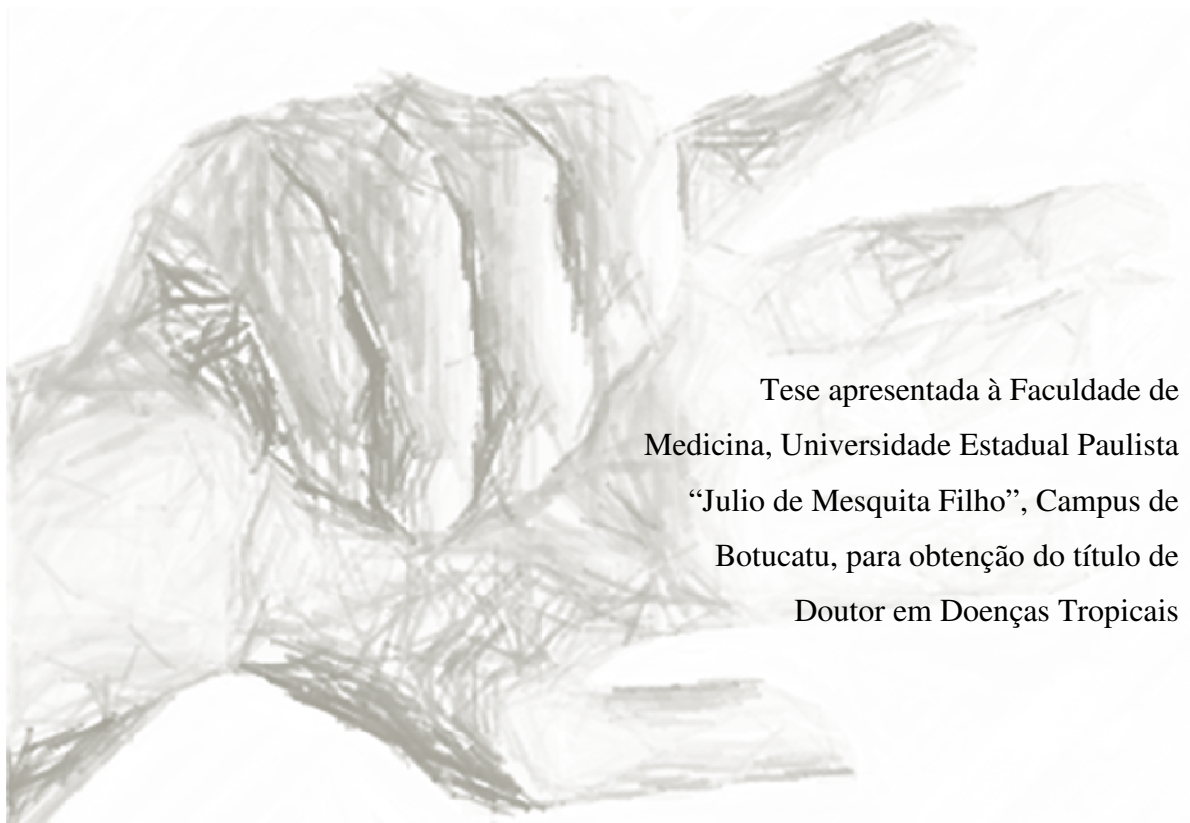


Larissa Lumi Watanabe Ishikawa

**Estratégias tolerogênicas antígeno-específicas visando profilaxia e  
terapia na artrite autoimune experimental**



Tese apresentada à Faculdade de  
Medicina, Universidade Estadual Paulista  
“Julio de Mesquita Filho”, Campus de  
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Doutor em Doenças Tropicais

Orientadora: Profa. Dra. Alexandrina Sartori

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*Dedicatória*

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*Epígrafe*

*“Uma mente que se abre a uma nova ideia nunca volta ao seu tamanho original”*

*Albert Einstein*



*Resumo*

## RESUMO

ISHIKAWA, L.L.W. Estratégias tolerogênicas antígeno-específicas visando profilaxia e terapia na artrite autoimune experimental. 2014. 86 f. Tese (Doutorado) – Faculdade de Medicina de Botucatu, Universidade Estadual Paulista, Botucatu, 2014.

A artrite reumatoide (AR) é uma doença autoimune que compromete as articulações. A maioria das terapias utilizadas para o seu tratamento é baseada na inibição global da resposta imune, podendo aumentar a susceptibilidade a agentes infecciosos. O objetivo geral deste projeto foi definir estratégias tolerogênicas específicas para profilaxia ou terapia da AR. Para isso, em uma primeira etapa, estabelecemos um modelo de artrite induzida por proteoglicano (PG) bovino. Camundongos BALB/c fêmeas *retired breeders* imunizados com PG bovino associado ao adjuvante brometo de dimetil dioctadecil amônio apresentaram os sinais clínicos característicos da artrite, como eritema e edema decorrentes da inflamação articular de uma ou mais patas. A análise histopatológica mostrou a presença de hiperplasia sinovial, infiltrado inflamatório (formação de *pannus*), destruição da cartilagem e erosão óssea. A incidência da doença foi de 100% e os animais artríticos produziram níveis significativos de citocinas pró e anti-inflamatórias e anticorpos IgG1 e IgG2a anti-PG. Em uma segunda etapa, testamos o potencial profilático do PG. A inoculação de três doses de 50 µg de PG determinou um efeito profilático caracterizado pela diminuição significativa da incidência da artrite e do escore clínico dos animais. A diminuição da produção de IFN-γ e IL-17, bem como o aumento da produção de IL-4 e IL-10 por células esplênicas estimuladas com PG, podem estar contribuindo para o efeito profilático observado neste grupo. Em uma terceira etapa, avaliamos o potencial terapêutico da associação da vitamina D ativa (VitD3) com o PG. A associação VitD3+PG determinou um efeito terapêutico na artrite experimental caracterizado por diminuição significativa do escore clínico. Este feito foi confirmado pela análise histopatológica que revelou que a maioria das patas do grupo tratado apresentou estrutura articular bem preservada, semelhante a dos animais saudáveis. Tanto as citocinas pró quanto anti-inflamatórias estavam diminuídas neste grupo. Não foram observadas diferenças no percentual de células dendríticas e T reguladoras no baço dos diferentes grupos experimentais.

Palavras-chave: Artrite experimental, proteoglicano, vitamina D, imunomodulação, tolerância imunológica.



*Abstract*

**ABSTRACT**

ISHIKAWA, L.L.W. Specific tolerogenic strategies for prophylaxis and therapy in experimental autoimmune arthritis. 2014. 86 f. Thesis (Doctorate) – Botucatu Medical School, Universidade Estadual Paulista, Botucatu, 2014.

Rheumatoid arthritis (RA) is an autoimmune disease that affects the joints. Most of the therapies used for RA treatment are based on general suppression of immune response, which may increase the susceptibility to infectious agents. The main objective of this work was to establish specific tolerogenic strategies for prophylaxis or therapy of RA. For this purpose, we first established a model of arthritis induced by bovine proteoglycan (PG). Female BALB/c retired breeder mice immunized with bovine PG associated with dimethyl-dioctadecyl ammonium bromide adjuvant developed a typical arthritis characterized by erythema and edema resulting from joint inflammation of one or more paws. Histopathological analysis showed the presence of synovial membrane hyperplasia, inflammatory infiltrates (*pannus* formation), cartilage destruction and bone erosion. Disease incidence was 100% and the arthritic mice produced significant levels of pro and anti-inflammatory cytokines and IgG1 and IgG2a anti-PG antibodies. Further, we tested the prophylactic potential of PG. Three doses of 50 µg of PG determined a prophylactic effect characterized by a significant decrease in both, arthritis incidence and clinical scores. The decrease in IFN- $\gamma$  and IL-17, as well as the increase in IL-5 and IL-10 production by spleen cells stimulated with PG may be contributing to the prophylactic effect observed in this group. Lastly, we evaluated the therapeutic potential of the combination of active vitamin D (VitD3) with PG. The VitD3+PG association determined a therapeutic effect in experimental arthritis. There was a significant decrease in the clinical scores after VitD3+PG treatment that was confirmed by the histopathological analysis. Most mice paws from the treated group presented well preserved joint structures that were similar to the ones present in healthy animals. Both pro and anti-inflammatory cytokines were decreased after this treatment. No differences were observed in the percentage of dendritic and regulatory T cells quantified in the spleen of the different experimental groups.

**Keywords:** experimental rheumatoid arthritis, proteoglycan, vitamin D, immunomodulation, immunological tolerance.



## *Sumário*

## SUMÁRIO

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# *Capítulo 1*

## *Introdução*

## **CAPÍTULO 1**

Este capítulo é constituído pelo artigo de revisão “Immunomodulation in human and experimental arthritis: including vitamin D, helminths and heat shock proteins”, publicado em uma edição especial sobre meio-ambiente na revista *LUPUS*. Trata-se de uma revisão sobre a imunopatogênese da artrite reumatoide, modelos experimentais da doença e estratégias imunomoduladoras.

## SPECIAL ARTICLE

# Immunomodulation in human and experimental arthritis: including vitamin D, helminths and heat-shock proteins

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Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that is mainly directed to the joints, affecting the synovial membrane, the cartilage and also the bone. This disease affects 1% to 2% of the world population and is associated with significant morbidity and increased mortality. RA experimental models have allowed a great deal of information to be translated to the corresponding human disease. This review summarizes some of the most relevant findings targeting immunomodulation in arthritis. Some general guidelines to choose an adequate experimental model and also our experience with arthritis are supplied. *Lupus* (2014) **23**, 577–587.

**Key words:** Rheumatoid arthritis; regulatory T cells; dendritic cells; vitamin D; helminths; heat-shock proteins

## General considerations

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease involving the synovial membrane, cartilage and bone. Evidences indicate contribution of the innate and acquired immunity including humoral and cellular types of response.<sup>1</sup> This plethora of effector immune mechanisms associated with a variety of etiopathogenic determinants such as genetic susceptibility, environmental stimuli, physical stress and defective immune responses, are probably causing the accentuated heterogeneity in clinical manifestations and severity observed in RA.<sup>2</sup> Arthritis patients suffer from persistent joint inflammation, characterized by joint pain, stiffness and swelling, resulting in progressive disruption of cartilage and bone in multiple joints.<sup>3</sup> If inadequately treated, the inflammatory process can promote permanent destruction and deformity of the joints. Systemic symptoms such as fatigue, anemia and low-grade fever can also be present. Patients with more severe disease can also exhibit extra-articular manifestations such as pericarditis, myocarditis, vasculitis and

pulmonary fibrosis.<sup>4</sup> Overall, the disease is associated with considerable disability and loss of work capacity.

There is not a single experimental model that encompasses all the heterogeneity observed in human RA. However, there is a myriad of experimental models, mainly in rodents, that allows the addressing of the most relevant aspects of the human disease including immunopathogenesis, predictor markers for disease evolution and also approaches to treatment. Briefly, the most classical models for immune-mediated arthritis include administration of adjuvants, oils, bacterial cell components and cartilage-specific antigens, genetic engineering and spontaneous arthritis. A detailed description of these models can be found in recent reviews.<sup>5,6</sup>

## Immunopathogenesis

Despite of the unknown etiology of RA, it is generally accepted that autoreactivity plays a pivotal role in the pathogenesis of this disease. The most relevant autoantigens involved include immunoglobulin (Ig)G, collagen, proteoglycan, citrulline, BiP, Sa-antigen, hnRNP A2, calpastatin, calreticulin, p205, fibrin, fibrinogen and the shared human leukocyte antigen (HLA)-DR epitope.<sup>7</sup>

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In the last years, citrullinated proteins have emerged as the most likely autoantigens involved in RA. Citrullination is a post-translational modification that occurs during the terminal differentiation of cells, apoptosis and also in the course of inflammation. The presence of citrullinated type II collagen and the enzyme that catalyzes the arginine conversion to citrullin correlated with severity of inflammation in experimental arthritis. This citrullinated protein also presented increased immunogenicity and arthritogenicity in rat joints and promoted an earlier onset of the disease than native collagen II.<sup>8</sup> Citrullinated fibrinogen was also able to trigger or increase the severity of arthritis.<sup>9</sup> Interestingly, citrullinated fibrinogen alone or complexed with specific antibody was significantly more potent to stimulate tumor necrosis factor (TNF)- $\alpha$  production by human and murine macrophages.<sup>10</sup> Even though monoclonal antibodies specific for citrullinated collagen II can also induce arthritis in mice, disease severity was enhanced by combining anti-citrullinated and non-citrullinated collagen antibodies.<sup>11</sup>

The precise pathogenesis of RA still remains unclear because it is characterized by an enormous and challenging complexity. Human epidemiological and experimental animal data indicate its association with genetic factors, infectious agents and environmental conditions.

Most of the genetic susceptibility to RA development is related to gene variants in the human HLA system. However, other genes located in non-major histocompatibility complex (MHC) regions, such as protein tyrosine phosphatase non-receptor type 22 (*PTPN22*), TNF receptor-associated factor 1-complement 5 (*TRAF1-C5*), peptidyl arginine deiminase, type IV (*PADI4*) and signal transducer and activator of transcription 4 (*STAT4*), are potentially able to increase their susceptibility. Also, other polymorphisms in TNF, interleukin (IL)-1, IL-6, IL-4, osteopontin (OPN) and profilin (*PRF*) genes are associated with more aggressive disease phenotype.<sup>12</sup> Gene variants in *PTPN22* and *STAT4* seem to confer susceptibility to RA through effects on thymic selection, leading to a repertoire that contains potentially self-reactive lymphocytes.<sup>13</sup> A very clear example of this possibility is observed in the SKG mice strain that spontaneously develops arthritis. These animals present a mutation in the tyrosine kinase protein zeta-associated protein (ZAP)-70 that results in a less strong signal transduction through the T cell receptor (TCR). This reduces thresholds for deletion of autoreactive T cells in the thymus and their release to the periphery.<sup>14</sup>

Infections are also suspected to be involved in triggering or aggravation of RA. The initial evidence of viral association with this disease was, however, indirect, i.e. it was suggested by elevated levels of specific antibodies, higher blood viral load in RA patients and presence of the virus itself or its specific antibodies in the synovial fluid.<sup>15–17</sup> More direct evidence for virus implication in RA was recently reported. Kuwana et al.<sup>18</sup> described the induction of an erosive arthritis in humanized mice inoculated with Epstein-Barr virus. Anaerobical periodontal Gram-negative bacteria have also been linked to RA. Exacerbation of experimental arthritis was observed in mice with periodontitis induced by oral gavage with *Porphyromonas gingivalis*.<sup>19</sup>

Smoking is considered the most prominent environmental risk factor for RA development.<sup>20</sup> Experimental data obtained from rat adjuvant-induced arthritis (AA) indicated that nicotine administration before AA induction is associated with more severe arthritis, increased T cell proliferation and also augmented production of interferon (IFN)- $\gamma$  and IL-17.<sup>21</sup>

Once established, RA is characterized by a vigorous and continuous inflammatory process in the joints that is mediated by both innate and acquired immunity. It has been suggested that this process is initiated by activation of innate immunity via interaction with Toll-like receptors (TLRs). Pathogen-derived molecules such as peptidoglycans and bacterial DNA capable of interacting with different types of receptors were identified in rheumatoid synovium.<sup>22</sup> Experimental models of arthritis sustain the role of TLR in disease induction and also in inflammation.<sup>23,24</sup> In this innate immunity context, it is believed that an unknown antigen (self or non-self) is presented by an antigen-presenting cell (APC) to a T helper cell that secretes a large array of inflammatory cytokines. TNF- $\alpha$ , IL-1 and IL-6 are considered the most relevant mediators of the inflammatory process in RA. The pivotal contribution of TNF- $\alpha$  in the pathogenesis of arthritis was observed by amelioration of classical clinical symptoms such as pain, joint swelling, and stiffness after administration of TNF- $\alpha$ -blocking biologics.<sup>25</sup> This cytokine is also able to promote the expression of other pro-inflammatory cytokines such as IL-17 and IFN- $\gamma$ , which amplifies the contribution of TNF- $\alpha$  in the pathogenesis of RA.<sup>26</sup>

The contribution of CD8+ T cells in the pathogenesis of RA is still controversial. Although they secrete well-known cytotoxic molecules that lead to inflammation and joint destruction, they can also

mediate a regulatory effect through IL-10 production.<sup>27</sup>

For many years the immunopathogenesis underlying RA was mainly attributed to the polarized Th1 lymphocytes. However, following the identification of Th17 cells, strong evidence indicates that this cell subset also plays a key role in mediating synovitis and articular damage.<sup>28</sup> IL-17, which is the Th17 signature cytokine, activates several cell types located in inflamed rheumatoid joints: monocytes, macrophages, fibroblasts, osteoclasts and chondrocytes. This activation leads to secretion of a wide range of molecules such as pro-inflammatory cytokines, chemokines, prostaglandin E<sub>2</sub>, cyclooxygenase-2 and matrix metalloproteinases, all known to be related to joint destruction.<sup>29</sup> In addition, IL-17 promotes osteoclastogenesis with consequent bone erosion.<sup>30</sup> This essential but also extremely complex role of IL-17 in RA pathogenesis is being carefully investigated to disclose possible new therapeutic targets.<sup>31</sup>

Many down-regulatory mediators such as IL-10, receptor antagonists and soluble cytokine receptors are also found in the synovial joint.<sup>32</sup> However, the activity of these molecules is not strong enough to control the inflammatory process and joint damage goes on. This has been, at least partially, attributed to a deficit associated with regulatory T cell function. Many modern immunomodulatory strategies to treat arthritis are, consequently, based on stimulation of their activity as will be considered below.

Several lines of evidence implicate B lymphocytes in the pathogenesis of RA. They contribute to auto-antibodies and cytokines production, antigen presentation to T cells and also to ectopic lymphoid organogenesis in the joints.<sup>33</sup>

## Immunomodulation in RA

### *Regulatory T cells*

Regulatory T cells (Tregs) are a subset of T lymphocytes that express CD25 and the transcription factor Foxp3.<sup>34</sup> They play an essential role in the immune system homeostasis, avoiding the development of autoimmune diseases and also restraining chronic inflammation.

