# Characterization of two experimental rodent models for evaluating novel drugs for Rheumatoid Arthritis

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## ABSTRACT

Rodent models are widely used to evaluate molecular mechanisms of rheumatoid arthritis (RA) and potential new therapeutic drugs. In our context, we developed and characterized two experimental models in Lewis rats, adjuvant arthritis (AA) and collagen-induced arthritis (CIA), to assess novel biotechnology drugs for RA treatment. We evaluated clinical signs and histological damage in animals. Specific antibody production and cytokine profile of T cells were also determined. All the Lewis rats developed arthritis following a subcutaneous injection of type II collagen (CII) or *Mycobacterium tuberculosis* and showed a swelling rate of more than 100% at the hind paws. Data showed a faster course of arthritis and a more severe inflammatory response in the AA model compared to the CIA model. In both models, the formation of the *pannus* and osteoclast hyperplasia was observed. A strong antibody response to CII was detected in the CIA model. We also observed a pronounced skewing of the cytokine balance towards Th1 in both experimental models. These findings suggest that inflammatory response in the AA model is more severe than in the CIA model but both are useful in assessing new biotechnology drugs for RA treatment.

*Keywords:* Rheumatoid arthritis, adjuvant arthritis, collagen-induced arthritis, rat, autoimmunity

## ABSTRACT

**Caracterización de dos modelos experimentales en roedores para evaluar nuevas drogas biotecnológicas para la Artritis Reumatoide.** Diversos modelos en roedores son empleados para evaluar los mecanismos moleculares de la Artritis Reumatoide (AR), así como nuevas drogas terapéuticas para su tratamiento. Nuestro grupo ha desarrollado y caracterizado dos modelos experimentales en ratas Lewis: la artritis adyuvante (AA) y la artritis inducida por colágeno (AIC) a través de la administración subcutánea de *Mycobacterium tuberculosis* o el colágeno tipo II bovino, respectivamente. La evaluación de los signos clínicos y el daño histológico demostró una inflamación de un 100% en las patas traseras de todos los animales de ambos modelos, con la formación del pannus e hiperplasia de los osteoclastos. Sin embargo, en el modelo AA el curso de la artritis fue más rápido y la repuesta inflamatoria más severa. A diferencia del modelo de AA, en el modelo de AIC se detectó una fuerte repuesta de anticuerpo contra el colágeno tipo II bovino. En ambos modelos experimentales observamos un aumento en la producción de citocinas Th1 y una disminución de los niveles de citocinas reguladoras. Estos resultados sugieren que la repuesta inflamatoria en el modelo de AA es más severa que en la AIC pero que ambos modelos son útiles para evaluar nuevas drogas biotecnológicas para el tratamiento de la AR.

*Palabras claves:* Artritis Reumatoide, artritis adyuvante, artritis inducida por colágeno, rata, autoinmunidad

## Introduction

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology, which is described as a symmetric polyarticular arthritis that primarily affects the small diarthrodial joints [1]. The clinical manifestations and course of RA are extremely variable and characterized by exacerbations and remissions. Several experimentally induced rat models of autoimmune erosive arthritis are widely used to examine potential etiopathogenetic mechanisms in RA as well as new therapeutic drugs for RA treatment [2]. Depending on the inducing agents used, rat models of erosive arthritis can be classified into three major groups. In the first group, arthritis is induced with autoantigens such as type II collagen (CII) or cartilage oligomeric matrix protein (COMP) in incomplete Freund's adjuvant (IFA) [3]. In the second group, arthritis can be induced with oil-based adjuvants, of which heat-killed *Mycobacterium tuberculosis* (MT) in IFA is most widely used adjuvant [4]. The last group includes several forms of bacterial cell-wall components-induced arthritis; being the streptococcal cell wall (SCW) arthritis, the best characterized model [5].

