract Juvenile idiopathic arthritis (JIA) is a heterogeneous group of diseases characterized by autoimmune arthritis of unknown cause with onset before age of 16 years. Methotrexate provides clinical benefits in JIA. For children who do not respond to methotrexate, treatment with anti-tumor necrosis factor (TNF)-α is an option. However, some patients do not respond or are intolerant to anti-TNF therapy. Induction of peripheral tolerance has long been considered a promising approach to the treatment of chronic autoimmune diseases. We aimed to evaluate the potentialities of two altered peptide ligands (APLs) derived from human heat-shock protein 60, an autoantigen involved in the pathogenesis of autoimmune arthritis, in JIA patients. Interferon (IFN)-γ, TNF-α and interleukin (IL)-10 levels were determined in ex vivo assays using peripheral blood mononuclear cells (PBMC) from these patients. Wild-type peptide and one of these APLs increased IFN-γ and TNF-α levels. Unlike, the other APLs (called APL2) increased the IL-10 level without affecting IFN-γ and TNF-α levels. On the other hand, APL2 induces a marked activation of T cells since it transforms cell cycle phase's distribution of CD4+ T cells from these patients. In addition, we evaluated the therapeutic effect of APL2 in collagen-induced arthritis model. Therapy with APL2 reduced arthritis scores and histological lesions in mice. This effect was associated to a decrease in TNF-α and IL-17 levels. These results indicate a therapeutic potentiality of APL2 for JIA.

Keywords Altered peptide ligand · Juvenile idiopathic arthritis · Collagen-induced arthritis · Tolerance · Interleukin-10

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Introduction

Juvenile idiopathic arthritis (JIA) comprises the most common chronic autoimmune arthropathies of childhood. The autoreactive immune response in JIA is assumed to be triggered initially by an adaptive (T cell or B cell) response toward a self-antigen. This hypothesis is supported by the fact that joint inflammation in JIA patients is characterized by selective accumulation of activated memory T cells in the synovium, which are clustered around dendritic cells [1].

Current treatment focuses on reduction of inflammation through anti-inflammatory and immunosuppressive strategies, including methotrexate and anti-TNF-α therapies [2]. However, some children with JIA do not respond or are intolerant to treatment with disease-modifying antirheumatic drugs [3].

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In this context, the main challenge in the treatment of autoimmune diseases as JIA is the development of therapeutic strategies that could eliminate the pathogenic T cells with specificity, without affecting other non-related T cells. The induction of peripheral tolerance using autoantigens involved in autoimmune disease pathogenesis constitutes an alternative approach. This therapy may facilitate the restoration of the tolerance loss in the course of autoimmune diseases, depending on the doses, the route and frequency of administration of the antigen [4].

Human heat-shock protein 60 (Hsp60) is an antigen that can be used in the induction of tolerance in autoimmune arthritis. This protein belongs to the family of the Hsps, which are immunogenic proteins with exceptionally evolutionary conservation. The antibodies against these proteins can be abundant in healthy people and in patients with autoimmune diseases [5]. In particular, several evidences suggest that HSP60 may be significant in the course of JIA [6–8]. Remarkably, T cell reactivity to autologous HSP60 is associated with a favorable prognosis in patients with oligoarticular JIA subtype [9].

On the other hand, epitopes derived from autoantigens can be modified in order to modulate their immunological properties. These peptides are called altered peptide ligands (APLs). They are similar to immunogenic peptides but with one or several substitutions in the essential contact positions with the T cell receptor (TcR) or with the major histocompatibility complex (MHC) molecules interfering the cascade of necessary events for the activation of T cells [10–13].

Previously, we predicted a novel T cell epitope (E18-12) from human HSP60 by bioinformatics tools. This epitope was modified to design several APLs. One of these APLs (called APL2) increased the IL-10 levels in culture of peripheral blood mononuclear cells (PBMC) from rheumatoid arthritis (RA) patients [14].

In this paper, we report that APL2 induces the activation of CD4+ T cells and increases the IL-10 levels in PBMC from JIA patients, irrespective of JIA subtypes. In addition, APL2 inhibits efficiently the course of collagen-induced arthritis (CIA) in mice. These results indicate a therapeutic potentiality of this APL and support further investigation of this candidate drug for treatment of JIA.