There is increasing evidence that the suppressive ability of Tregs is somehow impaired in RA patients. At least partially, this defective activity is due to a reduced level of Foxp3 transcription. Fontenot *et al.*<sup>35</sup> detected low expression of this factor in CD4<sup>+</sup> CD25<sup>+</sup> high Treg cells isolated from RA patients. A lot of data obtained from

experimental arthritis models have shed some light on this promising field. It has been demonstrated, for example, that depletion of CD25<sup>+</sup> cells before or after induction of arthritis is associated with disease exacerbation and also with increased humoral and cellular immune responses. Interestingly, transfer of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells during arthritis induction but not during chronic established disease decreased the severity of arthritis.<sup>36</sup> These findings suggest that therapies based only on increasing the number of Tregs will probably not be enough to down-modulate ongoing inflammation in RA. These strategies need to consider the relevance of the cytokine microenvironment on Th17 and Treg cell differentiation: Th17 requires IL-6 and transforming growth factor (TGF)- $\beta$  whereas Tregs depend only on TGF- $\beta$ . Also important is the fact that IL-6, which is expressed in the RA synovium, was able to inhibit TGF- $\beta$  generation of Foxp3<sup>+</sup> Treg cells.<sup>37</sup> It is also important to keep in mind that Th17 and Tregs are endowed with plasticity, which seems to be mainly determined by cytokine pressure.<sup>38</sup>

The diminished expansion and/or activity of Tregs in RA had been imputed to an inhibitory effect carried out by TNF- $\alpha$  and B cells. Interestingly, this compromised function of Tregs in RA can be reversed by anti-TNF- $\alpha$  therapy and B cell depletion.<sup>39,40</sup> In the case of TNF- $\alpha$  neutralization, it is important to highlight that this effect depends on the chosen drug. Adalimumab, a human monoclonal antibody that binds directly to TNF, was associated with inhibition of Th17 and increased levels of Foxp3<sup>+</sup> T cells. However, this modulation was not observed in RA patients treated with etanercept, which is a TNF receptor-IgG fusion molecule.<sup>41</sup> Compared to control antibody-treated animals, mice submitted to proteoglycan-induced arthritis and whose B cells were eliminated by specific antibody showed a much less severe disease and a higher proportion of CD4<sup>+</sup> T cells expressing CD25 and Foxp3. These cells also presented a more accentuated suppressive activity than the ones without B cell depletion.<sup>40</sup>

In addition to these *in vivo* approaches targeting restoration of Tregs function, very recent strategies suggest an alternative *ex vivo* expansion of these cells. These methodologies are being optimized by using different products as vitamin D, rapamycin and intravenous Ig.<sup>42–44</sup> This procedure has been able to increase Treg cell number many fold, from 100 to 2000. Interestingly, these cells maintain their phenotype and display enhanced suppressive ability than fresh Treg cells.<sup>45</sup>

# Dendritic cells (DCs)

DCs are able to sense harmful pathogens and a profusion of molecules derived from host tissues. In the first case, DCs prime the immune system to induce effector cells to eliminate the pathogen. In the second case, DCs modulate the immune system to prevent self-destructive autoimmunity. These two functions are usually related to distinct states of DC maturation. Mature DCs (mDCs) are involved in body defense against pathogens whereas immature DCs (iDCs), also called tolerogenic DCs (tDCs), are involved in peripheral tolerance. For the sake of simplicity, we will refer to iDCs or tDCs as cells that are morphologically and phenotypically not fully mature. These iDCs are capable of capturing self-constituents as soluble protein and also apoptotic bodies from dying cells.<sup>46</sup> These tissue DCs that interact with apoptotic cells maintain an immature phenotype characterized by reduced secretion of IL-1 ( $\alpha$  and  $\beta$ ), IL-6, TNF- $\alpha$  and IL-12p70 and also by a marked reduction in their ability to stimulate naïve T cells.<sup>47,48</sup> Similarly to mDCs, iDCs that internalize self-antigens also migrate to proximal secondary lymphoid organs where they induce tolerance via T cell apoptosis, anergy or generation of Treg cells.<sup>49</sup>

A great scientific effort has been devoted to generating tDCs in order to modulate autoimmune conditions. Updated and complete reviews of the strategies to induce this type of DCs are available.<sup>50,51</sup> Briefly, these procedures can be classified considering three aspects: expression of molecules involved in DC maturation, exposure to apoptotic cells and genetic engineering. For example, down-modulation of the relB component of the transcription factor (nuclear factor kappa-light-chain-enhancer of activated B cells) (NF- $\kappa$ B) or neutralization of TNF- $\alpha$ -blocked DC maturation *in vivo*.<sup>52</sup> On the other hand, up-regulation of phospholipase D1, the Ig-like transcripts ILT2 and ILT4 and indoleamine were associated with generation of tDCs.<sup>53–55</sup>

The physiological process of programmed cell death by apoptosis is associated with the release of apoptotic bodies, which prevents the leakage of potentially toxic or immunogenic cellular components from dying cells.<sup>56</sup> The uptake of these bodies preferentially induces tolerance.<sup>57</sup> Recently, it was demonstrated that the specific uptake of apoptotic bodies polarized DCs into tDCs. These tDCs secreted high levels of TGF- $\beta$ , and, interestingly, they were able to trigger the differentiation of naïve T cells into Foxp3 Treg cells.<sup>58</sup>

Genetic manipulation is a promising strategy to induce tDCs. Both viral and non-viral vector-based technologies can be used to develop this type of DCs using genes encoding immunoregulatory molecules.<sup>59</sup> The administration of bone marrow-derived DCs cultured with recombinant lentivirus vectors inserted with mouse IL-10 cDNA prevented and treated experimental asthma.<sup>60</sup>

Some of these tDC manipulations have been studied in experimental arthritis, and the most relevant results observed in the last years are exemplified in Table 1.

# Vitamin D

Traditionally, vitamin D (vitD) has been considered a hormone able to increase calcium and phosphate absorption from the intestine.<sup>66</sup> However, in the last years there have been increasing data indicating that this hormone displays several immunoregulatory activities by interacting with the vitamin D receptor (VDR) that is expressed in different cells from the immune system.<sup>67</sup> Concisely, the myriad of investigations conducted in this area revealed that this immunoregulatory potential is toward immunosuppression, mainly on T cell-mediated immunity.  $1\alpha,25(\text{OH})_2\text{D}_3$ , the active form of vitD, inhibits T lymphocyte activity, mainly cytokine production, by Th1 cells.<sup>68</sup> Active vitD also affects the production of Th2 cytokines *in vitro* but the final effect is still uncertain. Boonstra *et al.*<sup>69</sup> demonstrated the development of a clearly Th2-polarized lymphocyte population, characterized by IL-4, IL-5 and IL-10 production after *in vitro* addition of this hormone. However, Staeva-Vieira and Freedman<sup>70</sup> reported an equally inhibitory activity of vitD in both Th1 and Th2 cytokine production. These effects have been more recently linked to vitD effects over DCs and Tregs. Data from the past few years clearly indicated that vitD or VDR agonists are able to modify not only the phenotype but also the function of APCs, especially DCs. Strong evidence indicates that vitD up-regulates the tolerogenic properties of myeloid DCs, down-regulating IL-12 and Th1 development.<sup>71</sup> Concomitantly, this hormone promotes the differentiation of CD4+ T cells with a T regulatory phenotype.<sup>72</sup>

Concerning this immunoregulatory potential, two aspects that are of overwhelming relevance have emerged: the vitD status in patients with autoimmune diseases and the possibility of modifying DC *in vitro*. Many clinical studies indicated an inverse correlation between vitD levels and disease severity, including cancer, musculoskeletal,

**Table 1** Strategies to generate tolerogenic DCs (tDCs) and their effects on experimental arthritis

| Strategy                                                                                      | Effects                                                                                                                 | Model    | Reference                          |
|-----------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------|----------|------------------------------------|
| IL-12 silencing in DCs                                                                        | Decreased clinical scores                                                                                               | Mice CIA | Li <i>et al.</i> <sup>61</sup>     |
| Histone deacetylase inhibition with trichostatin                                              | Prophylactic and therapeutic efficacy; Th17 reduction and Treg induction                                                | Mice SKG | Misaki <i>et al.</i> <sup>62</sup> |
| Treatment with tDCs generated <i>in vitro</i> by addition of vitamin D and dexamethasone      | Inhibition of disease progression and severity; decreased Th17 cells number; increased CD4+T cells IL-10 producers      | Mice CIA | Stoop <i>et al.</i> <sup>63</sup>  |
| Knockdown of CD40, CD80 and CD86 by si-RNA-transfected DCs prior to loading DCs with collagen | Disease suppression; downregulation of IL-2, IFN- $\gamma$ , TNF- $\alpha$ and IL-17; increased Foxp3+Treg cells number | Mice CIA | Zheng <i>et al.</i> <sup>64</sup>  |
| Blockage of B7/CD28 by using CTLA4-Ig                                                         | Disease suppression; increased proportion of CD4+CD25+Foxp3+ Treg cells in spleen and joints                            | Mice CIA | Ko <i>et al.</i> <sup>65</sup>     |

DCs: dendritic cells; IL: interleukin; Treg: regulatory T cells; IFN: interferon; TNF: tumor necrosis factor; CIA: collagen-induced arthritis; SKG: spontaneous chronic arthritis.

cardiovascular, infectious and autoimmune diseases.<sup>73,74</sup> Furthermore, other diseases that are somehow related to autoimmunity, such as polycystic ovary syndrome and tuberculosis, are also associated with lower serum levels of vitD.<sup>75,76</sup> A stronger correlation between disease severity and lower vitD levels has been detected in some autoimmune diseases such as type 1 diabetes, systemic lupus erythematosus, mixed connective tissue disease, multiple sclerosis and also RA.<sup>77–80</sup> Studies with experimental models have endorsed the contribution of low vitD levels to disease severity. Cantorna *et al.*<sup>81</sup> demonstrated that active vitD supplementation before arthritis induction in DBA/1 LacJ mice with collagen II significantly decreased disease symptoms and was also able to arrest disease progression in this model. The mechanisms by which vitD determines arthritis suppression *in vivo* are not fully elucidated. However, its effect over RA and also experimental encephalomyelitis and lupus has been linked to increased production of IL-4 and TGF- $\beta$ .<sup>68</sup> Treatment of experimental autoimmune diabetes with vitD induced a population of CD4+ CD25+ Treg cells that coincided with protection against the disease.<sup>82</sup> This presumed vitD Treg induction and its correlation with protection in experimental arthritis have not yet been investigated.

The increasing knowledge of vitD interaction with the immune system and the more recently revealed relevance of iDCs to tolerance induction disclosed a new strategy to modulate autoimmune

pathologies. This promising new immunotherapeutic procedure is focused on the attenuation of pathogenic T cell response by treatment with autologous tDCs. In this sense, it was initially demonstrated that active vitD and dexamethasone were able to act synergistically inhibiting lipopolysaccharide (LPS)-induced maturation of DCs.<sup>83</sup> The therapeutic efficacy of this proposal was recently proved in collagen-induced arthritis (CIA). Stoop *et al.*<sup>63</sup> demonstrated that type II collagen-pulsed tolerogenic DCs significantly inhibited disease severity and progression. Mechanistically, they found coincidence of this protection with an increased number of IL-10-producing CD4+ T cells and reduction of Th17 cells.

Although there is a clear correlation between vitD deficiency and autoimmunity in clinical and experimental studies, there is not a consensus about the indication of vitD supplementation to treat arthritis or other autoimmune diseases.<sup>84–86</sup>

#### *Helminths and heat-shock proteins (hsp)s*

According to the hygiene hypothesis, the decreased prevalence of infections in Western countries and also in developing areas is, to a certain degree, responsible for the increasing incidence of allergies and autoimmune diseases.<sup>87,88</sup> This possibility, also called “Old friends hypothesis,” is based on the presumed ability of certain organisms such as helminths and environmental mycobacteria, or their soluble components, to interact with the innate

immune system and trigger a state of immunological tolerance. The immunomodulatory potential of these organisms has been imputed to induction of regulatory DCs that would be able to activate Treg cells.<sup>89</sup> Clinical trials employing helminth therapy have already been conducted in patients with multiple sclerosis, ulcerative colitis and Crohn's disease. Recent animal data revealed that this therapy may also be beneficial to RA.<sup>90</sup> The filarial nematode-derived glycoprotein ES-62 is present in the bloodstream of parasitized humans and it is clearly able to down-modulate the severity of experimental-induced arthritis. This suppressive effect was observed when ES-62 was administered during collagen priming and also after the disease had become clinically evident. In both cases, this activity was concomitant with decreased specific induction of IFN- $\gamma$ , IL-6 and TNF- $\alpha$ .<sup>91</sup> A great advance in this field was the discovery that the phosphorylcholine moiety of the filarial immunomodulator ES-62 was responsible for its anti-inflammatory activity in arthritis.<sup>92</sup>

Hsps are constitutively and abundantly present in all living beings, playing many important roles. One of the most interesting aspects of these proteins is their high degree of conservation during evolution. For example, hsp60 of different bacterial species displays more than 95% sequence homology at the DNA and protein levels. An overall 55% homology is detected by comparing bacterial and human hsp60.<sup>93</sup> Based on this structural similarity and also the antigenic immunodominance of some hsps during bacterial infections, the initial prevailing view was that these proteins were involved in autoimmunity because of molecular mimicry with the human counterpart.<sup>94</sup> However, further knowledge revealed that these molecules were endowed with strong immunoregulatory properties.<sup>95</sup> This potential duality of hsps is supported by many clinical and experimental reports involving cancer, infections, atherosclerosis and other autoimmune conditions.<sup>96,97</sup> As recently reviewed by Coelho and Faria,<sup>98</sup> the preferential direction of its immunoregulatory activity during therapeutic strategies is determined by a diversity of factors, such as route of administration, association with other modulatory agents and time point of the disease.

The mechanism by which hsps, especially the mycobacterial hsp (mhsp60), regulates inflammation was cleared up by testing purified mhsp60 and overlapping sets of 15-mer peptides spanning the complete mhsp60 sequence.<sup>99</sup> This study revealed nine distinct dominant T cell epitopes. Subsequent investigations made with T cell lines

with specificity for these epitopes revealed that only T cells that are able to recognize a highly conserved region located between 256 and 265 amino acids were able to transfer protection against RA.<sup>100</sup> Very interestingly, these T cells that confer protection were also able to recognize homologous peptide expressed by heat-shocked mammalian cells. The identity of these Tregs has been intensively investigated. It was initially discovered that these T cells were able to produce the immunoregulatory IL-10 cytokine.<sup>101</sup> Later on, other studies suggested that natural Tregs could recognize hsps as antigens.<sup>102</sup> Very recently, Ohue *et al.*<sup>103</sup> demonstrated that bacterial hsp60 was able to convert naïve T cells into CD4+CD25+Foxp3+ T cells. The discovery of this natural system of immunoregulation allowed the appearance of new artificial strategies to control inflammation. One of these strategies is the administration of hsp, hsp-derived peptides or altered peptide ligands. Nasal administration of a microbial hsp60-derived peptide, immunization with an altered peptide ligand derived from human hsp60 and immunization with DNA vaccines encoding *Mycobacterium leprae* hsp65 prevented experimental arthritis development in mice.<sup>104–106</sup> In some of these studies, the potentiality of the adopted procedure was also tested with human cells. For example, stimulation of peripheral blood mononuclear cells (PBMCs) from RA patients with an hsp60-altered peptide ligand increased the proportion of the CD4+CD25+ Foxp3+ T cells.<sup>104</sup>

Another strategy based on the decreased stress-induced expression of hsps in immune disorders is to boost endogenous hsp expression.<sup>107</sup> Even though this seems a very promising strategy, it has not been extensively investigated in RA. One of the few works in this area was performed by Wieten *et al.*<sup>108</sup> These authors demonstrated that intragastric administration of carvacrol increased hsp expression and, concomitantly, increased T cell response to hsp70. In addition, this procedure was associated with an increased number of CD4+CD25+Foxp3+ T cells in the spleen and the joint. Furthermore, this treatment significantly suppressed proteoglycan-induced arthritis.

As we stressed before, Treg function in RA has been shown to be compromised.<sup>39</sup> A newly emerging approach that could be successful in autoimmune diseases, including RA, is the *ex vivo* expansion of autologous Treg cells followed by their return to the patient.<sup>45</sup> Hsp60 or its peptide p277 added to fresh human T cells before activation with  $\alpha$ -CD3 were able to enhance Treg

function by cell-to-cell contact and secretion of TGF- $\beta$  and IL-10.<sup>109</sup>

### Considerations in choosing an experimental model

Choosing an arthritis model to study human RA is a decision that demands a careful analysis of the literature. First of all, human arthritis has a very complex molecular pathogenesis involving almost all branches of immunity. In addition, it is a very heterogeneous human disease in terms of evolution and clinical manifestations. Considering that the most classical clinical manifestation of RA is joint inflammation, there is a particular interest in the articular cartilage. Cartilage matrix components represent a source of specific antigens that are highly arthritogenic such as collagen and proteoglycan.<sup>110</sup> Lately, one of the most used experimental models of RA is induced by the human type II collagen. However, a quick search on this subject in the literature clearly shows the availability of a plethora of other arthritis animal models, each one probably resembling better one aspect or one subset of human RA. Very informative, complete and updated reviews are available concerning this variety of animal models.<sup>5,111</sup> Experimental models were initially more explored to investigate immunological and histopathological aspects. Later on, however, the focus has shifted toward the search for earlier diagnosis biomarkers and for new pharmaceuticals.

What aspects need to be considered to choose an adequate RA model? Above all, it is mandatory to have a very clear idea of the major question that is being asked and also at what extension it will be investigated. For example, a more general questioning as the evaluation of a potential new anti-inflammatory drug could be conducted using classical models such as the arthritis induced by adjuvant

(AIA) or oil in rats. Otherwise, if the purpose is to test an immunomodulatory protocol for specific tolerance induction, the models triggered by collagen or proteoglycan (cartilage-direct autoimmunity) would be more indicated. If the main objective is based on immune complex deposition and cartilage destruction, CIA and the KRN serum transfer model would then be preferable. A few examples that illustrate these aspects are summarized in Table 2.

The second factor to be considered is the reproducibility of the model. In some of them, the clinical manifestations will occur essentially at the same period. For example, in the AIA model, the rats will develop an acute disease by nine days after the initial adjuvant/oil mixture injection. Such predictable development allows any evaluation to be performed at a pre-determined time. This is especially relevant and time saving for preclinical tests with new anti-arthritogenic drugs. This will also provide more homogeneous immunological information. In contrast, in some models, the development of the disease can be spread over days or weeks, as happens for CIA in mice and rat models.<sup>120,121</sup>

Animal facility characteristics, animal colony-dependent differences and cost obviously need to be analyzed to choose a feasible and reliable model. Sex differences are also important to be considered either in spontaneous or induced animal models for RA.<sup>122</sup> Time required for disease development is another relevant issue and can be uncertain, depending mainly on the adopted model but also on the laboratory where it was induced. A clear demand for faster disease development exists in many arthritis models. How to abbreviate the required time is still an open question.

