RESEARCH

Suppression of the pathogenic responses in an animal model of adjuvant-induced arthritis by a peptide corresponding to a novel epitope from heat-shock protein 60

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ABSTRACT

Induction of peripheral tolerance has long been considered a promising approach to the treatment of chronic autoimmune diseases, including rheumatoid arthritis. The objective of this work is to induce tolerance by a peptide-specific immunotherapy in an Adjuvant-Induced Arthritis (AA) rat model. Based on an autoantigen involved in the rheumatoid arthritis pathogenesis, the heat shock protein 60 (HSP60), a novel T cell epitope was examined by bioinformatics; a tool used to design two altered peptide ligands (APL). The peptides were administered by intradermal route after inducing AA in Lewis rats. The therapy with the original peptide and an APL caused a significant reduction of clinical signs in AA and decreased histological damage of the joints. Thus, the effect was more significant using the APL. To investigate the effects of the immune mechanism induced by peptides in the suppression of pathogenic response, we observed changes of interleukin 10 expressions and tumor necrosis factor α in spleen of rats sacrificed when maximum severity of the disease was reached. We stated that therapy with peptides induced a significant reduction of tumor necrosis factor α and an increase of interleukin 10 gene transcription. Consequently, the protective effect induced by peptides was associated with functional changes from a proinflammatory to a regulatory phenotype.

Keywords: Rheumatoid Arthritis, adjuvant-induced arthritis, HSP60, tolerance

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RESUMEN

Supresión de la respuesta patogénica usando un nuevo péptido derivado de la proteína de estrés térmico de 60 kDa en un modelo animal de artritis inducida por adyuvante. La inducción de tolerancia periférica usando autoantígenos constituye una atractiva terapia para las enfermedades autoinmunes. El objetivo de este trabajo es la inducción de tolerancia usando un péptido derivado de la proteína de estrés térmico de 60 KDa (Hsp60) en un modelo animal de artritis inducida por adyuvante. Tomando como base la secuencia de la *Hsp60* de rata, se seleccionó un nuevo epitope de células T empleando herramientas de bioinformática. Dicho epitope fue usado para designar dos péptidos tipo APL. Los péptidos fueron administrados a las ratas Lewis por vía intradérmica una vez inducida la artritis en ellas. El tratamiento con el péptido original y uno de los APL provocó una reducción significativa de los signos clínicos y de los daños histológicos en las articulaciones, este último efecto fue más marcado con el APL. Se evaluaron los cambios en la expresión de la interleucina 10 (IL-10) y el factor de necrosis tumoral alfa (TNF-α) en los bazos de las ratas sacrificadas el día de máxima severidad clínica de la enfermedad. La terapia con los péptidos induce una reducción significativa de la expresión génica del TNF-α y un incremento significativo del gen que codifica para la IL-10. Estos resultados sugieren que el efecto protectivo de los péptidos está asociado con un cambio funcional de un fenotipo pro inflamatorio a regulatorio.

Palabras clave: Artritis Reumatoide, artritis inducida por adyuvante, HSP60, tolerancia

*I*ntroduction

Recently, relevant progresses have been achieved for the knowledge of immunological and molecular mechanisms of autoimmune diseases. This progress has been moved into a generation of biological therapeutic agents that target pro-inflammatory cytokines, with the aim at interfering with their mechanisms of action. This approach is expected to progressively complement or replace currently used immunosuppressive and anti-inflammatory therapies [1, 2]. Current anticytokine approaches remain vulnerable by limitations associated eminently with generalized immunosuppression and subsequent increase the occurrence of malignancies and infection diseases [3-5]

In this context, the main challenge in the treatment of autoimmune diseases 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 the autoimmune disease pathogenesis constitutes an alternative for this purpose.

Tolerance is the process that eliminates or neutralizes the autoreactive B and T cells [6]. The primary mechanism leading to self-tolerance is central tolerance that takes place in the thymus and bone-marrow [7, 8]. However, this mechanism is insufficient to control autoimmune events. Autoreactive lymphocytes are present in most healthy individuals, but only 5% of the population is affected by autoimmune diseases. This suggests the existence of mechanisms of peripheral tolerance that prevent the initiation of autoimmune

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diseases by limiting the activation of autoreactive lymphocytes [9].