Collagen-induced arthritis (CIA) is characterized by synovial cell proliferation, inflammatory cell infiltration, and erosion of cartilage and bone. Rheumatoid factor and antibodies against the HSP65 are also detected [6]. Previous studies have shown that anti-CII antibodies [6, 7] and CII-specific T-cell responses [8] participate in the pathogenesis of CIA. On the other hand, the adjuvant-induced arthritis (AA) is a T-cell-dependent disease where the main response is against the heat shock protein HSP60 of MT [9], while humoral immune mechanisms appear not to contribute to the disease process. In this model, arthritis progresses rapidly with an increase in proinflammatory cytokines synthesis such as tumor necrosis factor a (TNF-$\alpha$), interleukin 1 (IL-1), and interleukin 6 (IL-6) after adju-

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vant injection [6, 9]. Other advantages of these animal models are that: the evolution of the disease can be monitored carefully, the genetic background can be manipulated, and the environmental effects can be controlled. However, there are two disadvantages: the genetic differences between animals and humans and the possibility that known pathogenic pathways may diverge in humans [2]. RA is an inflammatory joint disease for which there is at present no effective treatment and therapy development is limited by an incomplete understanding of the causative mechanisms, as well as their enormous complexity. Thus, the animal models might provide valuable insights into the etiopathogenesis of RA. Since, RA is a clinically and genetically heterogeneous disease, it may be expected that no particular RA model will represent all aspects of the human disease, but some animal models can be used to describe different pathways leading to arthritis. In the present study we developed and characterized two experimental models in Lewis rats, AA and CIA, to test novel biotechnology drugs for RA treatment. The rat models developed display clinical and histological features resembling RA in humans; these models provide powerful tools to study the mechanisms leading to RA and to develop new therapies.

## **M**aterials and methods

### Experimental Animals

Female inbred Lewis rats, RT1.B$^L$ (5 to 8-weeks of age, weighing 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 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. All animal procedures were performed in accordance with the guidelines approved by the Ethical Committee and National Regulations for animal experiments.

### Indution of arthritis

The rats were divided into various groups, each containing 15 rats. Lyophilized Bovine CII (Sigma, USA) dissolved at a proportion of 4 mg/mL in 0.05M acetic acid, was mixed with an equal volume of IFA (Sigma, USA) and emulsified thoroughly. A total volume of 0.1 mL of the emulsion was injected subcutaneously 2-3 cm away from the base of the tail of the animals (Group I). A booster injection was given eight days later. Lyophilized MT (strain H37Ra) obtained from Difco (Detroit, MI), was thoroughly macerated with three drops of IFA at 30 second intervals. After 20 min a greater volume of IFA was added, always macerating to intervals of 30 s. Finally the emulsion at 10 mg/mL is gently stirred overnight at room temperature. A total volume of 0.1 mL of the emulsion was injected subcutaneously 2-3 cm away from the base of the tail (Group II).

### Evaluation of arthritis

The rats were observed daily for signs of arthritis from day 0 to day 35 and then, every three days from day 36 until the end of the experiment at day 50. Arthritic rats and healthy animals from the control group were sacrificed at days 15 y 21 (n=3 in each group). The severity of arthritis in each paw was determined according to a scoring system established in our laboratory: 0, no disease; 1, slight swelling of the anklebone or carpus areas, or visible redness and inflammation of a particular toe, regardless of the number of affected toes; 2, moderate redness and swelling of the anklebone or carpus areas; 3, severe redness and swelling of the whole paw including the toes; 4, maximum swelling and deformity of the paw involving multiple joints (total score: sum of 4 paws).

### Histopathology

For the histological assessment, hind legs were removed and fixed in 10% neutral buffered formalin (PANREAC, Spain) at room temperature between 5-7 days and were decalcified with formic acid (50% v/v) and sodium citrate (13% w/v). The tissues were then dehydrated in an 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 a system established in our laboratory: 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 in a completely blinded manner.