Availability of reagents for disease induction is another important aspect of experimental models

**Table 2** Experimental arthritis models used to perform studies in the immunological field

| General issue         | Subject                                             | Model                                              | Reference                                      |
|-----------------------|-----------------------------------------------------|----------------------------------------------------|------------------------------------------------|
| Innate immunity       | G-CSF and leukocyte trafficking                     | Mice CIA/G-CSF-/-                                  | Eyles <i>et al.</i> <sup>112</sup>             |
|                       | Toll-like receptor 4 (TLR-4)                        | Mice CIA/TLR-4 antagonist                          | Abdollahi-Roodsaz <i>et al.</i> <sup>113</sup> |
| Humoral immunity      | CD20 B cell depletion                               | Mice PGIA                                          | McGovern <i>et al.</i> <sup>41</sup>           |
|                       | Anti-citrullinated protein antibodies               | Mice CIA                                           | Kidd <i>et al.</i> <sup>114</sup>              |
| Cellular immunity     | Pathogenic role of IL-17                            | Mice SCW-induced arthritis/IL-17 KO                | Joosten <i>et al.</i> <sup>115</sup>           |
|                       | Apoptosis and Treg increase                         | Rats SCW-induced arthritis                         | Perruche <i>et al.</i> <sup>116</sup>          |
| Environmental effects | Arthritis regulation by <i>S. japonicum</i> protein | Rats AA                                            | Sun <i>et al.</i> <sup>117</sup>               |
|                       | Cigarette smoke condensate                          | Mice CIA                                           | Chujo <i>et al.</i> <sup>118</sup>             |
| Treatment             | Effect of fermented wheat germ extract              | Rats AA                                            | Telekes <i>et al.</i> <sup>119</sup>           |
|                       | Immunomodulation with DNahsp65                      | AIR <sub>max</sub> mice pristane-induced arthritis | Santos-Junior <i>et al.</i> <sup>106</sup>     |

G-CSF: granulocyte colony-stimulating factor; IL: interleukin; Treg: regulatory T cells; hsp: heat-shock protein; CIA: collagen-induced arthritis; PGIA: proteoglycan-induced arthritis; SCW: streptococcal cell wall; KO: knock-out; AA: adjuvant arthritis; AIR<sub>max</sub>: maximal acute inflammatory response.



**Figure 1** Clinical scores of female BALB/c retired breeder mice with bovine proteoglycan-induced arthritis. (a) Score 0: normal paw; (b) score 1: mild swelling and redness in one or two joints; (c) score 2: moderate swelling and redness in two or more joints; (d) score 3: severe swelling and redness in the entire paw and ankle; (e) score 4: severe swelling and redness in the entire paw and ankle and ankylosis. Arrows indicate inflamed joints and lines indicate increased ankles width.

of arthritis. Currently, the experimental model that we have been studying is the arthritis induced by proteoglycan (PG). This is a particularly interesting model because it is a non-usual experimental model concerning the source of the antigen. In this case, the PG is extracted from bovine nasal septum and co-administrated with an adjuvant for establishment of the disease. According to Glant *et al.*,<sup>123</sup> the association of human PG with a strong adjuvant such as Complete Freund's adjuvant, can induce a systemic experimental arthritis. However, there are few experimental data considering bovine PG for arthritis induction rather than human PG. On the contrary, most of these studies showed that bovine PG is poorly and even non-arthritisogenic. In our experience, bovine PG was highly arthritisogenic and 80%–100% of BALB/c female mice developed classical arthritis symptoms such as swelling, redness and also ankylosis (unpublished data). Clinical scores similar to the ones described by Glant *et al.*<sup>123</sup> were observed and are illustrated in Figure 1. This model is an alternative for experimental arthritis induced by PG because the antigen does not need to be synthesized in the laboratory and it is commercially available.

Experimental models are an exceptional tool for studying arthritis and many other autoimmune diseases. They have contributed to considerably enhancing our understanding of the immunopathogenic processes involved in joint destruction. They also have provided novel insights into treatment targets. Indeed, preclinical testing in experimental models is required by the United States Food and Drug Administration and the European Medical Agency for new therapeutics in RA.

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## Conflict of interest statement

The authors have no conflicts of interest to declare.

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## *Capítulo 2*

### *Caracterização do modelo experimental*

## **CAPÍTULO 2**

Este capítulo é constituído pelo artigo científico “Commercial bovine proteoglycan is highly arthritogenic and can be used as an alternative antigen source for PGIA model”, publicado em uma edição especial sobre doenças reumáticas autoimunes na revista *Biomed Research International*. Neste artigo, demonstramos que o proteoglicano bovino pode substituir, de maneira eficaz, o proteoglicano humano na indução da artrite experimental.

## Research Article

# Commercial Bovine Proteoglycan Is Highly Arthritogenic and Can Be Used as an Alternative Antigen Source for PGIA Model

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Rheumatoid arthritis (RA) is the most common systemic autoimmune disease. It affects mainly the joints, causing synovitis, cartilage destruction, and bone erosion. Many experimental models are used to study the mechanisms involved in immunopathogenesis and new therapies for this disease. Proteoglycan-induced arthritis (PGIA) is a widely used model based on the cross-reactivity of injected foreign (usually human) PG and mice self-PG. Considering the complexity of the extraction and purification of human PG, in this study we evaluated the arthritogenicity of bovine PG that is commercially available. Bovine PG was highly arthritogenic, triggering 100% incidence of arthritis in female BALB/c retired breeder mice. Animals immunized with bovine PG presented clinical symptoms and histopathological features similar to human RA and other experimental models. Moreover, bovine PG immunization determined higher levels of proinflammatory and anti-inflammatory cytokines in arthritic mice compared to healthy ones. As expected, only the arthritic group produced IgG1 and IgG2a antibodies against PG. Thus, commercial bovine PG can be used as an alternative antigenic source to PGIA for the study of many RA aspects, including the immunopathogenesis of the disease and also the development of new therapies.

## 1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects around 0.3 to 1% of the world population, with lower prevalence in developing countries [1]. It is considered the most common systemic autoimmune disease that usually affects the small joints, especially fingers. It may also involve larger joints, including shoulders, elbows, knees, and ankles. The inflammatory process in the joint is characterized by synovitis, cartilage destruction, and bone erosion. There is still no consensus on the autoantigens involved in this disease. Currently, it is known that some autoantigens such as

cartilage components, chaperone proteins, enzymes, nuclear proteins, and citrullinated proteins might be involved [2, 3]. Among several cell types found in the inflamed joint, CD4+ T-cells' subsets are considered the most important cells involved in synovitis and RA development [4]. Activated macrophages are also a very relevant source of inflammatory mediators, including proinflammatory cytokines [5]. TNF- $\alpha$  and IL-1, for example, promote the accumulation of inflammatory cells in the joints and the synthesis of other cytokines, chemokines, and matrix metalloproteinases [6]. Many cytokines, including IL-8, TNF- $\alpha$ , and IFN- $\gamma$ , have been detected in synovial fluid. These cytokines, especially

TNF- $\alpha$ , activate resident synovial cells that by producing proteolytic enzymes mediate the destruction of joint cartilage, ligaments, and tendons. Recently, the presence of IL-17 at the site of inflammation and its synergistic effect with TNF- $\alpha$ , exacerbating the inflammatory process, have been evidenced [7]. The participation of B cells in RA has been more investigated in recent years. The production of autoantibodies and cytokines, presentation of autoantigens to T cells, and ectopic lymphoid organogenesis are their possible roles [8]. Regulatory T cells have also been widely studied in both human and experimental arthritis because of their therapeutic and prophylactic potential [9].

There are several experimental models of arthritis being used to elucidate the mechanisms involved in the immunopathogenesis of RA. Also, the animal models are essential to study new therapy targets for this disease. Historically, the first experimental models of arthritis, which are called adjuvant-induced arthritis (AIA), were based on the inoculation of mycobacterial components suspended in mineral oil. Later, it was discovered that this model could be improved by using pristane, which is a purified component of mineral oil. The disease caused by pristane was more similar to human RA and this model has been widely used [10–12]. After that, there was an increased interest in experimental models based on the inoculation of cartilage components such as type II collagen and proteoglycan. The models presented clinical, immunological, histopathological, and genetic characteristics typical from human RA and, for that reason, they were considered the best models to study mechanisms and possible treatments for arthritis [13, 14]. In the 90s, the first arthritis transgenic models were described. By using immunogenetic tools, Keffer et al. [15] observed a spontaneous arthritis in mice overexpressing human TNF- $\alpha$  transgene. In this study, the animals developed a chronic inflammatory polyarthritis that evidenced the critical role of TNF- $\alpha$  in the immunopathogenesis of RA. Currently, collagen-induced arthritis (CIA) is a very reliable and reproducible experimental model that is being widely used for the study of all aspects of arthritis, including the immunopathogenesis of RA, the development of new drugs from natural extracts, the new molecular targets for treatment, and also gene therapy [16–19].

The experimental model chosen for this study was based on the immunization of BALB/c mice with proteoglycan (PG). Proteoglycan-induced arthritis (PGIA) was elegantly described by Glant et al. [13]. Briefly, the systemic autoimmune arthritis in this model is induced by intraperitoneal inoculation of BALB/c or C3H mice with PG isolated from various sources. Many genetic and immunological aspects of PGIA have already been studied in this model. For example, epitopes recognized by the arthritogenic T cells and the contribution of various cytokines such as IFN- $\gamma$ , IL-4, and IL-12 were determined [20–22]. Although human cartilage is the preferable source of PG, its extraction and purification is a complex and laborious process that includes a variety of biochemical steps. Besides, ethical issues and rules involving the utilization of biological samples from human and animals contribute to complicate PG purification. In this scenario, we investigated the possible arthritogenicity of bovine PG

in BALB/c mice. We considered that this evaluation could be very beneficial to researchers that are not able to purify human PG. Commercial availability of bovine PG could not only facilitate the experimental model implementation but also facilitate the comparison of results obtained by different laboratories.

## 2. Materials and Methods

**2.1. Animals.** Female BALB/c retired breeder (beyond the reproductive age) mice were removed from breeding colonies by the age of 8–11 months and purchased from CEMIB (Campinas, SP, Brazil). They were maintained in the Department of Microbiology and Immunology facility under controlled conditions of luminosity (12h light/12h dark) and temperature ( $22 \pm 2^\circ\text{C}$ ). Mice were allocated in ventilated cages with sterile pine shavings and received sterile food and filtered water *ad libitum*. The manipulation of the animals was in compliance with the local ethics committee (Protocol number 257-CEEA).

**2.2. Arthritis Induction and Score Evaluation.** As previously described [23], with minor modifications, a dose ( $100 \mu\text{L}$ ) containing  $100 \mu\text{g}$  of bovine proteoglycan extracted from nasal septum (Sigma Aldrich, St. Louis, MO, USA) and  $1 \text{mg}$  of emulsified (micelle form) dodecyl dioctadecyl ammonium bromide (DDA) adjuvant (Sigma Aldrich, St. Louis, MO, USA) was intraperitoneally injected three times with 21-day interval for arthritis induction. After the third injection, arthritis score was daily evaluated until euthanasia (70 days after the first immunization). Arthritis severity was determined using a standard visual scoring system based on the degree of swelling and redness ranging from 0 to 4 for each paw. The following system was used: 0 = normal; 1 = mild swelling in the paw or one joint; 2 = moderate swelling and redness in the paw and one or more joints; 3 = pronounced swelling and redness in the paw, all joints, and ankle; 4 = severe swelling and redness of the entire paw and ankle and movement limitation. This classification resulted in a total score that ranged from 0 to 16 for each animal.

**2.3. Histopathological Analysis.** After euthanasia, mice paws were collected and fixed in 10% formalin phosphate buffer for at least seven days at room temperature. The samples were thoroughly demineralized in 10% Titriplex EDTA disodium salt (Merck Millipore, Darmstadt, Germany) for one to two months. The decalcified tissues were trimmed, dehydrated in graded ethanol, and embedded in paraffin. Serial sections ( $5 \mu\text{m}$ ) were cut and mounted on glass slides precoated with 0.1% poly-L-lysine (Sigma Aldrich, St. Louis, MO, USA). Histological assessment was carried out following routine hematoxylin and eosin (HE) staining. The images were acquired by a digital camera attached to the optical microscope (Nikon, Kurobanemuko, Otawara, Japan).

**2.4. Immune Responses Evaluation.** For cellular immune response, spleens were collected after euthanasia and the cells resuspended in RPMI medium containing gentamicin

and fetal calf serum. Th cells were stimulated with ConA (5  $\mu$ g/mL) and PG (50  $\mu$ g/mL). After 48 hours incubation at 37°C/5% CO<sub>2</sub>, the supernatants were collected for detection of IL-2, IL-6, TNF- $\alpha$ , IL-17, IFN- $\gamma$ , IL-5, and IL-10. These cytokines were quantified using enzyme linked immunosorbent assay (ELISA), according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA, and RD Systems, Minneapolis, MN, USA). For humoral immune response, blood samples were collected by facial vein two days before each dose and seven days after the third dose of PG+DDA. 70 days after the first immunization, the blood was collected by cardiac puncture. The sera were obtained by blood centrifugation (6000 rpm for 15 minutes at 25°C). Briefly, Maxisorp plates (Nunc, Life Technologies, USA) were coated with 5  $\mu$ g/mL of bovine PG (Sigma Aldrich, St. Louis, MO, USA) and nonspecific protein binding was blocked with 0.1% bovine serum albumin in phosphate buffered saline. Subsequently, plates were incubated with serum samples diluted 1:1000. Biotinylated anti-mouse IgG1 and IgG2a antibodies (BD Biosciences, San Jose, CA, USA) were used to detect heterologous anti-PG antibodies. Plates were then incubated with streptavidin (RD Systems, Minneapolis, MN, USA) and revealed by adding H<sub>2</sub>O<sub>2</sub> and orthophenylenediamine (Sigma Aldrich, St. Louis, MO, USA).

**2.5. Statistical Analysis.** Results were presented as mean  $\pm$  standard deviation for parametric variables and the comparison among the groups was performed by *t*-test. For nonparametric variables, the results were presented as median and the comparison between the groups was performed by Mann-Whitney's test. Paired *t*-test was performed for antibody production. All data were analyzed using SigmaPlot software version 12.0 (Jandel Corporation, USA) and *P* < 0.05 was considered significant.

### 3. Results

**3.1. Arthritis Incidence and Clinical Score.** As expected, animals from control group did not develop experimental arthritis. However, all animals immunized with three doses of bovine PG+DDA adjuvant developed the disease (Figure 1(a)). Arthritis onset was observed at day 51 and total clinical score increased in the arthritic group until day 70 (Figure 1(b)). Moreover, the median of the maximum score in the arthritic group was statistically significant in comparison to the healthy control group (Figure 1(c)).

**3.2. Histopathological Analysis.** Figure 2 shows the differences among the clinical scores observed in mice hind paws and forepaws during arthritis development. HE stained paw sections revealed important histological changes in the arthritic joints compared to the healthy ones. According to the scoring system, all animals from control group presented score 0 and there was no signal of inflammation in these animals (Figures 2(a) and 2(a')). The joint structure was preserved and characterized by a well-defined synovial space, cartilage presence, thin synovial membrane, and compact bone (Figure 2(a'')). Mice from arthritic group

presented a variety of scores, ranging from 1 to 4 in each paw. Score 1 was characterized by only one inflamed joint (head arrows; Figures 2(b) and 2(b')). No differences were observed in histological sections from paws with score 1; that is, all animals presented well preserved joint structures (Figure 2(b'')). Score 2 was characterized by the presence of two or more affected joints in the paw (Figures 2(c) and 2(c')). In this score, there was an inflammatory cell infiltrate and a slight thickening of the synovial membrane. However, it was still possible to observe the presence of the synovial space and well-preserved cartilage and bone tissue (Figure 2(c'')). Score 3 was characterized by the inflammation of multiple joints including the ankle (lines; Figures 2(d) and 2(d')). In this score, there was an inflammatory cell infiltrate that characterizes the initial *pannus* formation, which is the inflammatory tissue that invades the synovial space and promotes cartilage destruction and bone erosion (Figure 2(d'')). However, bone tissue was still preserved in this score. Score 4 was characterized by accentuated erythema and edema throughout the foot and ankle, involving all joints, with consequent movement impairment (Figures 2(e) and 2(e')). Inflammation and joint destruction were evident and were characterized by *pannus* formation, synovial membrane thickening, cartilage destruction, and bone erosion in paws with score 4 (Figure 2(e'')).

**3.3. Production of Cytokines.** Compared to the control group, spleen cells from arthritic mice produced significantly higher levels of IL-2 and the proinflammatory cytokines TNF- $\alpha$ , IL-6, IFN- $\gamma$ , and IL-17 when restimulated *in vitro* with the specific antigen (Figures 3, 4, and 5). Interestingly, arthritic animals also produced significant levels of IL-5 and IL-10 anti-inflammatory cytokines in response to *in vitro* stimulation with PG (Figure 6). Results from nonstimulated cultures showed that there was spontaneous production of all cytokines in the arthritic animals, but not in the healthy ones (Figures 3, 4, 5, and 6). However, polyclonal stimulation of spleen cells with ConA triggered significant increase only in IL-6, IL-17, and IL-10 production by spleen cells from the arthritic group compared to the control one.

**3.4. Production of Anti-PG Antibodies.** The experimental arthritis induced by bovine PG determined production of both IgG1 and IgG2a anti-PG antibodies, with higher production of IgG1 (Figure 7). The levels of these specific antibodies increased significantly and progressively after the first immunization with PG+DDA (day 1). After reaching the maximum level around day 41, antibody production was maintained until euthanasia at day 70. As expected, control animals that were not immunized with PG+DDA did not produce specific antibodies against bovine PG (data not shown).