The induction of tolerance using autoantigens constitutes a therapeutic approach which facilitates the tolerance of loss restoration in the course of autoimmune diseases; also can be used by mechanisms of bystander suppression or anergy [10-14], depending on doses, route and frequency of administration of the antigen [15].

An antigen used in the induction of tolerance is HSP60, a protein that belongs to the family of heat shock proteins (HSP), which is formed by immunogenic proteins with exceptional evolutionary conservation. The antibodies against these proteins must be abundant in healthy people and in patients with autoimmune illnesses [16].

Cohen and Young stated that heat shock proteins could be part of the immunological homunculus, which include a few dominant self antigens encoded in a cellular regulatory network that constitute the immune system's picture of self [17]. Thus, Hsps are considered as candidate antigens to restore the tolerance based on the possibilities of triggering the activation of regulatory T cells [18-20]. In this sense, HSP60 epitopes involved in the mechanisms of regulation have been identified in animal models of adjuvant arthritis [21, 22] and have been selected with the objective of inducing tolerance in patients with autoimmune diseases [23, 24].

On the other hand, these epitopes can be modified in order to modulate their immunological properties. These modified peptides are called 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 [25, 26].

Novel candidate drugs are usually evaluated in animal model. The similarity between the inflammatory processes in AA and in human RA has made this model, a useful tool, for developing anti-inflammatory drugs for human patients [27]. This is an experimental model of autoimmune arthritis that can be induced in susceptible inbred strains as Lewis rats upon immunization with heat-killed *Mycobacterium tuberculosis* (MT) in incomplete freund adjuvant. The rats reproduced several clinical and histo-pathological characteristics of RA patients. Usually, the arthritis severity in this model is evaluated in association with inflammation levels in rats and the presence of pannus, which is a granulation tissue that spreads from the synovial membrane, invades the joints and is responsible for cartilage and bone erosion [28].

Here, we have selected a novel T cell epitope from HSP60 by bioinformatics' tools. This epitope was also used to design two APL. The clinical, histo-pathological and immunological effects of these peptides were compared to an AA animal model.

Materials and methods

Experimental Animals

Female inbred Lewis rats, RT1.B^{L} (5 to 8 weeks-old, weighting 101 to 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. All animal procedures were performed in accordance with the guidelines approved by the Ethical Committee and National Regulations for experiments with animals.

Selection of T cell epitope from hHSP60 of Lewis rats

A peptide binding to rat RT1.B^{L}MHC-II molecule was selected from the sequence of rat HSP60 and according to the sequence motif described in the database SYFPEITHI [29]. (http://www.uni-tuebingen.de/uni/kxi). A program that analyzes all possible peptides was designed to search those that fulfill with the motif. The chosen peptide was called $F_{19\text{-}6}$ and its sequence is shown in figure 1.

Design of APLs with modifications at amino acids involved in MHC binding site

The sequence DPTKVVRTA was identified in the peptide $F_{19\text{-}6}$, as the core residues directly involved in the interaction with RT1.B^{L} using the prediction server SYFPEITHI [18]. Analysis of the binding motif indicated that the substitutions K4/H and A9/E in the core peptide could improve the binding properties of the peptide [30, 31]. The peptide having these substitutions was named $F_{19\text{-}7}$ and its sequence is shown in figure 1.

Design of APLs with modifications at amino acids involved in TCR binding

In the core peptide sequence DPTKVVRTA, V5 is the main residue involved in TCR binding according to structural and immunological studies [32, 33]. An amino acid substitution was introduced at position 5 to modify but not avoid TCR recognition. This peptide was named $F_{22\text{-}29}$ and its sequence is shown in figure 1.

Antigens and adjuvants

Heat-killed *Mycobacterium tuberculosis* (MT, strain H37Ra) was obtained from Difco (Detroit, MI). Incomplete Freund's Adjuvant (IFA; Sigma) 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.

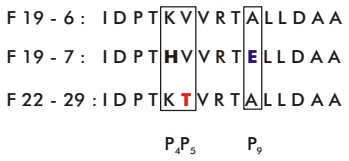

Figure 1. Modifications carried out in the peptides $F_{19\text{-}7}$ and $F_{22\text{-}29}$ starting from the original epitope $F_{19\text{-}6}$ (The amino acids modified in each APL have been represented with different colors). Positions P_4, P_5 and P_9 represent residues interacting with the RT1.B^{L} groove.