Materials and methods

Peptides, 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. 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), lyophilized and analyzed by reverse phase HPLC and mass spectrometry.

Patients

Fifteen patients fulfilling ILAR criteria for JIA (Edmonton, 2001) and treated in the Department of Pediatrics Rheumatology, Pedro Borrás Hospital, Havana, Cuba, were included in the study (Table 1). Peripheral blood sample collection and the investigation protocol were approved by the Ethics Review Committees of the Center for Genetic Engineering and Biotechnology and Pedro Borrás Hospital. Among the 15 children who suffered from JIA, 27 % were diagnosed with oligoarticular, 27 % polyarticular, 20 % systemic, 20 % enthesitis-related arthritis and 6 % psoriatic arthritis. The age of children ranged from 3 to 18 years (mean 11.13 ± 6.02) and 66.7 % of them were girls. Informed consent was received from all parents/guardians.

Isolation of the peripheral blood mononuclear cells (PBMC)

Twenty millilitre of peripheral blood were extracted from each patient and diluted twice in phosphate buffer solution (PBS). Three millilitre of Ficoll-Paque (Amershan) were added to 5 mL of diluted blood and centrifuged in 15-ml tubes during 30 min at 1,200 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).

Cell cycle phase's analysis

PBMC were plated (4×10^6) in RPMI 1640 10 % FBS in 6-well plate and 40 µg/ml of APLs or wild-type peptide were added. PBMC were stimulated with phytohemagglutinin (PHA) (20 µg/mL, Sigma) 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 more than 96 % 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

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Table 1 Characteristics of patients with juvenile idiopathic arthritis (JIA) included in the study

JIA patients	Age (years)	Sex	Diagnostic	Disease duration	Treatment
P1	3	F	Polyarticular RF-	2 ½ years	MTX and prednisone
P2	4	F	Systemic	2 years	MTX, prednisone and folic acid
P3	14	M	Enthesitis-related arthritis	4 years	Azulfidine and prednisone
P4	17	F	Polyarticular RF-	5 years	Azulfidine
P5	10	F	Polyarticular RF-	3 months	–
P6	13	F	Polyarticular RF-	9 years	MTX, prednisone, INF-γ, Naproxen and folic acid
P7	19	F	Oligoarticular persistent	5 years	Azulfidine, folic acid, prednisolone, homatropine and vitamin C
P8	17	M	Psoriatic arthritis	1 years	Azulfidine, prednisolone, ciclosporine
P9	14	M	Enthesitis-related arthritis	3 years	–
P10	3	M	Oligoarticular persistent	1 years	Naproxen
P11	5	F	Systemic	4 years	MTX, prednisone and naproxen
P12	13	M	Enthesitis-related arthritis	3 months	Azulfidine
P13	19	F	Oligoarticular persistent	15 years	Chloroquine
P14	3	F	Oligoarticular persistent	6 months	Prednisone e ibuprofen
P15	13	F	Systemic	5	Prednisone and Imuran

MTX methotrexate; *INF-γ* interferon gamma

containing 100 mg/mL propidium iodide (PI, Sigma) and 50 mg/mL RNase (Sigma). All analyses were performed on a Partec Cyflow Space unit (Partec, Germany) by collecting a minimum of 20,000 events and analyzed using the FloMax software version 2.81.

Detection of cytokines in culture supernatant of PBMC

PBMC isolated as described above were cultured in triplicate (1 mL per well) in 24-well plate (Costar, Cambridge, MA) at 10^6 cells/well. Afterward, 40 µg/mL of each APLs or wild-type peptide was added. RPMI 1640 was used as baseline for each specific cytokines. The mononuclear cells were incubated during for 24 h at 37 °C in 5 % carbon dioxide with 100 % relative humidity, and cytokine concentration in supernatant was determined by specific kits (Quantikine, R&D Systems) according to the manufacturer's instructions.

Induction and clinical assessment of collagen-induced arthritis (CIA)

Each DBA/1 mouse was immunized intradermally with 50 µg/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. Therefore, each mouse can receive a maximum score of 16 points.