### 4. Discussion

There are several experimental models of rheumatoid arthritis that contribute to understand the mechanisms involved in this disease [24]. Experimental arthritis models induced

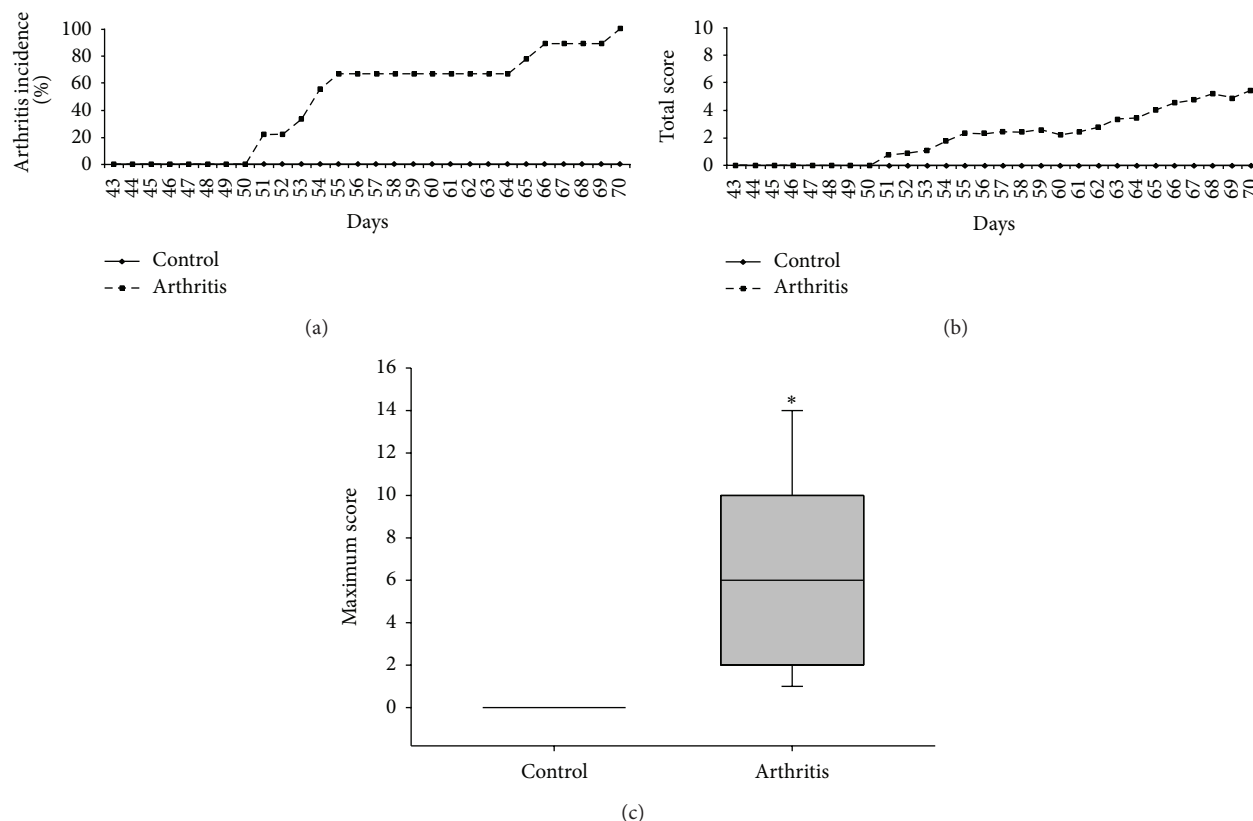


Figure 1: Arthritis incidence (a) total clinical score (b) and maximum clinical score (c) in mice with bovine proteoglycan-induced arthritis. Female BALB/c retired breeder mice were immunized with three doses of bovine PG associated with DDA adjuvant, 21-day interval. Clinical score was daily evaluated after the third immunization. \*  $P < 0.05$  compared to control.

by cartilage components have been extensively studied, primarily the induction of arthritis by collagen and proteoglycan (PGIA). Considering the clinical and histopathological characteristics of the disease, this model shares many similarities with human arthritis. The development of arthritis in PGIA is attributed to a cross-reaction against foreign PG and mice self-PG [25]. In this experimental model, the disease is usually triggered by injections of human PG associated with a strong adjuvant. The PG can be extracted from the cartilage of various origins, but human PG is considered the most arthritogenic one. In this context and considering that PG purification is a complex and laborious process, we determined the arthritogenicity of a commercial source of bovine PG. This evaluation was done by immunization of BALB/c retired breeders with three doses of bovine PG emulsified with DDA. In spite of the advanced age of these animals, no spontaneous arthritis was observed. According to Besenyei et al. [26], approximately 0.5 to 1.0% of retired breeder BALB/c mice can develop the disease spontaneously.

Immunization with the commercial bovine PG was very effective to induce arthritis. A 100% incidence was observed in the majority of the experiments as has been described with human PG [13, 23]. In terms of clinical disease, we observed slightly lower scores than the ones described for human PG. However, this finding was equally described by

other authors that employed bovine PG [23, 27]. In spite of this, the histopathological analysis revealed the presence of very typical arthritic histological alterations as inflammatory infiltrates, synovial membrane thickening, *pannus* formation, cartilage destruction, and bone erosion. The features are very similar to the ones described by other authors in PGIA and also in human arthritis [13, 28]. This parallelism indicates that bovine PG can be further explored as another source of antigen to study arthritis. The efficacy of this bovine PG to induce murine arthritis is probably related, among other things, to the adopted adjuvant. As nicely described by Hanyecz et al. [23], the arthritogenicity of bovine PG was significantly incremented when it was combined with DDA. We also believe that the employment of BALB/c retired breeders contributed a lot to this achievement. According to Tarjanyi et al. [29], these old animals are very prone to arthritis development.

Immunization with this commercial product also induced significant production of IgG1 and IgG2b anti-bovine PG antibodies. Even though we were not able to assess a possible cross-reactivity of these antibodies with murine PG, we believe that it exists and is underlying the arthritogenicity of bovine PG to mice. This assumption is mainly based on structural and comparative biochemical studies and on arthritogenicity for mice. In this context,

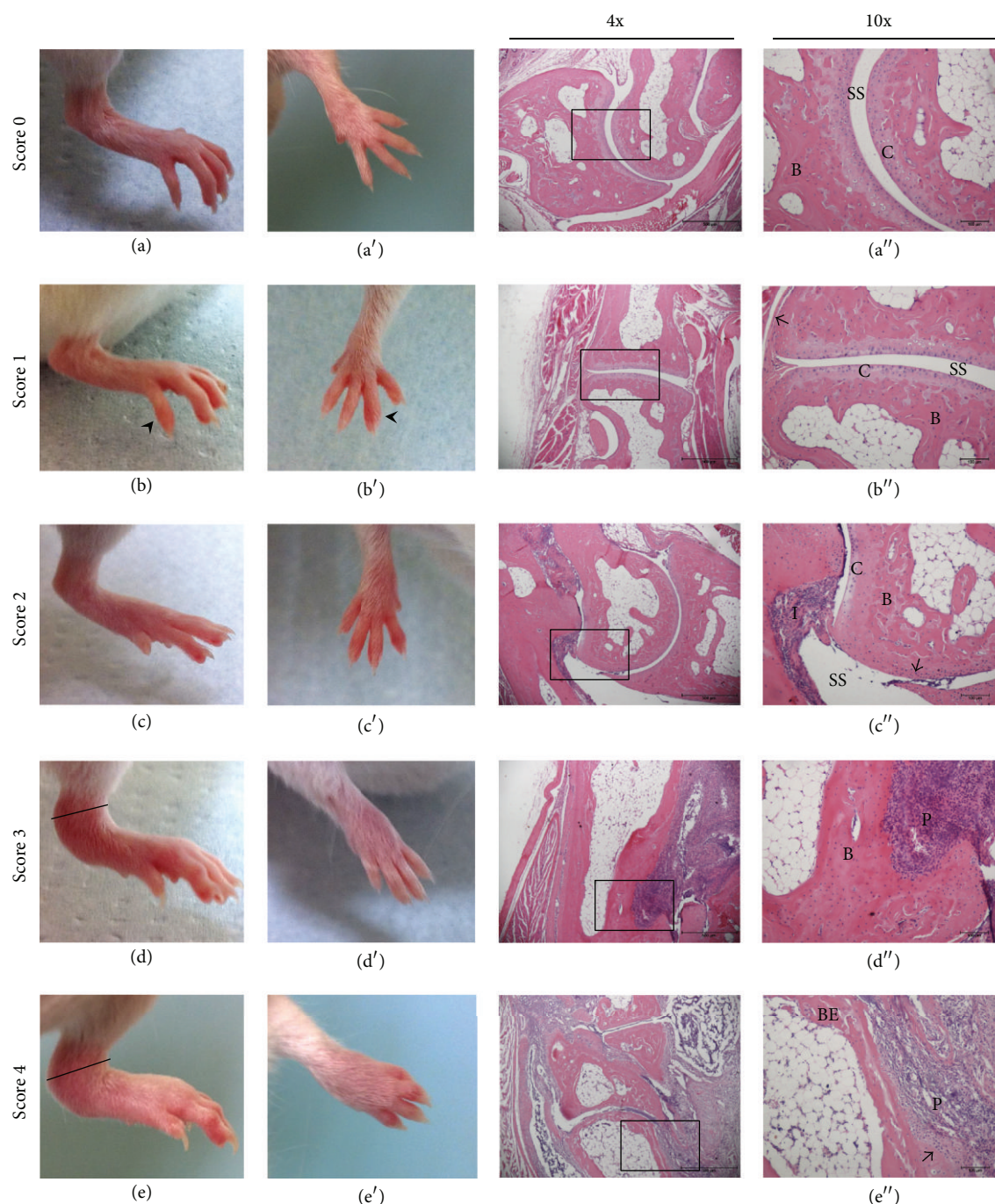


Figure 2: Representative clinical scores of hind paws (first column) and forepaws (second column) and histological sections of mice joints (third and fourth columns) with arthritis induced by bovine PG. Female BALB/c retired breeder mice paws were collected 70 days after the disease induction. The rectangles represent the regions highlighted in the fourth column. Head arrows indicate single joint inflammation; lines indicate ankle thickening; filled arrows indicate the synovial membrane. SS: synovial space; C: cartilage; B: bone; I: inflammatory infiltrate; P: pannus; BE: bone erosion.

Walcz et al. [30] demonstrated that murine and bovine PG core protein share 72.5% homology. The arthritogenic potential of distinct PG sources was checked in mice. Interestingly, arthritogenicity or its absence was associated with the ability to induce or not, respectively, the production of cross-reactive antibodies [31].

An aspect that deserves further elucidation is the degree of glycosylation present in this commercial PG. It has been strongly emphasized that PG deglycosylation is fundamental to achieve arthritogenicity [13, 32]. However, we believe that this preparation is not devoid of polysaccharides. This hypothesis is based on references specified by the company

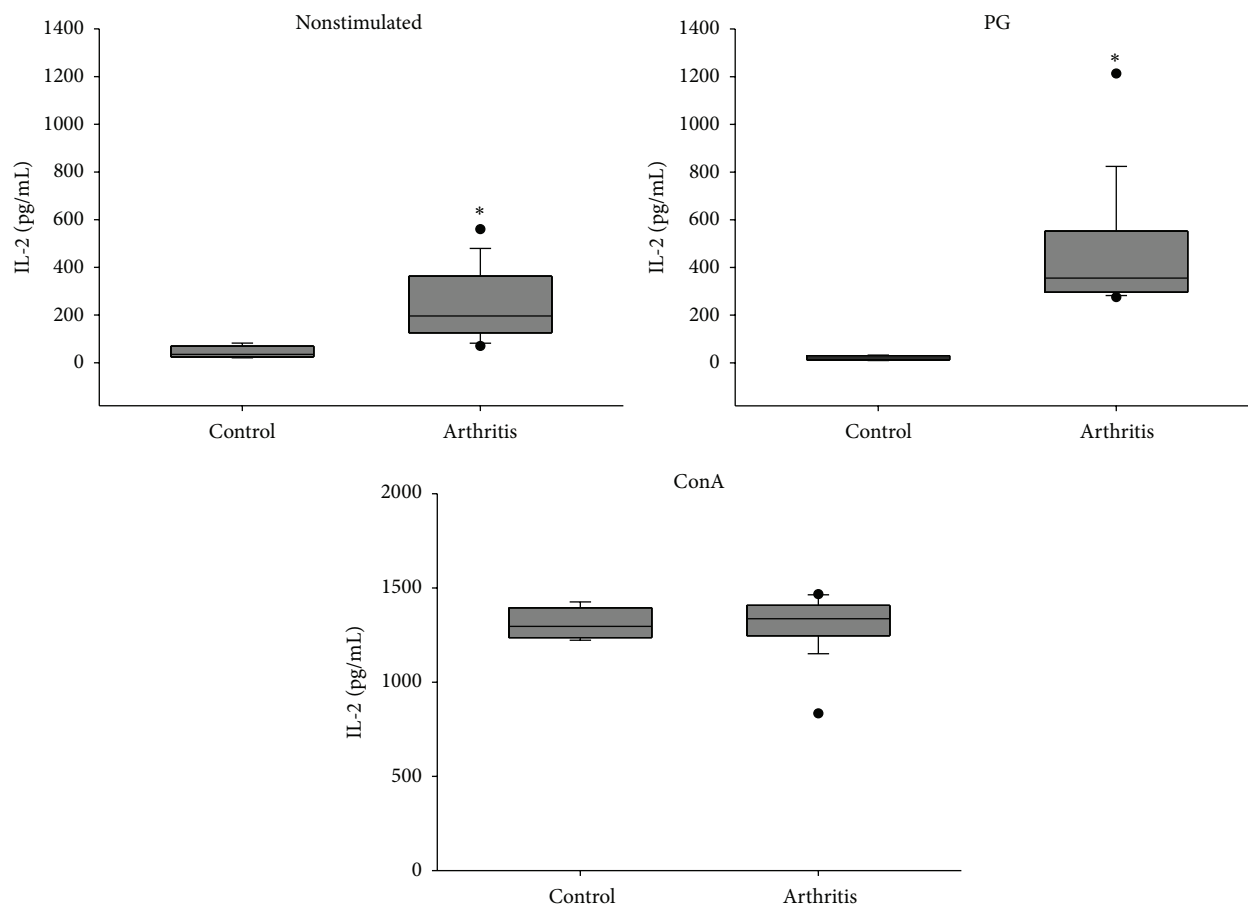


Figure 3: IL-2 production by spleen cells from BALB/c retired breeder mice with bovine proteoglycan-induced arthritis. Spleen cells were *in vitro* stimulated with PG and ConA and incubated for 48 hours. \* $P < 0.05$  compared to the respective control.

that commercializes the product and also in information described by authors that utilized this product. According to Tha et al. [33], this commercial product contains 86% of chondroitin sulfate, 8% protein, 6% keratan sulfate, and less than 1% hyaluronic acid and it was able to enhance the survival of neural stem cells. In the central nervous system, chondroitin sulfate proteoglycan (CSPG) is the most prevalent PG and CS removal with chondroitinase reduces neural stem cells proliferation and neurogenesis. Also, according to Antonopoulos et al. [34], PG isolated by urea procedure is probably found in PG subunits instead of PG complexes form due to its gel chromatographic pattern. PG subunits could expose the G1 domain and the link protein, which are highly arthritogenic [27, 35]. Antonopoulos et al. [34] also demonstrated that urea did not cause PG degradation. It is possible to think that this organic compound could interfere in PG structure and protein solubility exposing some core protein epitopes and, therefore, become able to induce experimental arthritis.

Results from cytokine production by spleen cells *in vitro* stimulated with PG showed that arthritic animals, but not

healthy ones, produced high levels of IL-2, TNF- $\alpha$ , IL-6, IFN- $\gamma$ , and IL-17. Spleen cells from arthritic mice were already producing higher levels of IL-2 than the healthy ones. Also, addition of PG to the cultures determined a significant increase in the production of this cytokine in the arthritic group. As a very good correlation has been established between IL-2 level and T-cell proliferation index, in either up- [36] or downregulation [37], our results indicate the occurrence of a specific proliferative process in the spleen. The higher production of TNF- $\alpha$ , IL-6, IFN- $\gamma$ , and IL-17 was expected and corroborates with their arthritogenic potential observed in humans [38] and in animal models [24]. The production of IL-6 and TNF- $\alpha$  is related to the immunopathogenesis and maintenance of RA. These cytokines are also responsible for hyperalgesia caused by mechanical stimulus in this disease. According to Schaible et al. [38], these proinflammatory cytokines act on nerve cells responsible for the nociceptive stimuli during joint movement. The authors showed that drugs which neutralize the action of TNF- $\alpha$  promoted reduction of pain and inflammation in rats with adjuvant-induced arthritis. A similar result was found concerning IL-6 neutralization.

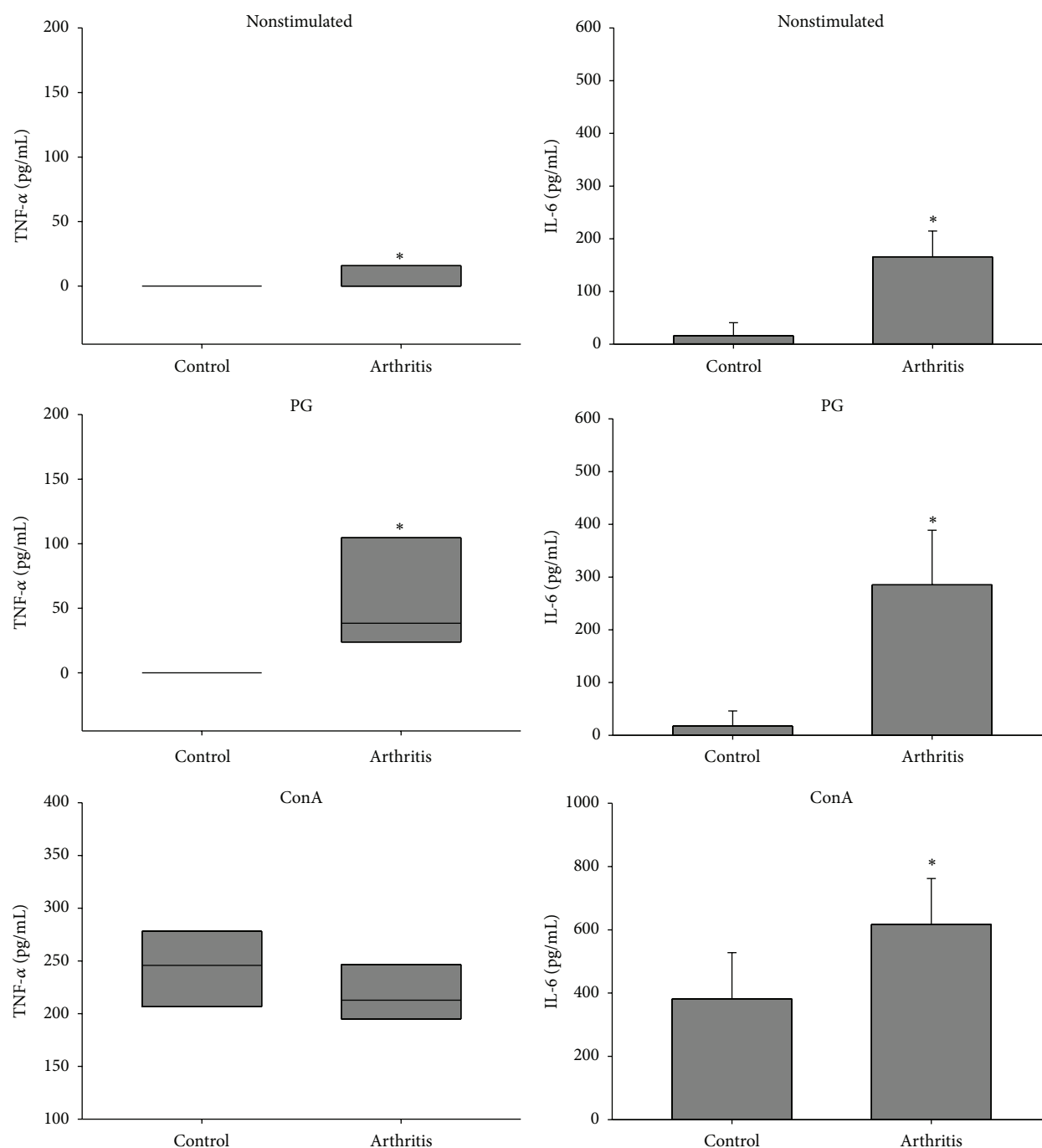


Figure 4: TNF- $\alpha$  and IL-6 production by spleen cells from BALB/c retired breeder mice with bovine proteoglycan-induced arthritis. Spleen cells were *in vitro* stimulated with PG and ConA and incubated for 48 hours. \*  $P < 0.05$  compared to the respective control.