### Splenocyte isolation

The spleen from three rats of each group was removed and homogenized 15 and 21 days after immunization. The splenocytes were washed once with PBS 1X, erythrocytes were lysed with 0.83% NH4Cl and 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, and 25 mM/L HEPES (all from Gibco BRL, England).

### Isolation of RNA and RT-PCR

Total RNA was extracted from splenocytes with TRI-REAGENT$^{TM}$ (Sigma, USA) as specified by the manufacturer. Relative quantities of mRNA for TNF-$\alpha$, IL-10 and GAP-DH were determined by a 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-$\alpha$ forward 5'-GTTCCATGG CCCAGACCC TCACA-3', reverse 5'-TCCCAGGTACATGGC TCATACC-3'; IL-10 forward 5'-CCAGTTTTAC CTGGTAGAAGTGATG-3', reverse 5'-TATTT ATGTC CTGCAGTCCAGTAGAC-3'. PCR conditions were as follows: 94 ℃ for 3 min, 94 ℃ for 1 min, annealing temperature specific for each gene for 1 min, and 1 min primer extension at 72 ℃, subsequently 35 cycles of 1 min at 94 ℃, GAP-DH: 58 ℃, TNF-$\alpha$: 64 ℃, and IL-10: 54 ℃ for 1 min and 72 ℃ for 1 min, an extension step of 72 ℃ for 3 min. PCR products were analyzed with the ID Image software program

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(Kodak, USA). The results were expressed as relative levels of mRNA for each cytokine.

## Detection of collagen type II-specific antibodies

Anti-collagen antibodies were detected by an ELISA test optimized for this purpose. Briefly, high-binding plates (Costar, USA) were coated with 2 mg/mL of collagen bovine type II in PBS pH 7.2 for 45 min at 37 °C in a humid chamber. After washing with PBS/0.05% Tween 20 buffer six times, plates were incubated for 1 h at 37 °C in a humid chamber with serum diluted 1/20 in PBS + 0.05% Tween 20. Specific antibodies were detected with protein A conjugated peroxidase and anti-rat IgG conjugated peroxidase diluted 1/20 000 and 1/10 000, respectively. Finally, ortho-phenylendimine substrate (Sigma, USA), was added and the plate was incubated 10 min at room temperature. Plates were read at 492 nm using a multiscan (Labsystem, Finland).

## Statistical analysis

Statistical analysis was performed 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 $P<0.05$.

## *R*esults

### Evaluation of the clinical signs in AA and CIA

We investigated initially if both models reproduced essential clinical signs of human RA. The development of the inflammatory process began with a redness of the joints of the hind limb that progressively extended towards the remaining limbs, followed by a slight swelling that can end up in severe inflammation in some animals, which causing degradation in locomotion and exhaustion of the general clinical condition of rats. In the AA model (group II) the first swelling signs were observed around day 15 after the induction of the diseases, while in the CIA model (group I) the first clinical signs were observed around day 21 (Figure 1). These results indicate that the course of arthritis in the AA model was much faster than in the CIA model. On day 21 AA model rats presented the most severe signs in the inflammatory
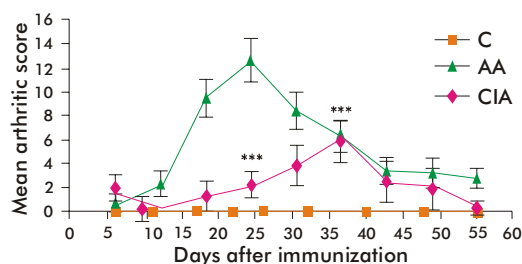