Peptide immunotherapy protocols

On day 23, CIA mice were randomly divided into three groups (12 mice per group). One group was inoculated with APL2 by subcutaneous injection and other group with PBS, as control using the same procedure. The peptide (50 µg) was administered on days: 28, 31, 34, 37, 40, and 43 after disease induction. The third group corresponds to healthy mice.

Histopathological analysis

Ankle joints were harvested on day 60 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 (2–3 mm) were stained with hematoxylin 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 histopathological procedures were performed totally blinded.

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Detection of cytokine levels in serum of mice

Blood samples from four mice of each group were collected at day 60, and cytokines were determined in serum. IL-10, TNF-α and IL-17 concentrations were measured with commercially available ELISA kits (Quantikinew, R&D Systems) according to the manufacturer's instructions.

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. Two-way ANOVA was used in clinical assessment of mice. Kruskal–Wallis and Dunn's post-test were also performed where appropriate. P values less than 0.05 were considered statistically significant.

Results

Peptides affect cell cycle progression of CD4+ T cells from JIA patients

The effect of peptides on activation of CD4+ T cells from JIA patients was characterized through cell cycle analysis by PI staining. Cells stimulated with PHA were used as positive control. This control represents an excellent physiological condition of antigenic activation of T cell functions.

Cell cycle analysis showed that E18-12, APL1 and APL2 peptides increased the number of CD4+ T cells in S and G2/M phases, similarly to that observed after PHA stimulation (Table 2). On the other hand, APL3 did not affect the cell cycle phase's distribution of CD4+ T cells. For these experiments, APL3 was used as a control because this peptide did not affect cell cycle progression of CD4+ T cells from RA patients [14].

These results suggest that E18-12, APL1 and APL2 peptides stimulate the CD4+ T cells inducing exit from G0/G1 and entry into S and G2/M phases of the cell cycle.

APL2 induced an increment of IL-10 levels in PBMC from JIA patients

Quantitative changes in cytokines response induced by peptides were evaluated in ex vivo assays using PBMC from JIA patients. The concentrations of IFN-γ, TNF-α and IL-10 were measured. In total, 15 patients were included in this study. Information about the patients is provided in Table 1. Wild-type peptide and APL1 induced an increment of IFN-γ (Fig. 1a) and TNF-α (Fig. 1b) secretion and

Table 2 Effects of E18-12 and APLs on cell cycle phase's distribution of T CD4+ cells from juvenile idiopathic arthritis (JIA) patients

Stimulation	% cells in G0/G1	% cells in S	% cells in G2/M
Unstimulated	98.53	0.43	0.86
E18-12	90.42	2.53	7.03
APL1	90.60	2.65	6.72
APL2	89.80	2.52	7.65
APL3	97.89	0.45	1.02
PHA	81.98	7.03	10.95

Representative results obtained by propidium iodide (PI) staining of CD4+ T cells from a patient are shown