Thus, drugs whose action mechanisms are based on TNF- $\alpha$ , IL-6, and IL-1 neutralization have been extensively studied and some of them such as TNF- $\alpha$  and IL-6 are already used for the treatment of RA clinical symptoms [39].

Many studies have considered the balance between the production of IL-17 and IFN- $\gamma$  as the key to understand the main immunopathogenic mechanisms involved in arthritis development. Considered a major proinflammatory cytokine

in human arthritis and in most experimental models, IL-17 plays an important role in the establishment, maintenance, and progression of this disease [40–42]. Regarding this, studies have shown that the absence of IL-17 decreased the clinical symptoms of arthritis in different experimental models [43, 44]. The role of IL-17 in PGIA is not clearly evaluated yet. However, it has been suggested that in this case the IFN- $\gamma$  is more important than IL-17 in the establishment

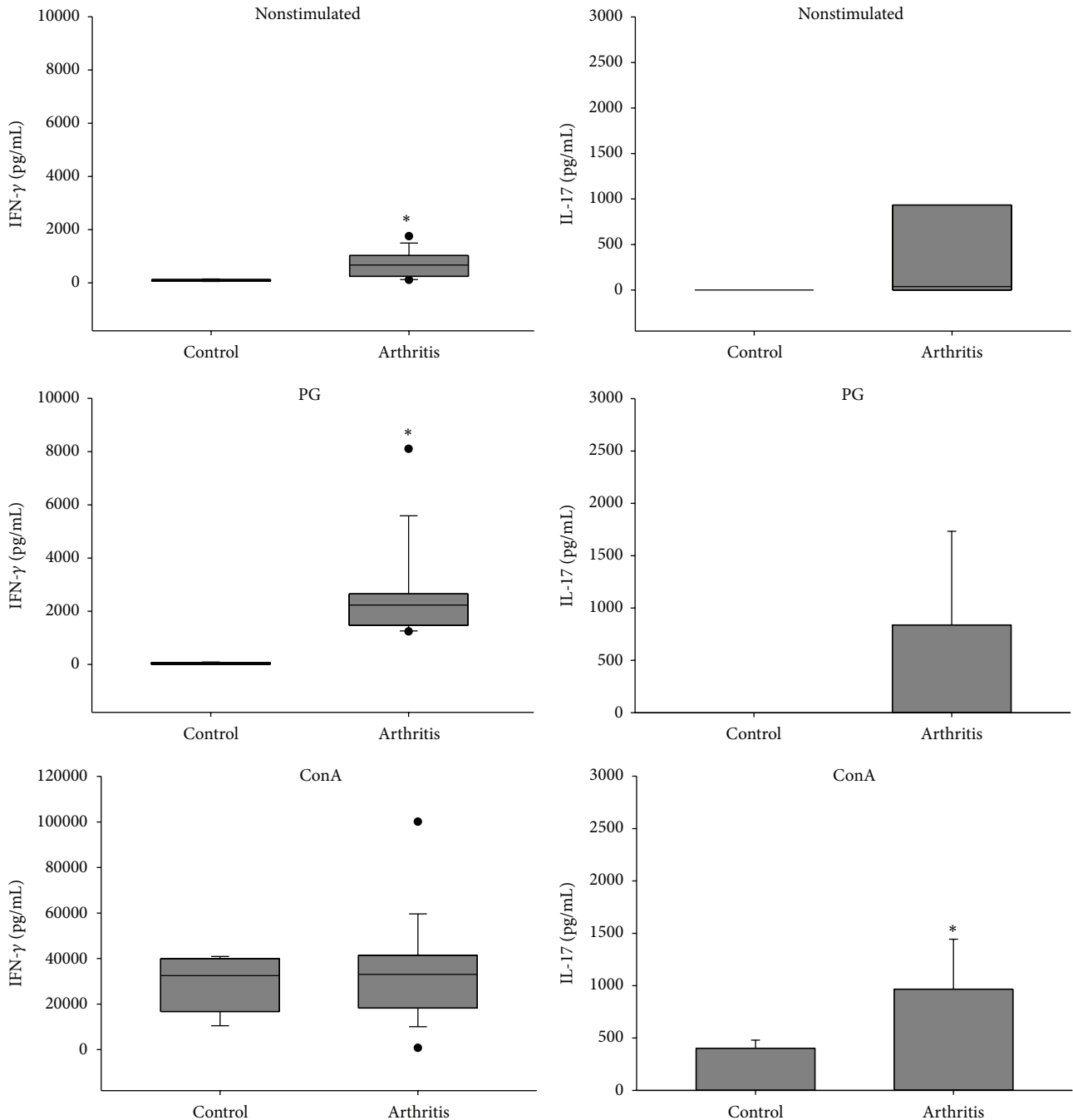


Figure 5: IFN- $\gamma$  and IL-17 production by spleen cells from BALB/c retired breeder mice with bovine proteoglycan-induced arthritis. Spleen cells were *in vitro* stimulated with PG and ConA and incubated for 48 hours. \*  $P < 0.05$  compared to the respective control.

of the disease. Doodes et al. [21], using knockout mice, showed that IFN- $\gamma$  is essential for PGIA triggering in an IL-17 independent manner. The IFN- $\gamma$  represents a paradox in autoimmune arthritis. Although its pathogenic effect is well described, recent studies showed a protective effect of this cytokine in arthritis. Alzabin and Williams [45] carefully reviewed the role of effector T cells in autoimmune arthritis. By analyzing the results of several experimental models, the authors demonstrated the protective role of IFN- $\gamma$ .

The administration of this cytokine that is, theoretically, proinflammatory, decreased clinical signals in different arthritis models. For example, genetically modified animals which were not able to produce IFN- $\gamma$  presented an exacerbated collagen-induced arthritis [46]. However, it has been also reported that animals that did not produce this cytokine were less susceptible to PGIA [47].

The specific *in vitro* stimulation of spleen cells also triggered production of anti-inflammatory cytokines such

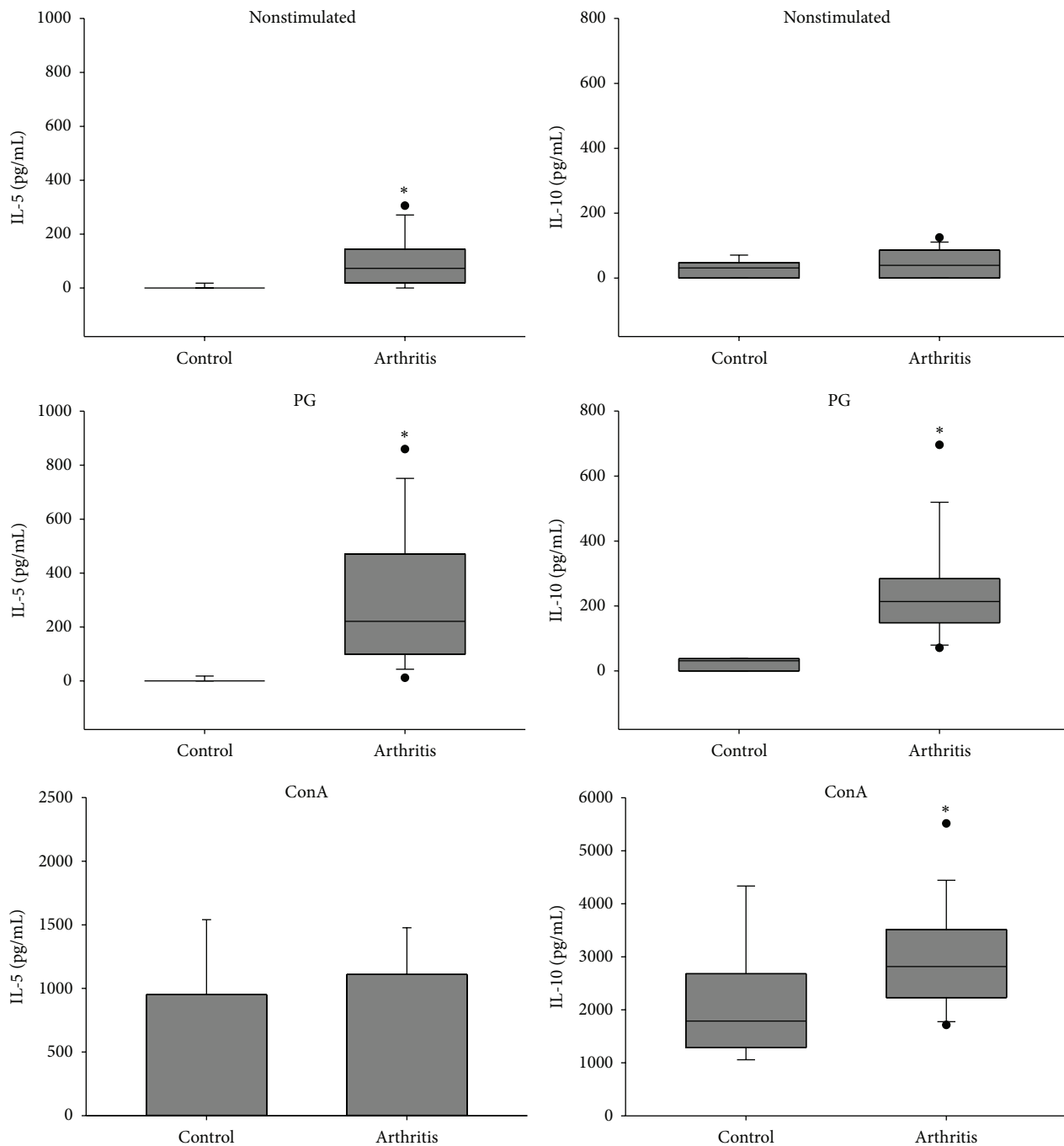


Figure 6: IL-5 and IL-10 production by spleen cells from BALB/c retired breeder mice with bovine proteoglycan-induced arthritis. Spleen cells were *in vitro* stimulated with PG and ConA and incubated for 48 hours. \*  $P < 0.05$  compared to the respective control.

as IL-5 and IL-10. Although RA is considered a disease characterized by predominant Th pattern, studies indicate that Th cytokines such as IL-4 and IL-10 also contribute to the immunopathogenesis of the disease and may also be related to the stage of disease development [48]. According to Gerli et al. [49], there is a high production of IL-4 and IL-10 by T cells from peripheral blood of patients in earlier stages of arthritis, but this production decreases significantly

in later stages, contributing to disease progression and joint destruction in the chronic phase. Our results are, therefore, similar to the mixed Th1/Th pattern already shown in humans and in PGIA model [31]. An interesting aspect was observed in nonstimulated spleen cell cultures from arthritic animals when compared to control group. The arthritic group produced detectable levels of IFN- $\gamma$ , TNF- $\alpha$ , IL-6, IL-17, IL-5, and IL-10 even in the absence of the speci

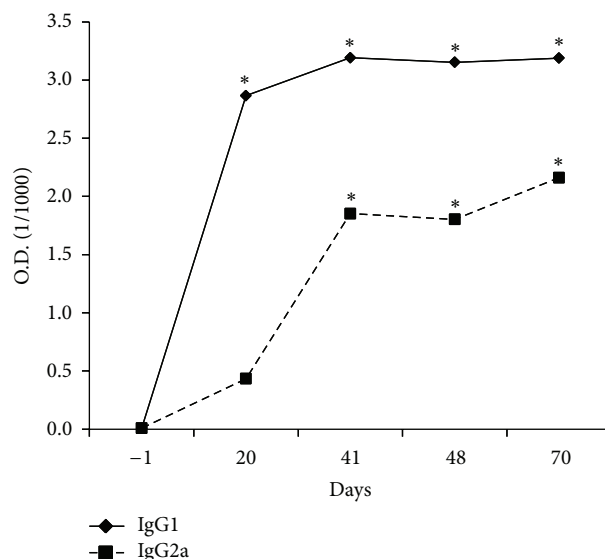


Figure 7: IgG1 and IgG2a serum levels from BALB/c retired breeder mice with bovine proteoglycan-induced arthritis. Blood samples were obtained two days before each PG+DDA immunization (days -1, 20, and 41), seven days after the third immunization (day 48), and after euthanasia (day 70). \*  $P < 0.05$  compared to day -1 of the same group.

stimulus. This spontaneous production, which might be more properly called endogenous production, could result from the intense immune response activation and cytokine secretion by effector cells that are significantly occurring.

Some interesting results mainly related to IL-17 were also detected in spleen cell cultures stimulated with ConA. In this case, we highlight the fact that the production of this cytokine after polyclonal stimulation was very similar to that induced by specific antigen stimulation. This finding is different from the ones usually obtained after polyclonal activation. The stimulation with mitogens is usually associated with induction of significantly higher cytokine production than the specific stimulus. However, recently, Doodes et al. [21] observed that the production of IL-17 and IFN- $\gamma$  in response to specific stimulus is extremely high, reaching levels greater than 2000 pg/mL in the PGIA model. Similarly high levels of IL-17 were observed in human studies. Leipe et al. [50] evaluated the importance of IL-17 in autoimmune arthritis and found that purified T cells from the peripheral blood of patients, in the early stage of the disease, produced very high levels of this cytokine. Furthermore, analysis of the production of IL-17 by mononuclear cells from peripheral blood of healthy individuals, in response to different mitogens, revealed that the level of this cytokine in response to ConA did not exceed 500 pg/mL [51].

## 5. Conclusions

Our results indicate that this commercial bovine PG is highly arthritogenic for BALB/c retired breeder mice. In addition, the disease induced by this reagent presents clinical symptoms and histopathological features that are very similar to those found in other arthritis models and also the human

corresponding pathology. Taken together, these results suggest that this bovine PG can be used as an alternative source in PGIA for the study of many aspects of RA, including the immunopathogenesis of the disease and also the development of new therapies.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgments

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## *Capítulo 3*

*Estratégia profilática na artrite experimental*

### **CAPÍTULO 3**

Este capítulo é constituído pelo manuscrito “Systemic administration of proteoglycan protects BALB/c mice from experimental arthritis” decorrente deste projeto de pesquisa. Neste manuscrito demonstramos que a administração sistêmica de um antígeno específico da cartilagem (proteoglicano) induz uma tolerância específica que pode ser utilizada como uma estratégia profilática na artrite autoimune experimental.

***Systemic administration of proteoglycan protects BALB/c mice from experimental arthritis***

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**Abstract:** It is well known that soluble proteins can induce specific tolerance when administered systemically. This study aimed to evaluate the prophylactic potential of proteoglycan (PG), which is a specific cartilage antigen, in experimental arthritis. Female BALB/c retired breeder mice received two (2xPG50 and 2xPG100 groups) or three (3xPG50 group) intraperitoneal (i.p.) doses of bovine PG (50µg or 100µg) with three days interval. One week later, the animals were immunized with three i.p. doses (21 days interval) of bovine PG associated with dimethyl-dioctadecyl ammonium bromide adjuvant for arthritis induction. After the third immunization, disease severity was daily assessed by score evaluation. The 2xPG50 and 3xPG50 groups presented significant lower scores but disease incidence decreased only in the 3xPG50 group. Clinical downmodulation by previous 3xPG50 injection was concomitant with decreased production of IFN-γ and IL-17 and increased production of IL-5 and IL-10. Previous PG administration did not alter the frequency of dendritic and regulatory T cells, suggesting the contribution of other mechanisms to this prophylactic effect. Delayed-type hypersensitivity reaction made in mice previously injected with PG or ovalbumin confirmed the induction of a tolerance state and also indicated that this PG prophylactic effect was antigen-specific. Taken together, these data suggest that previous PG inoculation determined a specific tolerogenic effect that decreased the severity of subsequently induced arthritis.