Figura 1. Course of arthritis disease in rats immunized with CII or MT. The onset of arthritis in the AIA model was faster than in the CIA model, with a disease period of 10-46 days. Inflammatory signs of RA in animals were determined on days 6, 11, 15, 20, 26, 32, 41, 48 and 55. Values are shown as mean±standard error for 12 rats per group. Data were analyzed using the Mann Whitney Rank Sum test and the Student-Newman-Keuls test (***, P <0.001)

process, ending up with a maximum average of 13.75 of 16 points possible. This value decreased over time, although a complete remission of the disease was not observed. In general, these rats showed difficult locomotion due to the severity of the swelling. In animals of the CIA model, we observed arthritis of 6.1 points around day 32. This value decreased over time and a complete remission of the inflammatory process was observed. Although these rats developed a less severe inflammatory process than those belonging to the AA model, redness and swelling of the four joints were evidenced. Their movements and clinical status were severely affected. As expected, the animals of group III (control) did not presented any signs of inflammation.

### Histopathological analysis

In order to corroborate the evaluation of the clinical signs observed in both models and to compare them with the control group, joints of all sacrificed animals were histopathologically analyzed. Animals of the control group did not present pathological alterations (Figure 2A). In the CIA model the development of the disease was less severe compared to the AA model (Figures 2D). The animals of group I sacrificed at days 15 and 21 after the induction of arthritis did not present histopathological alterations. These results are in agreement with the evaluation of signs of RA in this group, since on days 13 and 21 animals reached an average of 2.6 and 6.1, respectively (Figures 1). Alterations in this group were observed around day 28 after the induction of arthritis (Figures 2D).

The inflammatory process in both arthritis models was characterized by a distortion of the architecture of the paw with peri-articular fibrosis and cartilage erosion. We observed the formation of the *pannus* and osteoclast hyperplasia, which are destructive cells related with adjacent areas of bone resorption. There was also a granulomatous reaction with infiltration of the bone marrow (Figure 2B-C). Beside this analysis, the results in the AA model revealed the presence of the *pannus*, which causes the erosion of the cartilage and the infiltration of the bone marrow in all animals sacrificed on day 15 (Figures 2C). The granules typical of arthritis induced with MT were observed, as well as the massive osteoclasts hyperplasia and polymorphonuclear infiltration of the synovial membrane. The data indicated that the inflammatory stage was directly associated with the histological damage. The arthritis index was higher in the AA model compared to the CIA model, which is in agreement with the clinical differences observed in both groups.