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Induction of Arthritis by adjuvant in Lewis rats and assessment of arthritic damage

Each animal was inoculated subcutaneously at the base of the tail with a freshly prepared emulsion (100 µL) containing 1 mg of MT. The rats were observed daily for signs of arthritis from day 0 until day 35; then, every three days from day 36 until the end of the experiment on 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 could receive a maximum score of 16 points.

Peptide immunotherapy protocol

On day 10, rats were randomly divided into 3 treatment groups. The peptides were administrated on days: 12, 16 and 20 after disease induction, each dose contained 200 µg of peptide in PBS. The rats were anaesthetized using ketamine (50 mg/kg, intramuscular) previously to the inoculation.

Histopathology

For histological evaluation, hind limbs were removed and fixed in 10% neutral buffered formalin (PANR EAC, 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 haematoxylin and eosin. The histological damage, evaluated microscopically, was defined according to an established scale as follows: 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 histopathology procedures were performed totally blinded.

Splenocyte isolation

Spleens from three rats of each group were removed and homogenized on day 21after arthritis induction. The splenocytes were washed once PBS 1X, erythrocytes were lysed with 0.83% NH_4Cl, washed three times and resuspended with RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL gentamycin, 25 mM/L HEPES (all from Gibco BRL, England).

Isolation of RNA and RT-PCR

Total RNA was extracted from splenocytes with TRI-REAGENT™ (Sigma, USA) as specified by the manufacturer. Relative amounts of mRNA for TNF-α, IL-10 and GAP-DH were determined by RT-PCR using the Gene Amp RNA PCR Kit (Perkin-Elmmer, USA). The following primers were used: GAP-DH, forward 5'-ATCTCTGCCCCCTCTGCTGAT-3', reverse 5'-AGTGTAGCCCAGGATGCCCTT-3'; TNF-α forward 5'-GTTCCATGGCCCAGACC CTCACA-3', reverse 5'-TCCCAGGTACATGGC TCATACC-3'; IL-10 forward 5'-CCAGTTTT ACCTGGTAGAAGTGATG-3', reverse 5'-TATTT ATGTCCTGCAGTCCAGTAGAC-3'. PCR conditions were: 94 ºC for 3 min, 94 ºC for 1 min, annealing temperature specific for each gene for 1 min, and 1 min primer extension at 72 ºC, subsequently 35 cycles of 1 min at 94 ºC, GAP-DH: 58 ºC, TNF-α: 64 ºC, and IL-10: 54 ºC for 1 min and 72 ºC for 1 min, an extension step at 72 ºC for 3 minutes. PCR products were analyzed with the ID Image software program (Kodak, USA). The results were expressed as relative levels of mRNA for each cytokine.

Statistical analysis

Statistical analysis was achieved with Sigma Stat software (version N°2, GraphPad Software, Inc). Data were analyzed using the Mann Whitney Rank Sum test and the Student-Newman-Keuls test. Statistical significance was established at $P<0.05$.

Results

Treatment with peptides has clinical efficacy in Adjuvant Arthritis

Initially, a peptide (named $F_{19\text{-}6}$) from rat HSP60 (518-533 aminoacids) was evaluated to be a T cell epitope using bioinformatics' tools. This peptide was modified trying to increase the affinity to MHC-II or varying the affinity to TCR. Several APLs were designed. These peptides shared MHC binding characteristics with the native peptide, but containing modifications in essential contact positions with the TCR or with the MHC-II molecule that could change the T cell activation.

Two of these APL were selected and their clinical effects as well as the original peptide were evaluated in an animal model. The sequences of these peptides are shown in figure 1. The peptide $F_{19\text{-}7}$ was modified in the essential contact positions with the MHC-II molecule. In the peptide $F_{22\text{-}29}$ the modification was at the position 5, corresponding with the essential contact site with the TCR [32, 33].

As it is shown in figure 2, 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 inflammation of the posterior joints. It is also observed that the administration of peptide $F_{19\text{-}6}$, the original hHSP60 epitote, and its variant APL $F_{19\text{-}7}$ induced a significant reduction of clinical signs of Arthritis in animals treated with both peptides. However, in group IV, the arthritis signs were expanding to the rest of the joints until became severe in all rats.