**Keywords:** Proteoglycan, immunological tolerance, experimental arthritis.

### *Introduction*

Rheumatoid arthritis (RA) is a systemic autoimmune disease that affects around 1% of population worldwide (WHO, 2003). It is an arthropathy characterized by a chronic inflammation that leads to progressive cartilage and bone destruction. The etiology of RA is multifactorial, involving both genetic and environmental factors (Klareskog, 2011). The mechanisms of the immunopathogenesis of RA are still not fully elucidated. However, much is known about the interaction of synovial pathological conditions during RA development, such as synovial membrane hyperplasia, chronic inflammation and increased osteoclastogenesis. The interaction of these processes contributes to the recruitment of several cell types from both innate and adaptative immunity with consequent cytokine and chemokine production (Scott et al., 2010). Cytokines are the main players in the arthritic joints, maintaining chronic inflammation and promoting autoimmunity. TNF- $\alpha$  is considered one of the most important cytokine involved in the synovitis. This cytokine stimulates the proliferation, metalloproteinase and adhesion-molecule expression, secretion of other cytokines, and prostaglandin production by the synovial tissue, which contribute to joint inflammation (Lee & Weinblatt, 2001). Anti-TNF therapy is currently the standard treatment for RA, however, the new promising target for arthritis treatment is IL-17. Hirota et al., 2007, showed that the development of spontaneous arthritis in SKG mice model is dependent on the generation of Th17 cells. Production of IL-17 acts synergistically with IL-1 $\beta$  and TNF to promote cartilage degradation (McInnes & Schett, 2007). The role of IFN- $\gamma$  in the immunopathogenesis of arthritis is still contradictory. This cytokine exerts paradoxal effects and can be both pathogenic and protective in RA. The pathogenic effect of IFN- $\gamma$  is related to its well-known inflammatory properties and the protective effect is related to its ability to suppress IL-17 production by Th17 cells (Schurgers et al, 2011). Vermeire et al., 1997, demonstrated that mice lacking IFN- $\gamma$  receptor developed a severe collagen-induced arthritis (CIA). However, the dual role of this cytokine in CIA is dependent on the stage of the disease. In early stages, IFN- $\gamma$  enhances the immune response and, in later stages, it exerts a protective effect (Boissier et al., 1995). On the other hand, IFN- $\gamma$  is crucial for the development of proteoglycan-induced arthritis (Finnegan et al., 2002) but it is dependent on the location of the antigen immunization during arthritis inducing. Intraperitoneal immunization with proteoglycan favored only the production of IFN- $\gamma$ , whereas subcutaneous immunization stimulated both IFN- $\gamma$  and IL-17 production by T cells (Rodeghero et al., 2013). Although

there are discrepant findings, most of human and experimental data demonstrated that both IFN- $\gamma$  and IL-17 are important to arthritis regulation (Kunz & Ibrahim, 2009). The imbalance between the synovial pro-inflammatory and anti-inflammatory cytokines is a typical feature in RA. Although anti-inflammatory cytokines such as TGF- $\beta$ , IL-10 and IL-4 are also detected in arthritic joints, their deficient expression is not enough to down-regulate the effects of pro-inflammatory cytokines and favors synovitis (McInnes and Schett, 2007).

Some of the autoantigens involved in the pathogenesis of RA include IgG immunoglobulin, collagen, proteoglycan, citrullinated proteins, fibrin, fibrinogen and the shared human leukocyte antigen (HLA)-DR epitope (Imboden, 2009; Ishikawa et al., 2014a). Currently, the most used experimental model of arthritis is the CIA, induced by a cartilage autoantigen. Susceptible mice immunized with type II collagen associated with Complete Freund's Adjuvant (CFA) develop an arthropathy that resembles the human RA (Brand et al., 2004). Another experimental model based on the immunization with a cartilage antigen is PGIA. According to Glant et al., 2003, BALB/c mice immunized with human proteoglycan (PG) associated with CFA also develop a disease that shares many similarities with human RA, including clinical manifestations, histopathology, genetics and immune regulation. More recently, we demonstrated that bovine PG can also induce experimental arthritis when associated with a potent adjuvant (Ishikawa, et al., 2014b).

It is well known that autoantigens have a paradoxical role in the context of autoimmune diseases. Several antigens that can be used to induce autoimmunity have also a potential to induce antigen-specific tolerance depending on the route (oral or dermal, for example), the dose and the physical form of the antigen. This type of tolerance can also be induced by systemic administration of soluble antigens. Initial studies demonstrated that intravenous or intraperitoneal routes are more effective in inducing tolerance (Liblau et al., 1997). The postulated mechanisms underlying this systemic tolerance are the classic ones, such as anergy and clonal deletion of effector cells, induction of regulatory T cells (Tregs) and immune response deviation (Harrison & Hafler, 2000).

Currently, most of RA treatment consists in general suppression of the immune response to control synovitis and prevent joint injury (Smolen et al., 2007). Unfortunately, these therapeutic interventions have some side effects and may increase the susceptibility to infections. Therefore, there is an increasing search for alternative immunomodulatory strategies, especially the ones that induce antigen-specific tolerance. Ideally, the

immunization with self-antigens to promote self-tolerance would be the safest way to avoid autoimmune diseases. This tolerogenic strategy is also called “negative vaccination”. Historically, transplantation studies were the first to use this expression to refer to the immunomodulatory strategy that induces specific self-tolerance (Moreau et al., 2012). Considering this “negative vaccination” approach, we expected that the systemic administration of a cartilage antigen (PG) could induce an antigen-specific tolerance that would prevent the development of arthritis induced by this same antigen.

### *Material and Methods*

#### *Animals*

Female BALB/c retired breeder (beyond the reproductive age) mice were removed from breeding colonies by the age of 8-11 months and purchased from CEMIB (Campinas, São Paulo, Brazil). They were maintained in the animal facility of the Department of Microbiology and Immunology under controlled conditions of luminosity (12h light/12h dark) and temperature ( $22\pm 2^{\circ}\text{C}$ ). Mice were allocated in ventilated cages with sterile pine shavings and received sterile food and filtered water *ad libitum*. The manipulation of the animals was in compliance with the local ethics committee (protocol number 257-CEEA).

#### *PG administration, arthritis induction and score evaluation*

Mice received two or three intraperitoneal (i.p.) doses of PG (50  $\mu\text{g}$  or 100  $\mu\text{g}$ ) with three days interval. One week later, experimental arthritis was induced as described elsewhere (Ishikawa et al., 2014). Briefly, mice received three i.p. injections (21 days interval) of bovine PG associated with dimethyl-dioctadecyl ammonium bromide (DDA) adjuvant. After the third injection, arthritis score was daily evaluated until euthanasia (70 days after the first immunization). Arthritis severity was determined using a standard visual scoring system based on the degree of edema and erytema ranging from 0 to 4 for each paw. The following system was used: 0 = normal; 1 = mild edema in the paw or one joint; 2 = moderate edema and erytema in the paw and one or more joints; 3 = pronounced edema and erytema in the paw, all joints and ankle; 4 = severe edema and erytema of the entire paw and ankle and movement limitation. This classification resulted in a total score that ranged from 0 to 16 for each animal.

#### *Cellular immune response*

Mice spleens were collected after euthanasia and the cells were resuspended in RPMI medium containing gentamicin and fetal calf serum.  $5,0 \times 10^6$  cells/mL were stimulated with PG (50 µg/mL). After 48 hours incubation at 37°C / 5% CO<sub>2</sub>, culture supernatants were collected for detection of TNF-α, IFN-γ, IL-17, IL-6, IL-5 and IL-10. These cytokines were quantified using enzyme linked immunosorbent assay (ELISA), according to manufacturer's instructions (BD Biosciences, San Jose, CA, USA and RD Systems, Minneapolis, MN, USA).

#### *Flow cytometry analysis*

Flow cytometry analysis was performed according to manufacturer's instructions (eBiosciences, San Diego, CA, USA). Briefly, for DCs immunophenotyping, splenic cells were incubated with FITC labeled anti-mouse CD11c (clone N418), APC labeled anti-mouse MHCII (clone M5/114.15.2) and PE labeled anti-mouse CD80 (clone 16-10A1) antibodies. The cells were washed and resuspended in flow cytometry buffer containing paraformaldehyde solution to avoid dye degradation until analysis. For Tregs, splenic cells were first incubated with FITC labeled anti-mouse CD4 (clone GK1.5) and APC labeled anti-mouse CD25 (clone PC61.5) antibodies. Intracellular Foxp3 transcription factor was detected using Foxp3 PE Staining Set (eBiosciences, San Diego, CA, USA) according to manufacturer's instructions.

#### *Delayed-type hypersensitivity reaction (DTH)*

Mice were injected with three i.p. doses of 50 µg of PG or chicken ovalbumin (OVA) (Sigma Aldrich, St. Louis, MO, USA) with three days interval. One week after the last dose, the animals were immunized with a single i.p. dose of PG associated to the DDA adjuvant. DTH was performed one week later by PG inoculation (10 µg) in the hind paw. Paw thickness was measured before and 24 hours after PG inoculation using a caliper (Starret, Athol, MA, USA).

#### *Statistical analysis*

Results were presented as mean ± standard deviation for parametric variables and the comparison among the groups was performed by ANOVA followed by Tukey's test. For non-parametric variables, the results were presented as median and the comparison among the

groups was performed by Kruskal-Wallis followed by Dunn's test. Incidence and severity were compared using Chi-square test. All data were analyzed using SigmaPlot software version 12.0 (Jandel Corporation, USA) and  $p < 0.05$  was considered significant.

## Results

### Disease incidence and score evaluation

As expected, animals from Control (-) group did not develop arthritis and the Control (+) group presented a very typical disease. The arthritis incidence in the 2xPG50 and 2xPG100 groups was similar to those in the Control (+) group. However, the group previously injected with 3xPG50 presented a significantly decreased disease incidence. Although there was no difference in the arthritis onset among the experimental groups, the maximum clinical score achieved in the 2xPG50 and the 3xPG50 groups was significantly reduced in comparison with the Control (+) group. Moreover, these two groups also presented a much less severe disease, with significant lower number of animals with clinical score above 8. These results are summarized in table 1 and figure 1.

Table 1: Arthritis incidence and severity in female BALB/c retired breeder mice previous inoculated with the specific antigen (PG).

|             | Arthritis incidence | Animals with score > 8 | Disease onset (days) |
|-------------|---------------------|------------------------|----------------------|
| Control (-) | 0/13 (0%)           | -                      | -                    |
| Control (+) | 22/22 (100%)        | 9/22 (41%)             | 43                   |
| 2xPG50      | 12/13 (92%)         | 0/13 (0%)*             | 44                   |
| 2xPG100     | 7/7 (100%)          | 0/7 (0%)               | 44                   |
| 3xPG50      | 12/16 (75%)*        | 0/16 (0%)*             | 43                   |

\* $p \leq 0,05$  compared to Control (+).

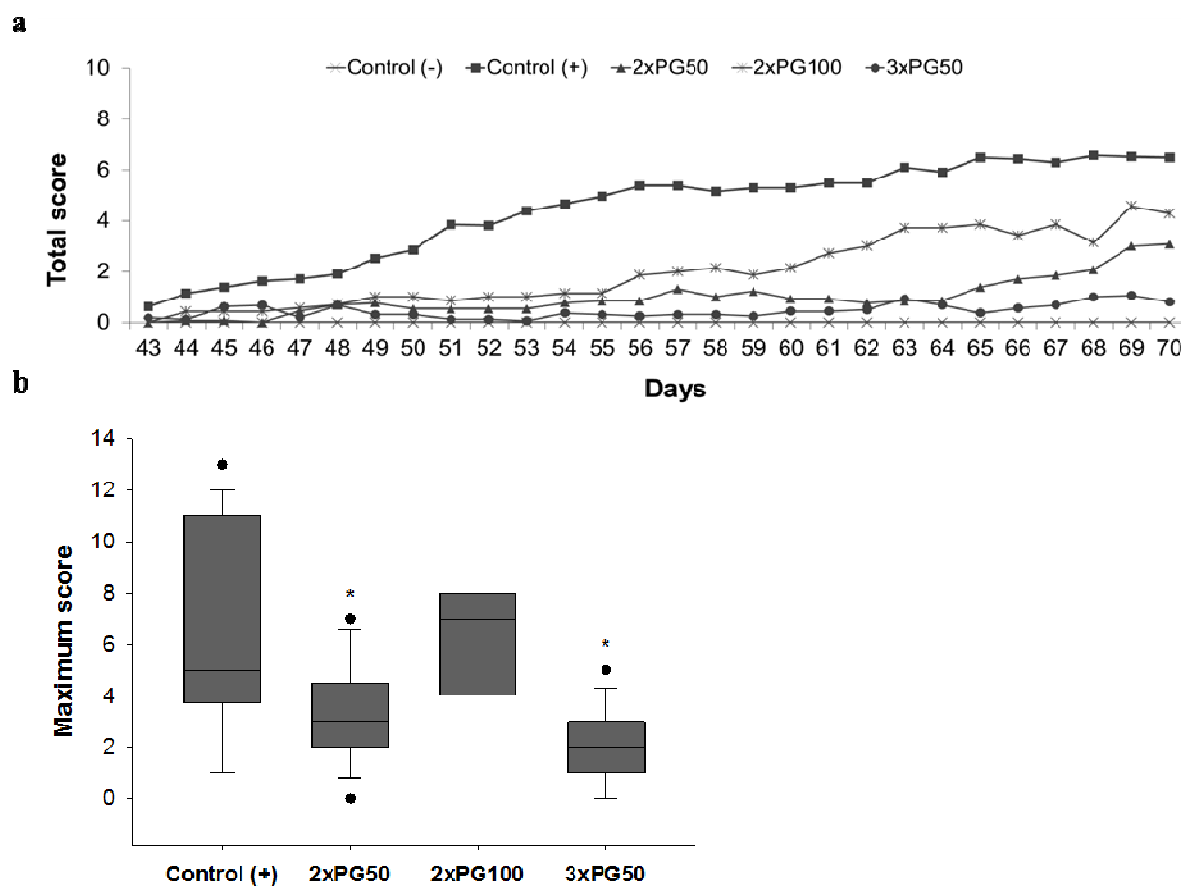


Figure1: Effect of previous inoculation with PG on experimental arthritis. Female BALB/c retired breeder mice were inoculated with different doses of PG and submitted to disease induction. (a) Kinetics of total score. (b) Maximum score per group. \* $p \leq 0.05$

### Cytokine production

Cytokine profile was assessed in spleen cell cultures stimulated with PG. There were no detectable levels of TNF- $\alpha$  in any experimental group (not shown). Figure 2 shows that IFN- $\gamma$  production was detected in nonstimulated cell cultures from all experimental groups. However, only the Control (+) group produced a significant level in comparison to the Control (-) group. In cultures stimulated with PG there was an increase in this cytokine levels in Control (+), 2xPG50 and 2xPG100 groups. The comparison of these PG stimulated cultures showed a significant reduction in IFN- $\gamma$  production in 2xPG50 and 2xPG100 groups compared to the Control (-) group. However, a significant reduction in this cytokine levels was only observed in the 3xPG50 group in comparison to the Control (+) group.

Figure 2 also shows a spontaneous production of IL-17 was detected in cultures from Control (+), 2xPG50 and 2xPG100 groups. After PG stimulation, there was a significant

increase only in the Control (+) group. Quantification of IL-17 levels in these PG stimulated cultures indicated a significant reduction of this cytokine in 2xPG100 and 3xPG50 groups in comparison to the Control (+) group. There was also a spontaneous release of IL-6 in all arthritic groups as documented in figure 2. These endogenous levels were significantly higher in 2xPG50 and 2xPG100 groups compared to the Control (-) group. Stimulation with the specific antigen increased its production only in the Control (+) group.

As illustrated in figure 3, nonstimulated cultures from all arthritic groups also produced detectable levels of IL-5. Even though the levels of this cytokine were higher in previously PG injected groups, only 2xPG50 and 2xPG100 presented significantly higher levels than Control (-) and Control (+) groups. Production of IL-5 in response to the specific antigen was significantly increased in Control (+), 2xPG50 and 3xPG50 groups. The comparison of these PG stimulated cultures showed a significant increase in IL-5 production only in 2xPG50 and 3xPG50 groups in comparison to the Control (+).

Production of IL-10 is also shown in figure 3. A significant spontaneous release of this cytokine was detected in 2xPG50 and 3xPG50 groups compared to both Control (-) and Control (+) groups. The addition of PG to the cultures significantly increased the production of this cytokine in all arthritic groups. Quantification of IL-10 levels in these stimulated cultures indicated that the 2xPG50 and 3xPG50 groups produced significantly higher levels than the Control (+) group.

Comparison between cytokine levels in nonstimulated and PG stimulated cultures is demonstrated in table 2.

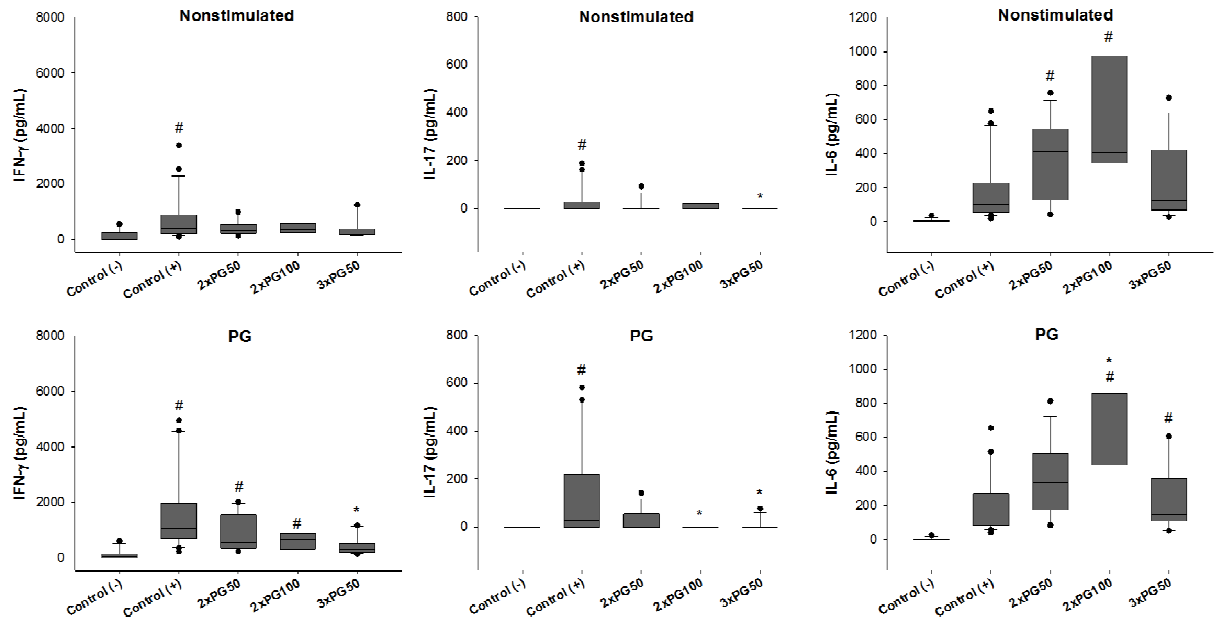


Figure 2: IFN- $\gamma$ , IL-17 and IL-6 production by female BALB/c retired breeder mice: effect of pre-inoculation with PG. Spleen cells were *in vitro* stimulated with PG and incubated for 48 hours. Cytokine levels were detected by ELISA. Control (-): not PG inoculated healthy group. Control (+): not PG inoculated arthritic group. \* $p \leq 0.05$  compared to the Control (+) group and # $p \leq 0.05$  compared to the Control (-) group.