### Anti-CII antibody response

In our model using Lewis rats we had obtained a significant antibody response to bovine type II collagen. We measured the levels of specific antibodies in sera on days 42 and 61 after the first immunization (data not shown). This response, in all animals, was higher in arthritic than non-arthritic rats and at the same time similar to positive controls employed in this assay. In this model we used IFA to emulsify type II collagen protein and obtain an antibody response to CII, this
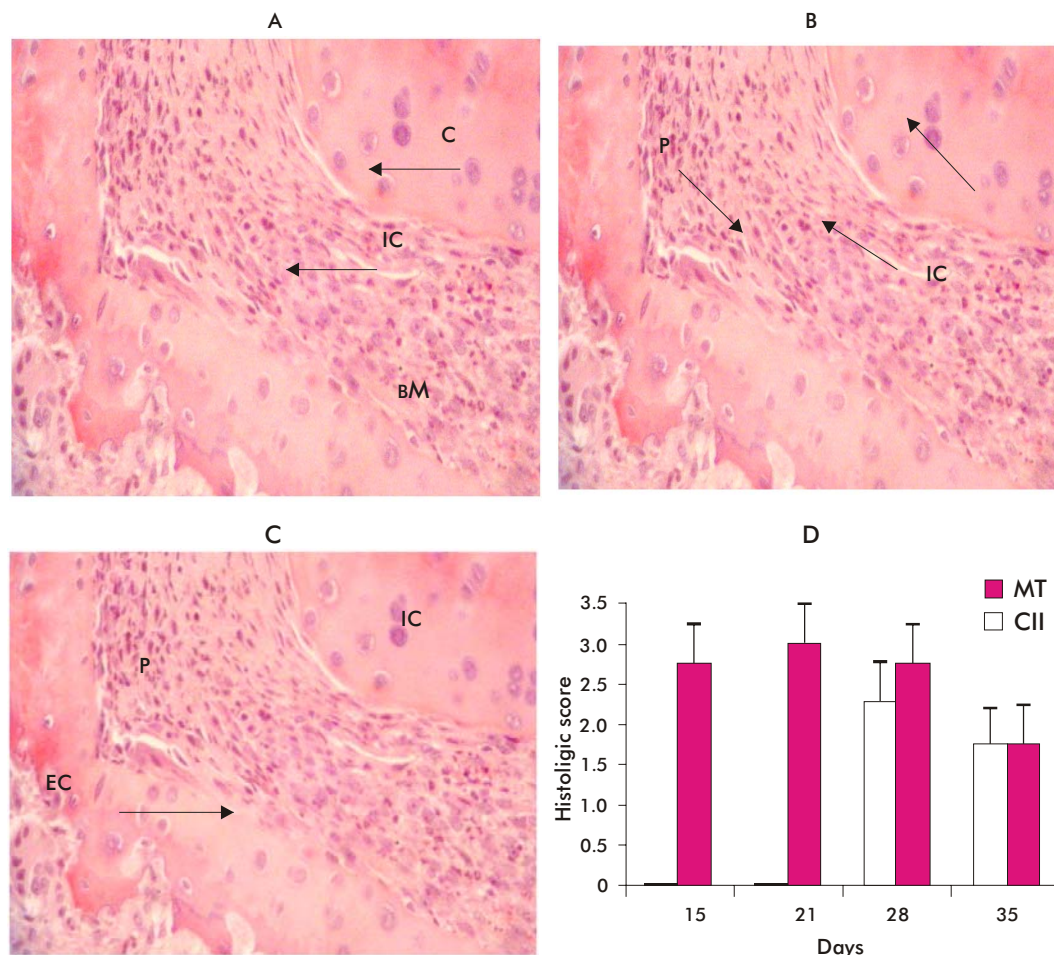
Figure 2. Histological examination of joints of animals immunized with CII or MT. Representative Hematoxilin and Eosin stained joint tissue sections from animals of control group (A), CIA (B) and AIA (C) models on day 21 after immunization. Cartilage erosion (EC), intra-articular cavity (IC) and pannus formation that penetrates into the bone (P) can be observed. The mean histological scores of the arthritic rats in two groups are also indicated for the four time points (D). Error bars indicate the standard error of the mean of 4 rats per group. The data are representative of four independent experiments

result resembles another model developed in our laboratory using CFA (unpublished results). On the other hand we could not detect antibody response to CII in AA model (data not shown).

### Cytokine production by autoreactive T cells involved in the pathogenesis of RA

To establish a correlation of the above data with cytokine secretion by T cells from rats of both models, we analyzed TNF-$\alpha$ and IL-10. Spleen cells obtained 15 days after immunization with MT produced high TNF-$\alpha$ mRNA levels regarding the control group. In contrast, the increase in TNF-$\alpha$ mRNA expression in rats immunized with CII was not significant (Figure 3A). In our experiment we were not able to detect significant levels of IL-10. However, on day 21 TNF-$\alpha$ mRNA levels in rats of both models increased compared with the control group (Figure 3B), while the relative levels of IL-10 were low (Figure 3C). In accordance with a predominant Th1 profile, the TNF-$\alpha$/IL-10 ratio were higher in both experimental models compared to the control group (data no shown).