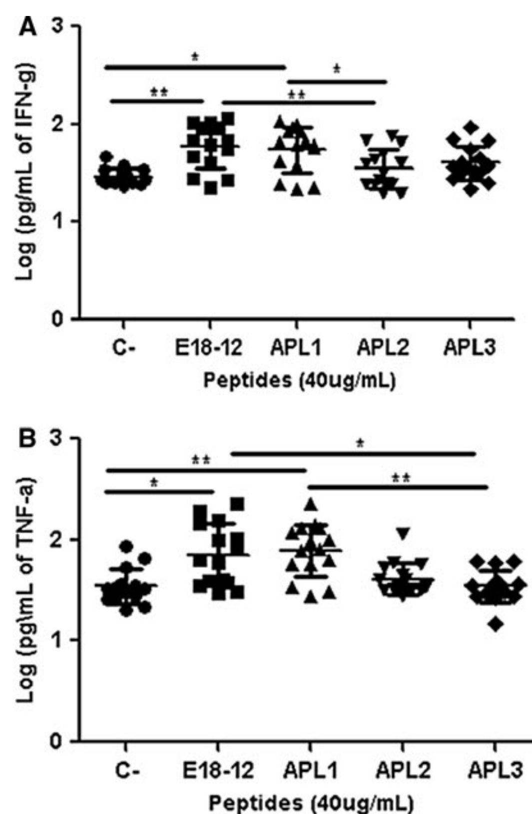

Fig. 1 Wild-type peptide and APL1 induced an increment of interferon (IFN)-γ (**a**) and tumor necrosis factor (TNF)-α (**b**) secretion in peripheral blood mononuclear cells (PBMC) from juvenile idiopathic arthritis (JIA) patients. PBMC were stimulated with 40 μg/ml of each peptide during 24 h. Concentrations of IFN-γ and TNF-α were measured in the culture supernatants by specific ELISA. PBMC without peptide stimulation were used as negative control (C−). Concentrations of IFN-γ and TNF-α were expressed as mean ± standard deviation and were analyzed using the ANOVA and Tukey's post-test (*$P \leq 0.05$, **$P \leq 0.01$). These results are representative of three similar experiments

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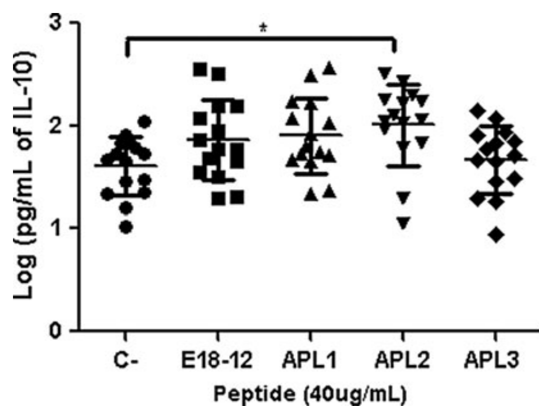

Fig. 2 APL2 induced an increment of interleukin (IL)-10 secretion in peripheral blood mononuclear cells (PBMC) from juvenile idiopathic arthritis (JIA) patients. PBMC were stimulated with 40 µg/mL of each peptide during 24 h. Concentrations of IL-10 were measured in the culture supernatants by specific ELISA. PBMC without peptide stimulation were used as negative control (C−). Concentration of IL-10 was expressed as mean ± standard deviation and was analyzed using the ANOVA and Tukey's post-test (*$P \leq 0.05$). These results are representative of three similar experiments

did not affect the IL-10 levels in these assays (Fig. 2). However, APL2 increased IL-10 levels compared with negative controls in all patients (Fig. 2), while did not induce any variation on IFN-γ and TNF-α concentration (Fig. 1a, b). APL3 did not affect the concentration of any cytokine evaluated.

The ratio of IL-10 to pro-inflammatory cytokines in PBMC from 15 JIA patients in response to the peptides is shown in Fig. 3. Both IL-10/INF-γ (A) and IL-10/TNF-α (B) ratios in response to the APL2 were more than 3 times higher with respect to the other peptides, reflecting that APL2 induces a regulatory phenotype. In some patients (P5, P9, P12 and P15), even these ratios were more than 5 times. Significant differences were not observed in the ratio of IL-10 to INF-γ and TNF-α induced by APL2 in the different subgroups of JIA due to possibly the few patients tested (Table 3).

APL2 reduced arthritis in mice

In order to confirm the therapeutic possibilities of APL2 for JIA, we evaluated healing effects of this peptide in the CIA model.

Collagen-induced arthritis was induced in male DBA/1 mice by two subcutaneous immunizations with CII. The mice were randomly divided into three treatment groups on day 23 after induction of arthritis: (1) ill animals without treatment (inoculated with PBS as control), (2) treatment with APL2 and (3) healthy animals. Three independent experiments were performed, with 12 mice per group.