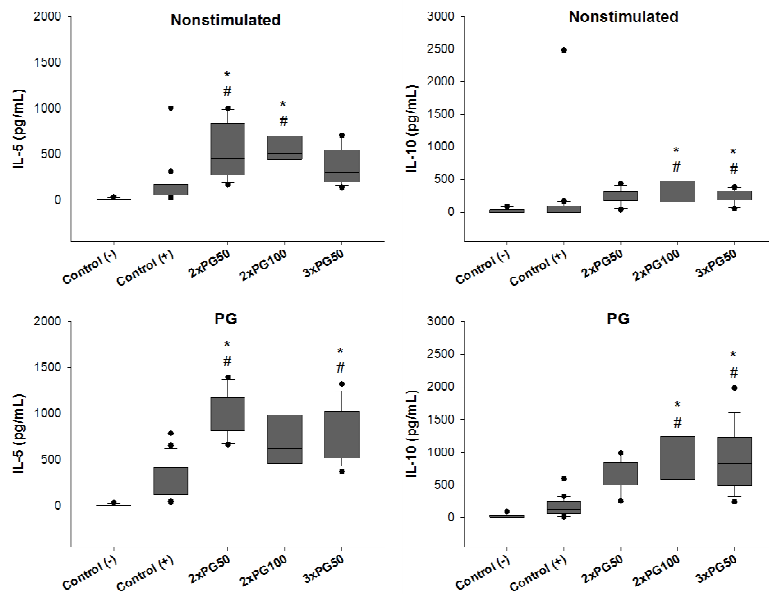


Figure 3: IL-5 and IL-10 production by female BALB/c retired breeder mice: effect of pre-inoculation with PG. Spleen cells were *in vitro* stimulated with PG and incubated for 48 hours. Control (-): not PG inoculated healthy group. Control (+): not PG inoculated arthritic group. \* $p \leq 0.05$  compared to the Control (+) group and # $p \leq 0.05$  compared to the Control (-) group.

Table 2: Cytokine production by spleen cell cultures from BALB/c retired breeder mice: effect of *in vitro* PG stimulation.

|                                |           | Control (-) | Control (+) | 2xPG50  | 2xPG100 | 3xPG50 |
|--------------------------------|-----------|-------------|-------------|---------|---------|--------|
| <b>IFN-<math>\gamma</math></b> | <b>NS</b> | 82,0        | 729,3       | 439,1   | 430,3   | 228,6  |
| <b>(pg/mL)</b>                 | <b>PG</b> | 43,0        | 1640,5*     | 854,9*  | 634,7*  | 328,7  |
| <b>IL-17</b>                   | <b>NS</b> | 0           | 0           | 0       | 10,4    | 0      |
| <b>(pg/mL)</b>                 | <b>PG</b> | 0           | 28,8*       | 0       | 9,6     | 0      |
| <b>IL-6</b>                    | <b>NS</b> | 0           | 102,3       | 355,8   | 629,2   | 251,9  |
| <b>(pg/mL)</b>                 | <b>PG</b> | 0           | 152,5*      | 355,2   | 623,9   | 250,2  |
| <b>IL-5</b>                    | <b>NS</b> | 0           | 102,8       | 531,7   | 513,7   | 361,5  |
| <b>(pg/mL)</b>                 | <b>PG</b> | 0           | 248,0*      | 1011,1* | 621,7   | 771,0* |
| <b>IL-10</b>                   | <b>NS</b> | 0           | 56,2        | 221,7   | 359,3   | 246,5  |
| <b>(pg/mL)</b>                 | <b>PG</b> | 0           | 131,0*      | 656,3*  | 995,4*  | 894,2* |

NS: nonstimulated; \*p  $\leq$  0,05 compared to the respective counterpart.

#### Flow cytometry analysis

The frequency of CD11c+ MHCII+ CD80+ mature DCs in the spleen of PG pre-inoculated animals is shown in figure 4a. Although the Control (+) group presented a higher frequency of mature DCs in comparison with the Control (-) group, previous PG inoculation did not alter DCs frequency in total CD11c+ cells. Evaluation of CD4+ CD25+ Foxp3+ Tregs in the spleen indicated that all experimental groups had similar frequencies of this subpopulation in total CD4+ cells (figure 4b).

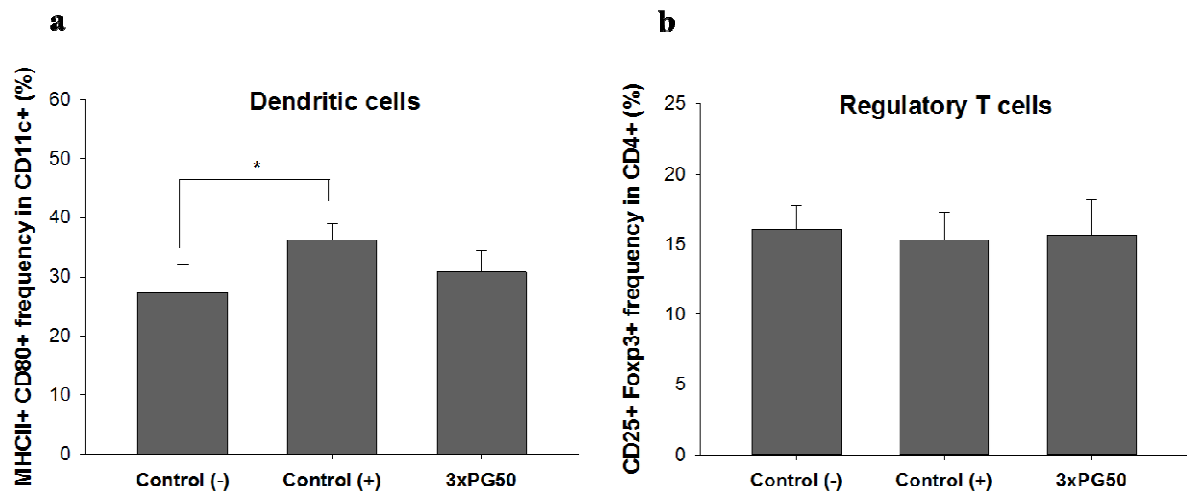


Figure 4: Splenic dendritic and regulatory T cells from female BALB/c retired breeder mice: effect of pre-inoculation with PG. Immunophenotyping was performed by flow cytometry. Control (-): not treated healthy group. Control (+): not treated arthritic group. (a) Frequency of MHCII+ CD80+ cells in total CD11+ cells. (b) Frequency of CD25+ Foxp3+ cells in total CD4+ cells. \* $p \leq 0.05$ .

### DTH

DTH was used to confirm tolerance induction by PG and also to evaluate the specificity of the tolerance triggered by previous PG inoculation. As expected, there was no increase in the paw thickness of animals from the Control (-) group (animals not previously immunized with PG+DDA and challenged with PG in the paw). However, all other experimental groups presented elevated paw thickness 24 hours after PG challenge in comparison to the negative control. Inoculation of OVA (an irrelevant antigen) before immunization with PG+DDA, did not trigger a nonspecific tolerogenic effect. Animals from this group presented paw thickness similar to the Control (+) group when challenged with PG. Previous inoculation with the specific antigen (PG) determined a tolerogenic effect demonstrated by a significant decrease in DTH response in comparison to the Control (+) group (figure 5).

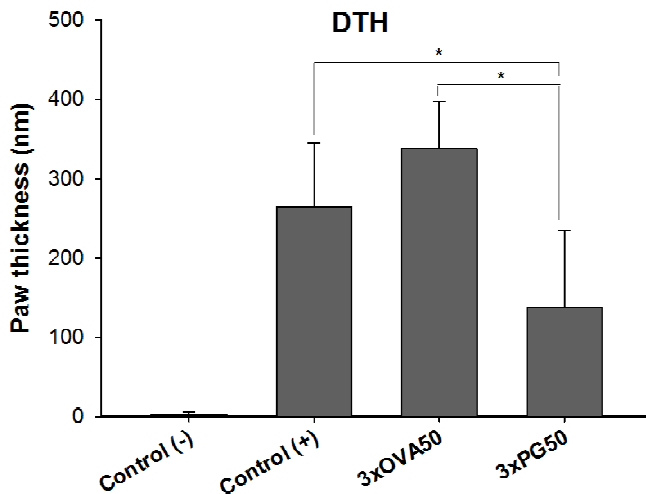


Figure 5: Delayed-type hypersensitivity reaction elicited by PG in BALB/c retired breeder mice previously injected with PG or OVA. Animals were injected with three doses of PG or OVA and then immunized with PG associated with DDA adjuvant. Paw thickness was measured after PG challenge. Control (-): animals not previously injected with PG nor immunized with PG+DDA. Control (+): animals not previously injected with PG and immunized with PG+DDA. \* $p \leq 0.05$ .

### *Discussion*

Autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and type 1 diabetes are in the list of the top ten most relevant cause of female death (Hunter et al., 2014). In this context, many efforts have been made to induce tolerance to self antigens as an alternative to current and usually global immunosuppressive treatments. Oral route, for example, has been successfully employed to induce tolerance to self-antigens. This strategy has been promising by showing a protective effect in experimental diabetes (Harrison et al., 2013) and encephalomyelitis (Peron et al., 2010). Intravenous administration of soluble antigen in the absence of adjuvants is another well documented procedure that is able to induce tolerance (Warren et al., 1997; Verbeck et al., 2007). Only few studies addressed this possibility in arthritis. The goal of the present study was, therefore, to evaluate if proteoglycan (PG) injected by a systemic route is able to induce a state of tolerance and to affect arthritis development. As an experimental model, we chose arthritis induced by immunization with PG associated with the DDA adjuvant in BALB/c retired breeder mice. In this study, we demonstrated that intraperitoneal PG inoculation, before arthritis induction by this same antigen associated with an adjuvant, was able to decrease both incidence and disease severity. The most effective dose regimen was the previous inoculation of three doses of 50 mg of PG given with three days interval. Two doses of 50 mg of PG triggered a similar but less accentuated protective effect. The tolerogenic effect observed in this work is consistent with the literature. Liblau et al., 1997, already discussed the available knowledge at that time and suggested that systemic inoculation of soluble proteins, synthetic peptides or peptide analogues, known as altered peptide ligands, could become an acceptable approach to prevent and or to treat human autoimmune diseases as multiple sclerosis and type 1 diabetes. The prophylactic effect of a cartilage specific antigen was described by Gumanovskaya et al., 1999. These authors demonstrated that intravenous administration of type II collagen was an effective way to induce tolerance and to suppress collagen-induced arthritis. At first sight, it could appear complicated to translate our findings to human arthritis. Differently from multiple sclerosis and type 1 diabetes whose involved autoantigens are very well established, RA is an even more complex pathology whose targeted autoantigens are still being debated. However, in a recent report it was demonstrated that the oral administration of shark type II collagen was able to suppress arthritis induced in rats by inoculation of Complete Freund's Adjuvant (Chen et al., 2012). We could, therefore, suppose that another specific joint antigen, as in the case of PG, could trigger a similar protective effect when administered by a systemic

route. The possible prophylactic use of bovine PG, that was used in our model, certainly needs further investigation because it shares epitopes with rodent and human PG (Walcz et al., 1994; Hering et al., 1997).

To elucidate the possible mechanism involved in the protection triggered by i.p. PG injection, we analyzed the production of some cytokines. Interestingly, there were no detectable levels of TNF- $\alpha$  in spleen cell cultures from any experimental group. Experimental studies indicate the pivotal role of this cytokine in RA. Butler et al., 1997, demonstrated that human TNF- $\alpha$  transgenic mice spontaneously developed severe arthritis. Moreover, the treatment with antibodies against TNF- $\alpha$  or soluble TNF- $\alpha$  receptor ameliorated experimental arthritis induced by type II collagen (Williams et al., 1992; Joosten et al., 1996). However, both human and experimental data show that, during RA, TNF- $\alpha$  production is mainly detected in sera and synovial fluid (Lee & Weinblatt, 2001; McInnes & Schett, 2007). Considering this, it is possible that the therapeutic effect of previous PG administration on TNF- $\alpha$  levels would be evidenced in serum or joint lesions from the arthritic groups. Conversely, all experimental groups produced detectable levels of IFN- $\gamma$  and IL-17 in spleen cell cultures. Even though there are discrepant findings in the literature considering the role of IFN- $\gamma$  in RA immunopathogenesis, several studies demonstrated the pro-arthritis effect of IFN- $\gamma$  and IL-17 (McInnes & Schett, 2007; Kunz & Ibrahim, 2009). Considering our experimental model, we believe that these inflammatory cytokines are probably contributing to arthritis development. Previous PG immunization prevented mice from a severe disease and significantly reduced the production of IFN- $\gamma$  and IL-17. The analysis of cytokine production by spleen cells stimulated with the specific antigen suggests that the protective effect is also mediated by an increased production of IL-5 and IL-10 anti-inflammatory cytokines in the pre-inoculated PG groups compared with the Control group (+). As IL-5 is described as a typical Th2 cytokine (Xu et al., 2012.; Park et al., 2012), it is possible that the administration of systemic PG has polarized the immune response towards Th2. Moreover, IL-10 has been described by some authors as being similar to IL-4 and IL-5 concerning the Th2 pattern (Cheng et al., 2012). However, in the context of autoimmune diseases and other inflammatory diseases, the production of this cytokine by Tregs is more extensively studied. IL-10 is endowed with great anti-inflammatory potential and its contribution to the tolerance induction to self and foreign antigens has also been described (den Hartog et al., 2011). A possible correlation between the increase of IL-5 and IL-10 and protection is also supported by the

literature. Our group has recently shown that immunization of C57BL/6 mice with *Strongyloides venezuelensis* antigens associated with complete Freund's adjuvant, followed by an infection with the same helminth, protected the animals against the development of diabetes induced by streptozotocin. This protection was associated with significant production of IL-5 and IL-10 in the spleen of these animals (Peres et al., 2013). In the context of multiple sclerosis, it was demonstrated that treatment of experimental autoimmune encephalomyelitis with a viral vector (herpes simplex) expressing IL-5 decreased the number of infiltrating lymphocytes in the brain and ameliorated the disease (Nygårdas et al., 2011). Although the production of IL-5 has been increased after PG immunization, IL-10 has been classically indicated as the most important immunoregulatory cytokine in the innate and adaptive immune responses (Hedrich & Bream, 2010). The relevance of IL-10 as an anti-catabolic, anti-apoptotic and anti-inflammatory molecule in joint diseases has also been widely explored (Schulze-Tanzil et al., 2009). The production of this immunoregulatory cytokine has been attributed to different cell types such as T and B lymphocytes and natural killer cells (Yao et al., 2013). Among these cells, the natural regulatory T cells (nTregs) characterized by the expression of CD4, CD25 and Foxp3, has been extensively studied for its involvement in the maintenance of peripheral self-tolerance (Dasgupta & Saxena, 2012). The levels of Tregs and DCs in the spleen were not influenced by previous PG immunization. It is possible, however, that the number of Tregs is increased in the arthritic lesions in animals previous inoculated with PG compared to the Control (+) group. To examine this possibility, some studies should be conducted to evaluate the presence of Tregs and other cell subsets in the joints.

To determine if the prophylactic activity elicited by PG administration was specific, we compared the effects of previous injection of PG and OVA on the intensity of DTH reaction. Previous administration of three doses of the specific antigen (PG) determined a tolerogenic effect characterized by a significant decreased DTH response in comparison to the Control (+) group. This effect was not observed in animals previous inoculated with the non-related cartilage antigen (OVA). This result showed that the tolerogenic effect on the experimental arthritis was specific for PG. Taken together, these data indicated that previous PG inoculation determined a specific tolerogenic effect strong enough to reduce the severity of subsequently induced arthritis.

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## *Capítulo 4*

*Estratégia terapêutica na artrite experimental*

## **CAPÍTULO 4**

Este capítulo é constituído pelo manuscrito “Vitamin D and proteoglycan association: a new strategy for arthritis treatment?” decorrente deste projeto de pesquisa. Neste manuscrito demonstramos que a associação de um adjuvante tolerogênico (vitamina D) com um antígeno específico da cartilagem (proteoglicano) pode ser utilizada como uma estratégia terapêutica na artrite autoimune experimental.

***Vitamin D and proteoglycan association: a new strategy for arthritis treatment?***

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**Abstract:** Rheumatoid arthritis (RA) is a chronic inflammatory systemic autoimmune disease that compromises the joints, causing cartilage destruction and bone erosion. Current RA treatment consists in general suppression of immune response which may increase the susceptibility to infections. Considering the immunomodulatory properties and the tolerogenic effect of active vitamin D (VitD3), this study aimed to evaluate if the association of the specific antigen (proteoglycan) with VitD3 has a therapeutic effect on experimental arthritis. For disease induction, female BALB/c retired breeder mice were intraperitoneally (i.p.) immunized three times (21 days interval) with bovine proteoglycan (PG) and dimethyldioctadecyl ammonium bromide adjuvant. One day after the first injection, mice received 0,1 µg of VitD3 by i.p. route every other day during 15 days. PG (50 µg) was co-administered on second and on eleventh day of treatment. After the third immunization, disease severity was daily assessed by score evaluation. Although there was no difference on RA incidence, VitD3, associated or not with PG, determined lower scores comparing to the non-treated group. However, histopathological analysis indicated that the VitD3+PG treated group was the only one to be clearly protected from developing a severe arthritis. In addition, this was the experimental group whose cytokine production was significantly downregulated and whose percentage of CD11c+ MHCII+ CD80+ dendritic cells was higher than the positive control. This study showed that VitD3+PG association was therapeutical in experimental arthritis. It also suggested that VitD3 has the potential to be combined with other antigens to be used as a new strategy to induce specific tolerance to treat autoimmune inflammatory diseases.

**Keywords:** Vitamin D, proteoglycan, experimental arthritis.

### *Introduction*

Rheumatoid arthritis (RA) is the most common arthropathy that affects around 1% of the world population, with higher prevalence in women (Quintero et al., 2011). It is characterized by a chronic inflammation that leads to cartilage destruction and bone erosion. The immunopathogenesis of RA is not fully elucidated; however, it is known that it involves both innate and acquired immunity components. Current RA treatment consists in combined therapies that suppress the immune response in general, which may increase the susceptibility to infections. In this sense, many efforts are made to develop more specific immunomodulatory strategies as, for example, the induction of antigen-specific tolerance and the induction/activation of regulatory T cells (Tregs).