### Discussion

In this study we developed two experimental models in female inbred Lewis rats taking into account the fact that rats are susceptible to both CIA and AA, which allows parallel studies of both diseases in the same strain. The clinical assessment showed that rats developed arthritis faster in the AA model than in the CIA model. Besides, in the AA model the animals developed a higher degree of inflammation. However, the histological features in both models showed the formation of the *pannus*, a tissue of vascular granulation rich in phagocytic cells, which is a responsible for cartilage and bone erosion [10]. In these animals the inflammatory process was characterized by a degradation of the architecture of the paw with peri-articular fibrosis. We speculate that the differences in the degree of swelling is due to the inducing agent used in the AA model, which produces a more potent immune response compared to CII immunization. The MT contains a large number of proteins, which seem to induce a synergic effect with HSP60 in the induction of the inflammatory physiological response. In

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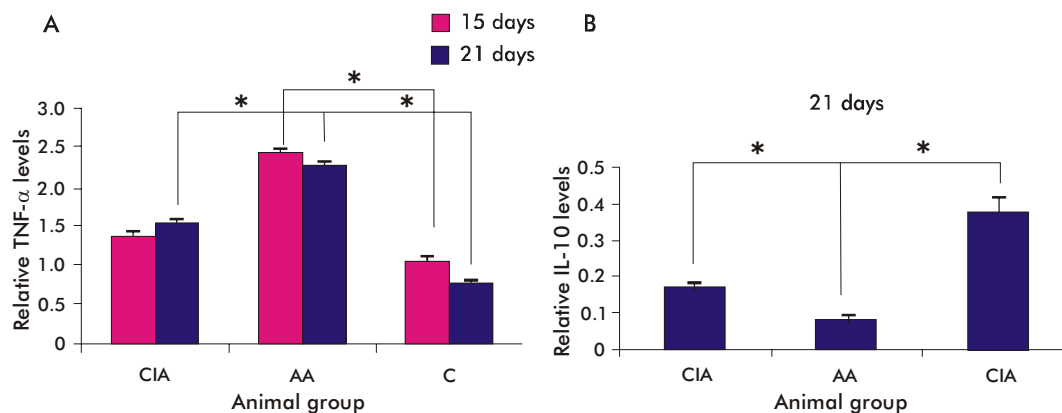
Figure 3. Relative cytokine levels. Administration of CII and MT led to significant increases in the TNF-$\alpha$ gene transcription, whereas it also led to significant reductions in the IL-10 transcription gene. The mRNA was isolated from spleens at day 15 and 21 after the induction of arthritis, and GAP-DH (440 bp), TNF-$\alpha$ (383 bp) and IL-10 (320 bp) mRNA levels were determined by RT-PCR analyses. A) Relative TNF-$\alpha$ levels at days 15 and 21 after induction of arthritis. (B): Relative IL-10 levels at day 21 after the induction of arthritis. CIA: Animals immunized with CII (collagen-induced arthritis model), AA: Animals immunized with MT (adjuvant-induced arthritis model) and C: healthy control group. Results are presented by mean + standard error of 4 rats per group. Data was analyzed using the Mann Whitney Rank Sum test and the Student-Newman-Keuls test (*, P < 0.05)

rats that received the MT inducing agent, an ulcer was observed at the administration site on the base of the tail. A similar ulcerative lesion was observed in humans that are vaccinated against tuberculosis. MT might therefore act as a potent inducing agent attracting components of the immune system of the first defense line towards the administration site. On the other hand, HSP60 epitopes involved in the activation of regulatory T cells have been identified in the AA model [11,12]. This function should be related to the spontaneous remission of arthritis observed in these models.

These findings are consistent with the results obtained by Cohen et al, who developed an animal model of RA with MT in female inbred Lewis rats [13]. In this study, the disease was induced by a single subcutaneous administration of 1 mg of the antigen in the base of the tail and the severity of arthritis was evaluated through a score similar to that used in our study. The most severe inflammatory signs in this model are also observed 21 days after the induction of arthritis. It is interesting to point out that we observed a spontaneous remission of arthritis. We had previously carried out a comparative study of the course of RA in female and male rats; there was no statistically significant difference for clinical signs and histo-pathological analysis regarding sex (data not shown). It is known that RA has more deleterious impacts on women [3], and we therefore developed both models in female rats, and we consider that the hormonal factor constitutes a critical element in the susceptibility to disease. However, Berent et al induced arthritis in male inbred Lewis rats by intracutaneous injections of MT at the base of the tail. In this model they observed the most severe signs of arthritis about 27 days after arthritis induction and animals remitted spontaneously until their total recovery [14].