As it is shown in Fig. 4, the signs associated with the development of arthritis began gradually in all animals

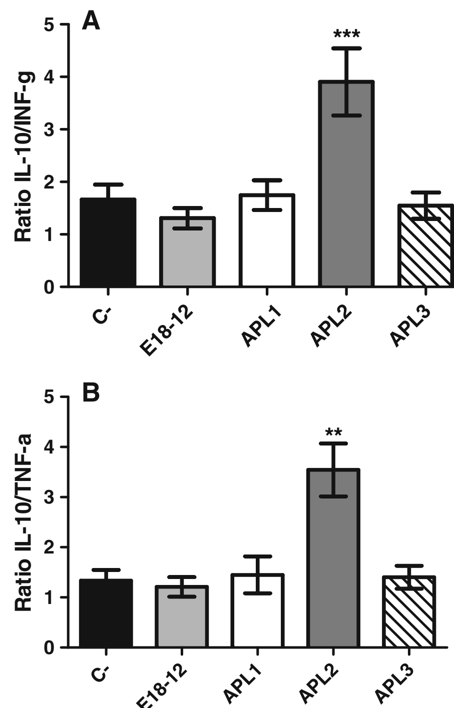

Fig. 3 APL2 induced relatively more interleukin (IL)-10 than interferon (IFN)-γ and tumor necrosis factor (TNF)-α. Ratio of IL-10 to INF-γ (A) and TNF-α (B) deduced from peptide-specific cytokine production of peripheral blood mononuclear cells (PBMC) from 15 patients with established juvenile idiopathic arthritis. PBMC without peptide stimulation were used as negative control (C−). *Bars* show the mean ± standard deviation. Data were analyzed using the ANOVA and Tukey's post-test (**$P \leq 0.01$; ***$P \leq 0.001$)

Table 3 Ratios of IL-10 to INF-γ and TNF-α deduced from APL2-specific cytokine production of PBMC from different subgroups of JIA patients. Ratios are expressed as median

JIA Subgroups	*n*	IL-10/INF-γ	IL-10/TNF-α
Polyarticular RF-	4	3.7	3.7
Oligoarticular persistent	4	5.3	2.9
Systemic	3	3.1	3.5
Enthesitis-related arthritis	3	5.3	5.1
Psoriatic arthritis	1	2.1	3.1

n number of patients; *IL-10* interleukin 10; *INF-γ* interferon gamma; *TNF-α* tumor necrosis factor alpha; *PBMC* peripheral blood mononuclear cells; *JIA* juvenile idiopathic arthritis

inoculated with CII. These signs were evident on day 23, characterized by a slight redness and inflammation of the posterior joints. It is also observed that the administration of APL2 induced a significant reduction of the clinical signs of arthritis in mice. However, in PBS-treated animals, the arthritis signs were expanding to the rest of the joints until became severe in all mice. A significant reduction of CIA mean arthritis score ($P < 0.05$) was achieved with APL2 compared with non-treated animals.

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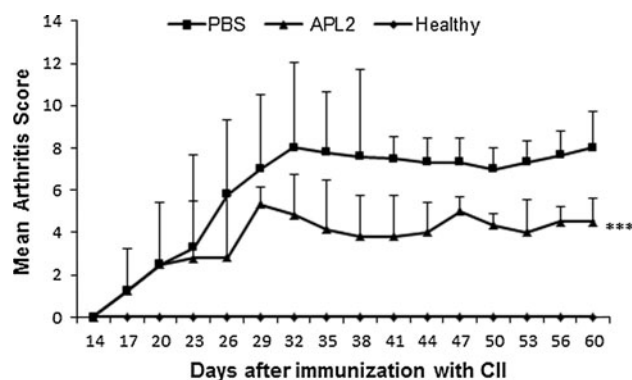

Fig. 4 Treatment with APL2 caused significant reduction of collagen-induced arthritis (CIA) in ill mice. Arthritis was induced in DBA/1 mice with chicken type II collagen (CII). APL2 was administered subcutaneously (s.c) on days 28, 31, 34, 37, 40 and 43 after immunization of CII. Phosphate buffer solution (PBS) was injected s.c as placebo in similar conditions. The clinical score of arthritis is expressed as mean for 12 mice per group + standard deviation. Data were analyzed using two-way ANOVA (***$P \leq 0.001$)

Table 4 Histopathology analysis of mice per group

Treatment groups	#Animal	Score histopathological
PBS	1	3
	2	3
	3	3
APL2	4	0
	5	2
	6	0
Healthy	7	0
	8	0
	9	0

Clinical improvement of CIA induced during therapy with APL2 was compared with decrease of the joint destruction by the arthritic process. Three animals were killed per group and ankle joints were collected on day 60 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 therapy with APL2 led to significant improvement of the histological score of the joints (Table 4). The mice inoculated with APL2 presented very slight damage, comparable to healthy animal (Fig. 5). 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 (Fig. 5).