The main parameter used in this study to measure clinical outcomes and evaluation of the effects of the peptides was the mean arthritis score on day 21 (the day of maximum arthritis severity). A significant reduction of AA mean arthritis score on day 21 ($p<0.05$) was examined with $F_{19\text{-}6}$ and $F_{19\text{-}7}$ peptides compared to non treated animals. Treatment with the $F_{22\text{-}29}$ peptide showed a trend towards reduction of arthritis, but statistically significant differences were

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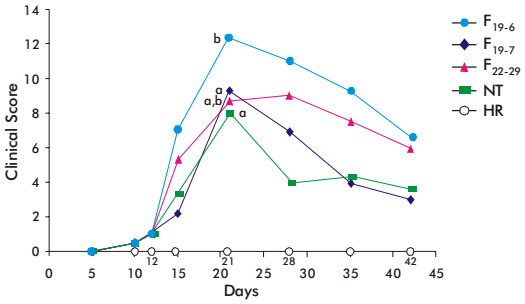

Figure 2. Evaluation of clinical signs. Treatment with F_{19-6} and F_{19-7} peptides led to a significant reduction of Adjuvant Arthritis (AA) in ill animals. Arthritis was induced on day 0 by immunization with heat-killed Mycobacterium tuberculosis (MT) in Incomplete Freund Adjuvant. On day 10, rats were randomly divided into five groups of 12 animals each, which were inoculated with peptides F_{19-6}, F_{19-7} or F_{22-29}, non-treated (NT) or healthy rats (HR), respectively. Arthritis scores were assessed on days 5, 10, 12, 15, 21, 28, 35 and 42. Mean arthritis scores are shown. Different letters mean statistical differences ($p<0.05$)

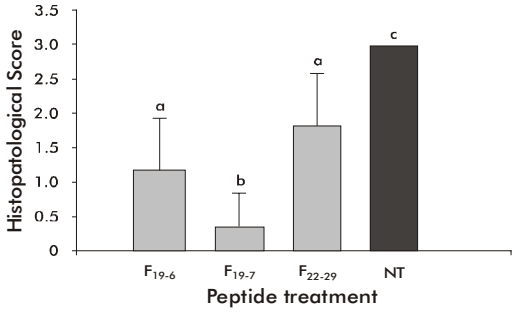

Figure 3. Histological analysis. Treatment with F_{19-7} peptide led to a significant reduction of histological damage in ankle joints. Joints were harvested on day 21 after induction of arthritis, the histopathological scores shown. The histological damage in ankle joints was defined as: Grade 0, normal; 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. All these hystopathological procedures were performed totally blinded, in six animals for the four groups of ill rats (inoculated with peptides F_{19-6}, F_{19-7} or F_{22-29}, or non-treated (NT). All the animals in the NT group reached the maximum histopathological score (3). Different letters mean statistical differences ($P=0.029$)

not observed versus non treated animals or treated with F_{19-6} and F_{19-7} figure 2.

Treatment with peptides and specially with F_{19-7} leads to a decrease of damage in the joints

Clinical improvement of AA, induced during therapy with peptides, was compared to decrease the joint destruction by the arthritic process. Six animals were sacrificed per group and ankle joints were collected on day 21 after the arthritis induction and scored for severity inflammation in the synovium, pannus formation, cartilage and bone erosion.

A considerable correspondence between the data obtained by the evaluation of the clinical signs and the histo-pathological report was showed. All rats without treatment presented a histological score of 3. The therapy with peptides led to a significant improvement of the histological score of the joints as shown in figure 3. Specially, the animals treated with F_{19-7} led to the best improvement of the histological score. Only two rats out of six sacrificed on day 21 presented histological damages which were limited to slight inflammation in the synovium.

Therapy with peptides led to a significant reduction and an increase of the expression TNF-α and IL-10 gene transcriptions

Qualitative changes in cytokine response induced by treatment were evaluated. In particular, the expressions of TNF-α and IL-10 were measured. The results were expressed as relative levels of mRNA for both cytokine (Figure 4).

Treatment with peptides led to a significant reduction of TNF-α gene transcription, also a significant increase of IL-10 transcription.