The selection of the administration site of the inducing agent is a crucial factor for the experimental model. Previously, we studied the influence of the administration site of the inducing agent on the development of arthritis. In this study, arthritis was in-

duced with a subcutaneous injection of the MT antigen at the base of the tail and at the hind footpad of female inbred Lewis rats. In both cases, the animals showed clinical signs of arthritis. However, in animals inoculated at the hind footpad it was not clear whether inflammation resulted from damage at the articulation caused by MT or whether it was due to an infection caused by the direct contact between inoculation site and the excrements of the animals, a factor that could induce a reactive arthritis. For this reason, we chose the base of the tail for the inoculation of the inducing agent.

Another hallmark of the CIA model is the strong B cell response specific for triple helical epitopes [15]. These B cells are autoreactive and produce arthritogenic antibodies, which cause a significant portion of the inflammatory effect on the joints. The antibody reaction to CII had been detected in different strain of rats with significant levels; it indicates that it is not strain-dependent [16]. Although the Lewis rat is not the most susceptible strain to CIA, we have obtained a similar antibody response to CII with a lower antigen concentration (0.4 mg) than that of other groups using the same adjuvant [17]. On the other hand, in the CIA model we used IFA to emulsified CII and obtained an antibody response resembling another model developed at our laboratory using CFA (unpublished results). Humoral responses to collagen have been demonstrated in patients with rheumatoid arthritis [6]. Our results confirm that CIA may be an appropriate animal model for rheumatoid arthritis in humans.

In patients with RA, there is an increase of activated mononuclear cells producing IL-1, TNF-$\alpha$, and IL-6, which are major contributors to inflammatory responses and joint destruction [18]. However, certain authors suggested that immunoregulatory cytokines, in particular IL-10 could inhibit TNF-$\alpha$ production. Furthermore, recent studies have shown that TNF-$\alpha$ down-modulates the function of human CD4$^+$CD25$^+$ regulatory T cells [19]. Notably, the treatment of RA patients with anti-TNF-$\alpha$ antibodies caused an increase in frequency of CD4$^+$CD25$^+$ Tregs

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and reversed their defect on inhibition of cytokine secretion by CD4$^+$CD25$^-$ T cells [20].

We observed that the TNF-$\alpha$ expression pattern is in line with the clinical evaluation and histo-patological analyses in both animal models. In the AA model the first swelling signs were observed at about day 15 after the induction of the disease, which was related with high TNF-$\alpha$ mRNA levels. In the group immunized with CII the TNF-$\alpha$ mRNA expression on day 15 was not significantly increased. In contrast, TNF-$\alpha$ mRNA expression increased significantly on day 21, which can be related to the clinical sings observed at this time point. Histo-pathological alterations in the CIA model were observed at about 28 days, while in animals of the AA model sacrificed at 15 and 21 days, *pannus* and cartilage erosion were observed. In both models, the increase of TNF-$\alpha$ mRNA in the spleen closely reflects acute disease and an inhibition the suppressive function of Tregs, as also reported in human arthritis. At the same time, we observed a pronounced skewing of cytokine balance towards Th1 in both models as indicated by the higher TNF-$\alpha$/IL-10 mRNA ratio. These results may explain the high arthritis index observed in the CIA and AA model since several studies have reported that Th1-associated cytokines strongly promote the type 1 inflamma-tory response in experimental arthritis, while Th2-associated cytokines may play a down-modulatory role [21]. These findings suggest that AA as well as CIA in Lewis rats is useful as an experimental animal model of RA. In our context, we induced autoimmunity breaking tolerance in these animals, which ena-bled the evaluation of novel drugs for RA treatment.

## Acknowledgments

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