Quantitative changes in cytokine response induced by APL2 were evaluated. In particular, TNF-α, IL-17 and IL-10 were measured in killed mice on day 60. Therapy with this peptide led to significant reduction of TNF-α and IL-17 levels. But, we do not find differences in the levels of IL-10 among the groups (Fig. 6).

Discussion

A remarkable progress in the understanding of the mechanisms of autoimmune arthritis has taken place in recent years and such understanding has been translated into novel therapeutic approaches. Particularly, significant is the success of therapies aimed at interfering the role played by TNF-α in JIA. The efficacy of several anti-TNF drugs [15, 16] has been shown in randomized controlled trials in the polyarticular form of disease. However, as in adult RA, some patients with JIA do not respond or are intolerant to anti-TNF therapy [17].

This work is focused on the induction of peripheral tolerance starting from an autoantigen involved in the autoimmune arthritis pathogenesis. Conceptually, this therapeutic intervention is based on modulation of T cell function, and therefore, higher specificity and lower toxicity are expected [18, 19]. The Hsp60 was selected as autoantigen. Several authors have reported that peptides derived from this protein are identified as "danger" signal and cause an inflammatory physiological response, which contributes in clearing a possible pathogen invasion but also induces T cells with regulatory function [20–22]. This last function is reduced in autoimmune arthritis.

In previous study, we predicted by bioinformatics tools a novel T cell epitope (called E18-12) located in the N terminal region (amino acids 55–75) of human HSP60. According to our prediction, this peptide would be a strong epitope directly involved in the interaction with HLA class II molecules, particularly those related to RA. 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. In theses assays, an increment of TNF-α and INF-γ levels was confirmed [14]. This epitope is different from that reported by Kamphuis et al. [23]. These authors found a potential pan-DR HSP60 epitopes using a computer algorithm, which induce a disease-specific anti-inflammatory T cell response in PBMC from JIA.

Starting from E18-12, we designed three APLs. Theses APLs share the binding characteristics to HLA class II molecules with the wild-type peptide, but containing one modification in an essential contact position with HLA class II molecules. The purpose of this modification in each case was transforming the T cell response induced by the wild-type peptide in order to induce tolerance in patients with autoimmune arthritis. E18-12, APL1 and APL2

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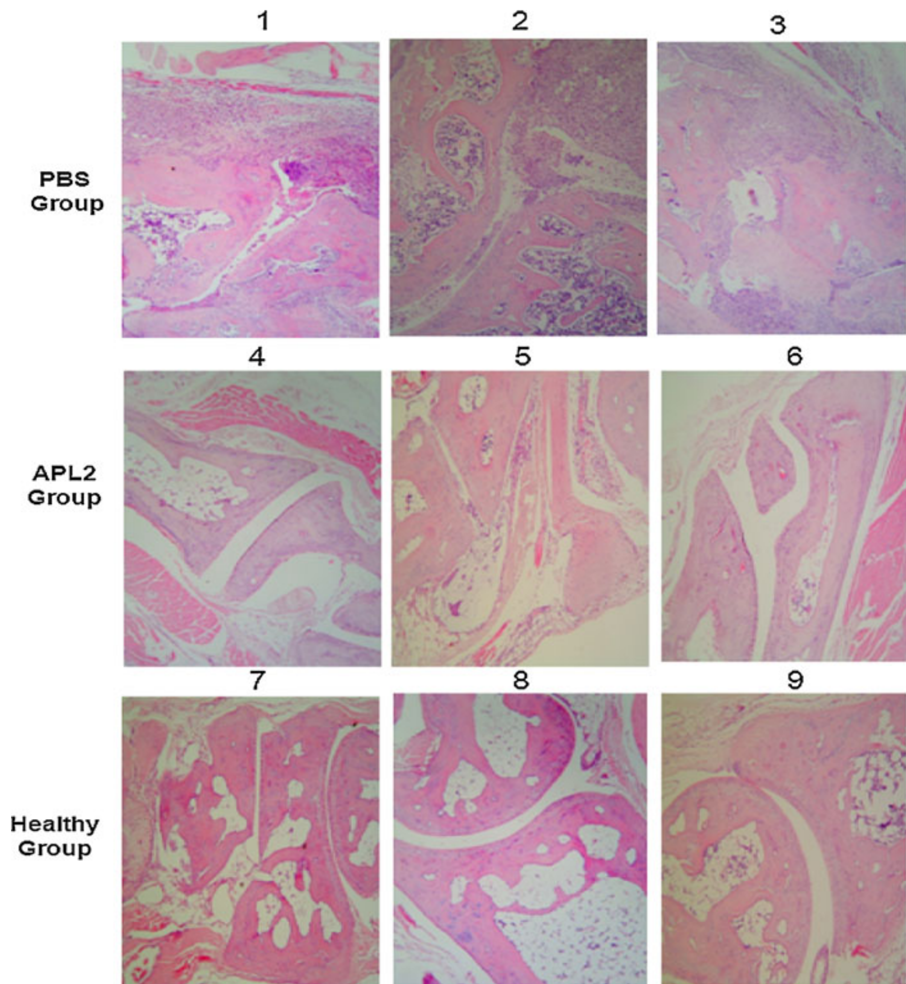