Discussion

Although the pathogenesis of many autoimmune diseases including RA remains unknown, a dramatic progress in the understanding of the mechanisms of autoimmune inflammation has taken place in recent years and has been used in novel therapeutic approaches. Particularly remarkable is the success of therapies aimed at interfering with the role played by TNF-α in RA. However, high cost, adverse drug events and unintentional concomitant immune suppression, leading to serious opportunistic infections, present limitations that might prevent the prescription of these biological drugs [3, 4]. Another one is the treatment outcome of the TNF inhibitors which remains insufficient in 40-60% of patients with rheumatoid arthritis [5].

This approach presented is based on the induction of peripheral tolerance using an autoantigen involved in the autoimmune arthritis pathogenesis. Concep-tually, a therapeutic intervention by modulation of T cell function; therefore higher specificity and lower toxicity is expected [34-37]. The HSP60 was selected as autoantigen. Several authors have reported that peptides derived from HSP60 may play a role in amplification of autoimmune process [14, 22, 38]. These peptides 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, 39]. This last function is reduced in autoimmune arthritis.

A novel HSP60 epitope from rat HSP60 (518-533 amino acids) was identified by bioinformatics' tools which demonstrated 100% similarity between human and rat but 56% similarity with mycobacterium. In theory, this peptide represents a strong epitope directly involved in the interaction with rat $RT1.B^{L}$ (MHC class II in rat) and could stimulate T cells participating in autoimmune inflammation. This concept is based on the modulation of this T cell response depending on the doses, route and frequency of administration of the peptide.

In addition, two peptides type APL were designed starting from this original epitope. Both peptides shared MHC-II binding characteristics with the native peptide,

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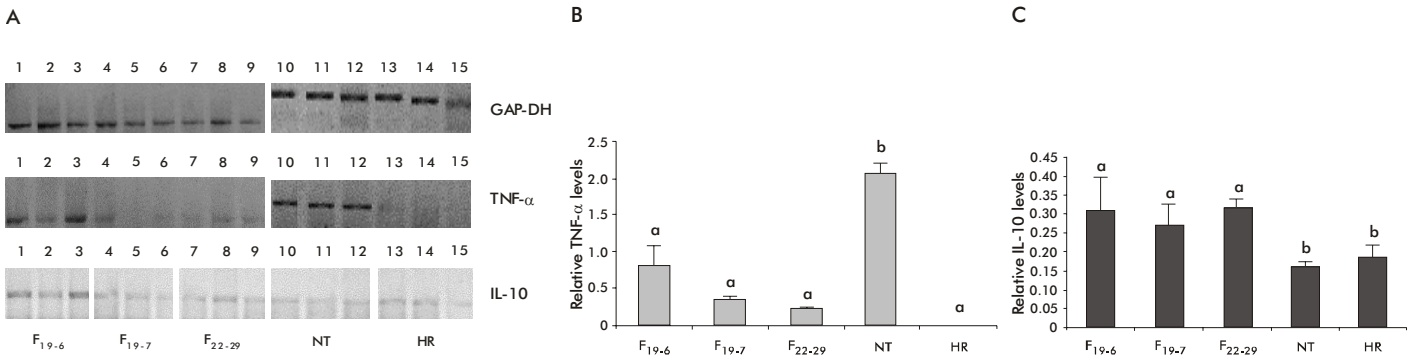

Figure 4. Relative cytokine levels. Treatment with peptides led to a significant reduction in TNF-α gene transcription, whereas it also led to a significant increase in IL-10 transcription. A) mRNA was isolated from spleens on day 21 after induction of arthritis, and GAP-DH (440 bp), TNF-α (383 bp) and IL-10 (320 bp) mRNA levels were determined by RT-PCR analysis. Represented are lanes corresponding to samples of three animals from each treatment group (peptides F_{19-6}, F_{19-7} or F_{22-29}, non-treated (NT) or healthy rats (HR), respectively), which were assayed for each cytokine. Data are representative for three independent experiments. The results were expressed as relative levels of mRNA for both cytokines. B) relative TNF-α levels. C) relative IL-10 levels. Different letters represent statistically significant differences

but contained modifications in essential contact positions with the TCR (F_{22-28} peptide) or with MHC-II (F_{19-7} peptide). The purpose of these modifications was to change the T cell response induced by the original peptide in order to reach a potent protection against arthritis in an animal model, as previous evaluation of this concept for the treatment of patients.