Fig. 5 Treatment with APL2 significantly prevented of histological damage in ankle joints from mice of collagen-induced arthritis (CIA) model. DBA/1 mice were immunized and boosted with chicken type II collagen (CII). Three mice per group were killed on day 60, joints were harvested and stained with hematoxylin and eosin and are represented original magnification of 10×. The histopathological damage score in ankle joints was assigned using values between 0 and 3. All these histopathological procedures were performed totally blinded. PBS group (mice 1–3): severe synovitis with pannus formation, extensive erosions of bone and cartilage. APL2 group (mice 4–6): mild synovitis with hyperplastic membrane, no inflammatory reaction. Healthy group (mice 7–9): joint with a conserved synovial space

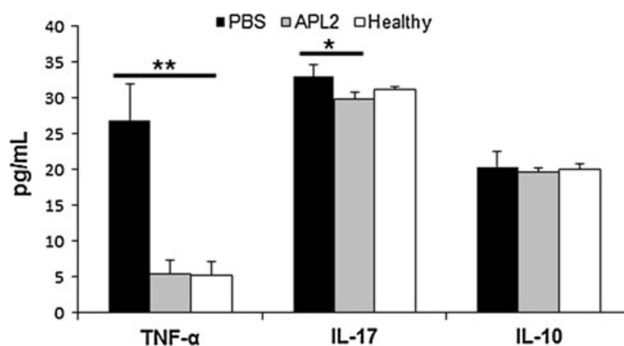

Fig. 6 Treatment with APL-2 led to significant reduction of tumor necrosis factor (TNF)-α and interleukin (IL)-17 in mice. DBA/1 mice were immunized and boosted with type II collagen (CII). APL2 was administered subcutaneously on days 28, 31, 34, 37, 40 and 43 after immunization of CII. TNF-α and IL-17 concentrations in serum from mice killed on day 60 were measured by ELISA. *Bars* show mean + standard deviation, and data were analyzed using Kruskal–Wallis and Dunn post-test (*$P \leq 0.05$; **$P \leq 0.01$)

induce the activation of CD4+ T cells, but only APL2 increases the IL-10 levels in PBMCs from RA patients [14].

In comparison with adult patients, experience in using TNF-α blocking agent in childhood is limited but it is known that not all subtypes of JIA respond successfully to treatment. For example, the rate of treatment failure is higher in the group with systemic-onset JIA, indicating that these patients in particular may require alternative treatments [15–17]. In this work, we evaluate the therapeutic possibilities of APL2 in JIA. In order to demonstrate that the effect of this peptide is independent of the disease subtypes, a small number of patients of different types of JIA were studied.