To state the therapeutic effect of the peptides, AA, a T cell HSP-dependent model of RA [40] was chosen. In this model, arthritis can be induced in Lewis rats with heat-killed MT suspended in IFA. Immunization with the MT HSP60 mycobacterium 60-kDa heat shock protein protects against subsequent arthritis induction and this protection is T cell mediated [41]. Protection was found to be mediated by T cells that recognized a conserved sequence of MT HSP60, peptide M256-270 and it was associated with the production of regulatory cytokines [42]

In the present work, the treatment with the original peptide (F_{19-6}) and APL F_{19-7} induced excellent clinical control of AA. This effect was correlated with improvement of the histological score in the joints induced by the peptides. Especially with $F_{19-7,}$ this peptide induced a very significant decrease of histological damage in the rat joints. The improvement induced by F_{19-7} was compared to healthy animals. This fact indicates that the modifications carried out to the original peptide reinforced its therapeutic potentialities.

In the case of the F_{22-29} peptide modified in the contact residue with the TCR, the rats treated with this peptide did not present a clinical improvement in comparison to the non treated animals neither with the group treated with the original peptide. Therefore, the modification carried out in the contact site with the TCR was not effective for attenuating the pathogenic inflammation compared to the original peptide.

The clinical efficacy achieved by the treatment with peptides, in special with F_{19-6} and F_{19-7}, could be caused by the induction of immune deviation [24, 43, 44], a switch from Th1 to a Th2 response or the development of IL-10/TGF-α producing cells because the therapy with the peptides led to an increase of the production of IL-10 and induced suppression of TNF-α. The increase of IL-10 secretion induced by these peptides could indicate a change in the systemic cytokine profile from an inflammatory to a regulatory response; thus, could explain the protection against inflammation by these peptides. IL-10 is a cytokine recognized for its immunosuppressive properties and secreted by regulatory T cells [45,46]

These results are with several studies reporting that the production of the anti-inflammatory IL-10 cytokine is enhanced following the acquisition of resistance to AA. Examples of experiments in which IL-10 is over-expressed are: priming of T cells with the aa 256-270 peptide [42]; induction of nasal tolerance by HSP65 peptide aa180-188[47]; DNA 188 vaccination against AA [48] and treatment of macrophages with protective anti-HSP65 antibodies [49]. These observations highly suggest that IL-10 plays a significant role in the suppression of inflammation in arthritis-resistant animals.

On the other hand, the peptides induced inhibition of TNF-α. This cytokine is known to be involved in stimulating inflammatory cytokine (including itself) production, enhancing the expression of adhesion molecules and neutrophil activation, and is also a costimulator of T-cell activation and antibody production by B cells [50]. The pivotal role of TNF-α in the induction and progression of rheumatoid synovitis is well established [51,52]

Recent studies have shown that TNF down-modulates the function of human $CD4^+CD25^+$ regulatory T cells [53]. Notably, treatment of RA patients with anti-TNF-α antibodies caused an increased frequency of $CD4^+CD25^+$ T regs and reversed their defect on inhibition of cytokine secretion by $CD4^+CD25^-$ T cells [54].

The proof that peptides down-module the expression of TNF-α provides a beneficial effect in the control of inflammatory process and may contribute to the restoration of tolerance by permitting the full expression of $CD4^+CD25^+$ T reg functions.

The immune deviation achieved during down-modulation of the pathogenic T cells by peptides could be associated with several mechanisms as inducing energy in these cells or indirectly through bystander suppression [24, 55-57]. Another indirect mechanism of regulation could be through modulation of APC by the induction of IL-10 to generate a Th2 type response to the autoantigens [58]

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In summary, the therapy with the original peptide and the APL $F_{19\text{-}7}$ significantly decreased the arthritis severity. In particular, $F_{19\text{-}7}$ induced the best therapeutic effect from histological point of view**.** The protective effect induced by peptides was associated with functional changes from a proinflammatory to a regulatory phenotype. Intervention on T cell function in a specific manner as shown here would give the possibility of focusing on one or more antigens involved in the autoimmune pathogenesis, thus avoiding the patients generalized immune suppression as happened with anti-TNF treatment. This study contributes to the knowledge of mechanisms and tools needed for induction of tolerance in humans using autoantigens or their variants.

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