Ex vivo assays confirmed that APL1 and APL2 maintained the ability of wild-type peptide to stimulate CD4+ T cells derived from all JIA patients. Our data suggest that these peptides provide a survival signal inducing the exit of G0/G1 phase and the progress through the G1 restriction point to the S and G2/M phases of the cell cycle.

On the other hand, E18-12 and APL1 significantly increased TNF-α and IFN-γ levels in vitro assays with PBMC from JIA patients. These findings suggest that both peptides induce a TH1 phenotype in CD4+ T cells from

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these patients. In contrast, APL2 increased IL-10 levels in all JIA patients in more than 3 times (Figs. 2, 3), suggesting that this peptide induces a regulatory phenotype irrespective of different subtypes of this disease. But, this peptide has not any effect on IFN-γ and TNF-α levels (Fig. 1). APL3 did not modify the concentration of any cytokines.

Persistent oligoarticular subtype of JIA has a benign clinical course, whereas the polyarticular subtypes are non-remitting in many cases and need aggressive immunosuppressive treatment. Notably, in the oligoarticular subtype does the presence of T-cell responses to self-HSP60 predict disease remission [24, 25]. The increase of IL-10 secretion induced by APL2 can indicate a change in the systemic cytokine profile from an inflammatory to a regulatory response. IL-10 is a cytokine recognized for its role in the control of autoimmunity [26]. These facts suggest a therapeutic potentiality of APL2 for treatment of JIA.

Quintana et al. [27] found similar results using fragments derived from Hsp60 in experimental autoimmunity models. For example, in type 1 diabetes mellitus, vaccination with HSP60 epitopes activates HSP60-specific regulatory T cells affecting the T cell response to disease-associated antigens by inducing a shift from secretion of INF-γ to IL-10. HSP60 vaccination induces a similar cytokine shift in the response to mycobacterium antigens [28].

In order to confirm the therapeutic effect of APL2 in vivo, we chose CIA model because it is the most commonly studied autoimmune arthritis model [29]. Several experiments have shown that APL derived from CII inhibits the progression of CIA [30–32]. We have previously reported that APL2 reduced the joint inflammation in CIA by inhibition of inflammatory response. These mice were inoculated by subcutaneous route with 50 μg of peptide, the days: 28, 31, 34, 37, 40 and 43 after disease induction and clinical score, histopathology and inflammatory response were monitored in the animals only until day 46 after CIA induction [14].

In the present work, the same therapy is used, but mice were monitored until day 60 after disease induction. Typically, in this animal model, mice are observed for 40–60 days [29]. In this study, we evaluated the arthritis course until day 60 to corroborate the efficacy of the therapy with APL2, obtained in the previous assay. The therapy with APL2 inhibits the progression of CIA. This effect correlated with improvement of the histological score and decrease in damage in the joints from mice killed on day 60, comparable to healthy animals.

On the other hand, APL2 treatment has not any effect on IL-10 levels in CIA. It is possible that APL2 acts through different molecular mechanisms in ex vivo experiments with PBMC from JIA patients and in CIA model. APL2 is derived from human HSP60; consequently, its binding affinity to MHC class II molecule could be different in human and mouse affecting also the interaction of the TcR with the APL2-MHC complex and thus altering the functional outcome of the immune response.

However, APL2 induced inhibition of TNF-α and IL-17. TNF-α is known to be involved in stimulating inflammatory cytokine (including itself) production, enhancing the expression of adhesion molecules and neutrophil activation, and it is also a costimulator of T cell activation and antibody production by B cells [33]. The pivotal role of TNF-α in the induction and progression of rheumatoid synovitis is well established [34, 35].

Several papers support the role of IL-17 in the pathogenesis of human RA and animal models such as CIA [36, 37]. The decrease of IL-17 and TNF-α secretion induced by APL2 could indicate that the therapeutic effect of this peptide is mediated by downregulation of inflammatory cytokines. The demonstration that peptide downmodulates the expression of theses cytokines represents a beneficial effect in the control of inflammatory process and may contribute to the restoration of tolerance in JIA patients.

The results shown here underline the potential of APL2 as a candidate for antigen-specific immunotherapy in autoimmune arthritis as JIA.

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

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