Self-tolerance can be induced by different strategies such as autoantigen administration via tolerogenic routes (oral mucosa, for example), concomitant blocking of costimulatory molecules or autoantigen targeting to resting antigen presenting cells, such as dendritic cells (DCs) (Harrison et al., 2000). Although traditionally characterized as strong immune response inducers, including autorreactive responses in autoimmune diseases, recent findings implicate the DCs in the induction and maintenance of tolerance to self-antigens (Torres-Aguilar et al., 2010). This functional dichotomy of DCs is determined by many factors, being some of the most important their stage of differentiation/maturation and their hematopoietic origin. The development of tolerogenic DCs mostly depends on the microenvironment where the DCs are differentiating. Regarding this, it is worth mentioning the presence of suppressive cytokines such as IL-10 and TGF- $\beta$  and other factors as histamine and prostaglandin E2 (Kaliński et al., 1998; Song et al., 2014). More recently, it was demonstrated that many pharmacological agents, including corticosteroids, cyclosporine A, rapamycin, mycophenolate mofetil, prostaglandin E2 and vitamin D confer tolerogenic properties to DCs (Hackstein & Thomson, 2004; Adorini et al, 2004).

The hormone 1,25(OH)<sub>2</sub>D<sub>3</sub>, also known as calcitriol, is the active form of vitamin D (VitD3) and it plays a central role in bone metabolism (Adorini & Penna, 2008). However, the biological effects of calcitriol are beyond calcium and phosphorus homeostasis and its effects in the immune system have been thoroughly studied. VitD3 activity is mediated by the vitamin D receptor (VDR) which is a member of the superfamily of nuclear hormone receptors. VDR acts as a transcription factor that binds to specific DNA sequences and it is found in many cell types including immune cells such as monocytes, macrophages, DCs and

activated B and T lymphocytes (Calberg & Polly, 1998). In the innate immunity, VitD3 decreases the TNF- $\alpha$  production when added to human monocytes. This effect is mediated through interaction with Toll-like receptors (TLR2 and TLR4) (Du et al., 2009). Calcitriol immunoregulatory activity has been partially attributed to its effects on DCs and subsequent induction of Tregs. Human DCs differentiated in the presence of VitD3 presented reduced expression of maturation markers such as CD1a, MHC II, CD40, CD80 and CD86 (Adorini & Penna, 2009). Moreover, the co-culture of DCs pre-treated with calcitriol and CD4+CD25+ T cells leaded to induction of CD4+Foxp3+Tregs (Penna et al., 2005). *In vitro* studies also indicate that VitD3 affects the adaptative immunity, clearly inhibiting the proliferation of T cells and IL-2 and IFN- $\gamma$  production (Dimeloe et al., 2010). However, the effect on Th2 cells is less clear. Some reports indicated higher levels of IL-4, IL-5 and IL-10 (Boonstra et al., 2001) while others demonstrated a decreased number of IL-4 producing cells or even no effect at all (Dimeloe et al., 2010). Th17 is another Th subpopulation affected by VitD3. In experimental autoimmune uveitis, calcitriol suppressed autoimmune response through inhibition of the activation and differentiation of Th17 cells and also of its cytokine production (Tang et al., 2009). Several studies have been demonstrated the beneficial role of VitD3 in other experimental models of autoimmune diseases. For example, treatment of adult NOD mice with calcitriol analogue inhibited the production of IL-12 and IFN- $\gamma$  and prevented the infiltration of Th1 cells into the pancreas, blocking the progression of insulitis. This blockage was associated with higher frequency of CD4+CD25+ Tregs in the pancreas and the lymph nodes. Besides, the adoptive transfer of these cells significantly reduced the disease development (Gregori et al., 2002). Spach et al., 2006, demonstrated that the inhibitory effect of calcitriol in the experimental autoimmune encephalitis is dependent on a signaling pathway involving IL-10. Recent findings showed a positive correlation between the serum levels of VitD3 and the frequency and function of Tregs in patients with multiple sclerosis (Smolders et al., 2009).

Considering the immunomodulatory and tolerogenic effects of calcitriol, this study aimed to evaluate if the administration of VitD3 associated to the specific antigen (PG) has a therapeutic effect on experimental arthritis. In this case, this specific strategy could polarize the immune response towards specific tolerance.

## Materials and Methods

### Animals

Female BALB/c retired breeder (beyond the reproductive age) mice were removed from breeding colonies by the age of 8-11 months and purchased from CEMIB (Campinas, São Paulo, Brazil). They were maintained in the animal facility of the Department of Microbiology and Immunology under controlled conditions of luminosity (12h light/12h dark) and temperature ( $22\pm 2$  °C). Mice were allocated in ventilated cages with sterile pine shavings and received sterile food and filtered water *ad libitum*. The manipulation of the animals was in compliance with the local ethics committee (protocol number 257-CEEA).

### Arthritis induction, vitamin D treatment and score evaluation

Experimental arthritis was induced as described elsewhere (Ishikawa et al., 2014). Briefly, mice received three intraperitoneal (i.p.) immunizations, with 21 days interval, containing bovine proteoglycan (PG) (Sigma Aldrich, St. Louis, MO, USA) and dimethyl dioctadecyl ammonium bromide (DDA) adjuvant (Sigma Aldrich, Saint Louis, MO, USA). One day after the first immunization, the animals received 0,1 µg of VitD3 (i.p.) every other day during 15 days. PG (50 µg) was co-administered on second and on eleventh day of VitD3 treatment. After the third immunization, arthritis score was daily evaluated until euthanasia (70 days after the first immunization). Arthritis severity was determined using a standard visual scoring system based on the degree of edema and erytema ranging from 0 to 4 for each paw. The following system was used: 0 = normal; 1 = mild swelling in the paw or one joint; 2 = moderate edema and erytema in the paw and one or more joints; 3 = pronounced edema and erytema in the paw, all joints and ankle; 4 = severe edema and erytema of the entire paw and ankle and movement limitation. This classification resulted in a total score that ranged from 0 to 16 for each animal.

### Histopathological analysis

After euthanasia, mice paws were collected and fixed in 10% formalin phosphate buffer for at least seven days at room temperature. The samples were thoroughly demineralized in 10% Titriplex EDTA disodium salt (Merck Millipore, Darmstadt, Germany) for one to two months. The decalcified tissues were trimmed, dehydrated in graded ethanol and embedded in paraffin. Sections (5 µm) were cut and mounted on glass slides precoated

with 0.1% poly-L-lysine (Sigma Aldrich, St. Louis, MO, USA). Histological assessment was carried out following routine hematoxylin and eosin (HE) staining. The images were acquired by a digital camera attached to the optical microscope (Nikon, Kurobanemuko, Otawara, Japan).

#### *Cellular immune response*

Spleens were collected after euthanasia and the cells were resuspended in RPMI medium containing gentamicin and fetal calf serum.  $5,0 \times 10^6$  cells/mL were stimulated with PG (50 µg/mL). After 48 hours incubation at 37°C / 5% CO<sub>2</sub>, culture supernatants were collected for the detection of IFN-γ, IL-17, IL-6, IL-5 and IL-10. These cytokines were quantified using enzyme linked immunosorbent assay (ELISA), according to manufacturer's instructions (BD Biosciences, San Jose, CA, USA and RD Systems, Minneapolis, MN, USA).

#### *Flow cytometry analysis*

Flow cytometry analysis was performed according to manufacturer's instructions (eBiosciences, San Diego, CA, USA) after euthanasia. Briefly, for immunophenotyping of DCs, splenic cells were incubated with FITC labeled anti-mouse CD11c (clone N418), APC labeled anti-mouse MHCII (clone M5/114.15.2) and PE labeled anti-mouse CD80 (clone 16-10A1) antibodies. The cells were washed and resuspended in flow cytometry buffer containing paraformaldehyde solution to avoid dye degradation until analysis. For Tregs, splenic cells were first incubated with FITC labeled anti-mouse CD4 (clone GK1.5) and APC labeled anti-mouse CD25 (clone PC61.5) antibodies. Intracellular Foxp3 transcription factor was detected using Foxp3 PE Staining Set (eBiosciences, San Diego, CA, USA) according to manufacturer's instructions.

#### *Statistical analysis*

Results were presented as mean ± standard deviation for parametric variables and the comparison among the groups was performed by ANOVA followed by Tukey's test. For non-parametric variables, the results were presented as median and the comparison among the groups was performed by Kruskal-Wallis followed by Dunn's test. All data were analyzed using SigmaPlot software version 12.0 (Jandel Corporation, USA) and  $p \leq 0.05$  was considered significant.

## Results

### Disease incidence and score evaluation

As expected, animals from Control (-) group did not develop arthritis whereas the ones from the Control (+) group developed very typical arthritis manifestations. Disease incidence in Control (+), PG, VitD3 and VitD3+PG groups was similar. However, all treated groups showed a less severe form of arthritis characterized by a lower percentage of animals with score above eight. Reduction in arthritis severity was significant in VitD3 and VitD3+PG. Analysis of maximum score confirmed the significant reduction in arthritis severity in VitD3 and VitD3+PG treated groups. The disease onset among the three arthritic groups occurred in the same period, i.e., around day 45. These results are summarized in table 1 and figure 1.

Table 1: Development of proteoglycan-induced arthritis in female BALB/c retired breeder mice treated with PG, VitD3 and VitD3+PG.

|             | Arthritis incidence | Animals with score > 8 | Disease onset (days) |
|-------------|---------------------|------------------------|----------------------|
| Control (-) | 0/11 (0%)           | -                      | -                    |
| Control (+) | 14/15 (93%)         | 11/15 (73%)            | 45                   |
| PG          | 15/16 (94%)         | 7/16 (43%)             | 45                   |
| VitD3       | 14/15 (93%)         | 3/15 (20%)*            | 46                   |
| VitD3+PG    | 6/7 (86%)           | 1/7 (14%)*             | 44                   |

\*p ≤ 0,05 compared to Control (+)

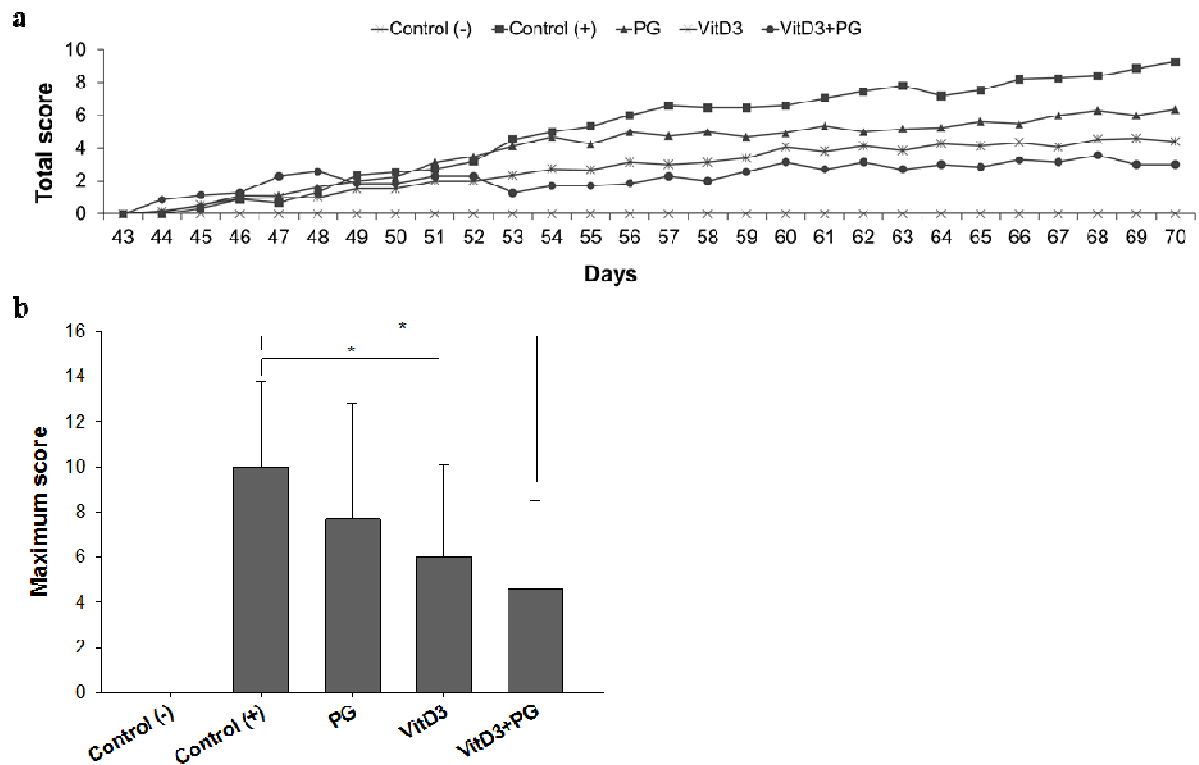


Figure1: Effect of treatment with VitD3+PG association on PG induced arthritis. Female BALB/c retired breeder mice were submitted to disease induction and early treated with VitD3+PG. (a) Kinetics of total score. (b) Maximum score per group. \* $p \leq 0.05$ .

### Histopathological analysis

The histopathological analysis was assessed by HE stained paw sections obtained after euthanasia. As anticipated, animals from the Control (-) group, who did not develop experimental arthritis, did not present any alterations in the joint structure. Figure 2b represents a score 0 hind paw of a healthy animal from the Control (-) group. The synovial space is well defined, with a very thin synovial membrane, and there are no inflammatory infiltrates. Also, cartilage and bone are clearly preserved (figure 2b'). All animals from the PG, VitD3 and VitD3+PG groups presented score paws ranging from 0 to 4. However, the frequency of these scores was distinct in each group (figure 2a). Animals from Control (+) group presented the majority of compromised paws in score 4. A representative histological section of a score 4 hind paw from the Control (+) group is shown in figure 2c. Score 4 paws from Control (+) group presented severe edema throughout the paw, involving all joints, with consequent movement impairment (not shown). Inflammation and joint destruction in this histological section were evident and were characterized by synovial membrane thickening,

*pannus* formation, cartilage destruction and bone erosion (figure 2c'). Interestingly, an evident higher frequency of paws with score 0 from the VitD3+PG group were observed. Figure 2d and 2d' represent the histological sections of a score 0 hind paw from the VitD3+PG group. The joint is well preserved and all structures are very similar to the Control (-) group (figure 2d'). Considering the four experimental groups, the comparison of the same clinical score (for example, the score 4) did not show any histopathological detail that could differentiate them.

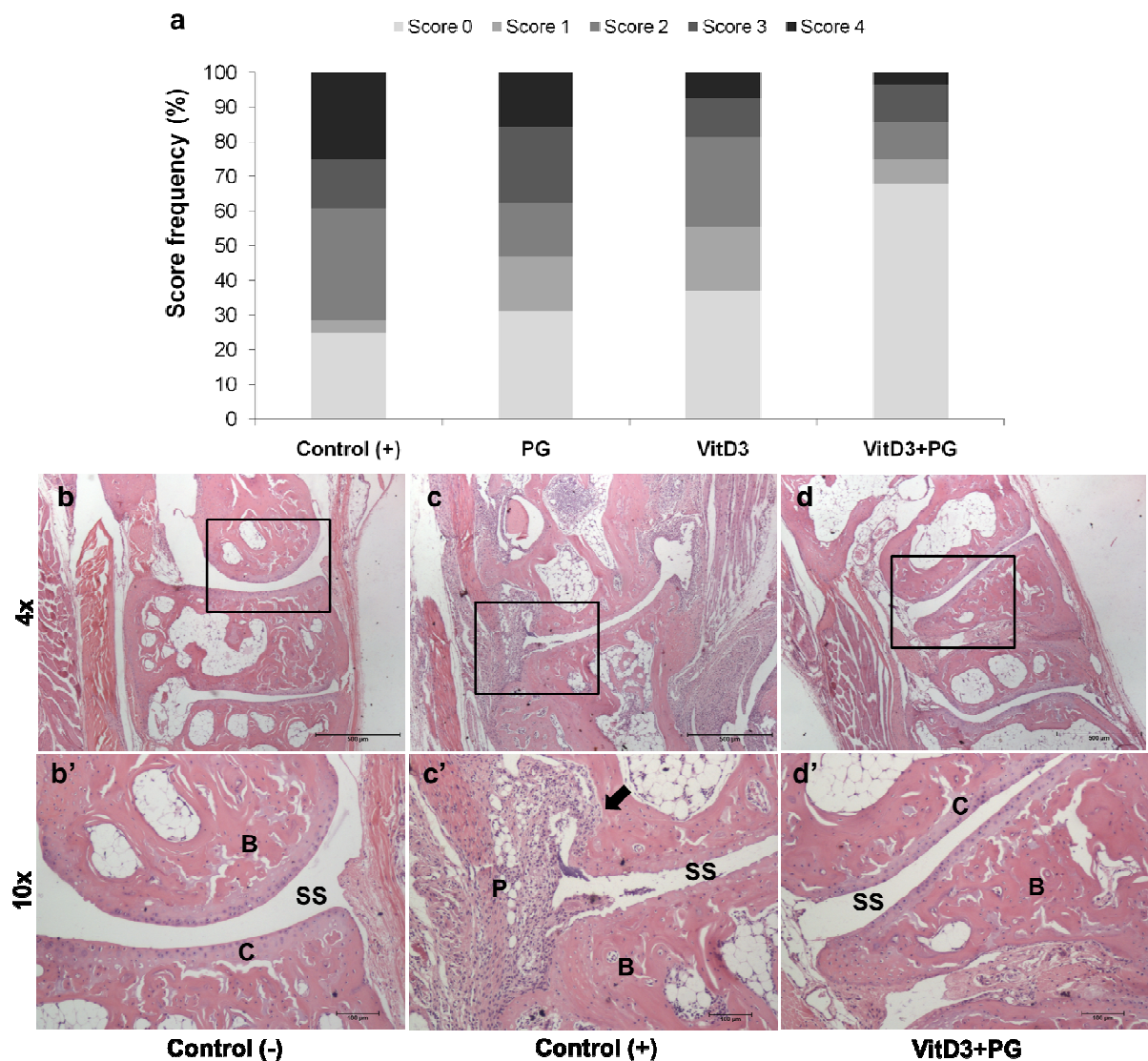


Figure 2: Score frequency and histopathological analysis of paws from mice treated with VitD3+PG association. (a) Frequency of the different scores of all mice paws per group. (b,c,d) Histopathological changes in samples obtained from Control (-), Control (+) and VitD3+PG groups. Female BALB/c retired breeder mice hind paws were collected 70 days after disease induction. Photomicrographs of histological sections was stained with HE and are shown in 4x (first line) and 10x (second line)

magnifications. The rectangles are the highlighted regions in the largest magnification and the arrows indicate the synovial membrane hypertrophy. SS: synovial space; C: cartilage; B: bone; P: *pannus*.

### *Cytokine production*

There was a spontaneous IFN- $\gamma$  production in all arthritic groups. In the Control (+) and PG groups, this cytokine levels were significantly increased compared to the Control (-) group. However, the VitD3+PG group produced lower levels of IFN- $\gamma$  compared to the Control (+) group. Specific antigen (PG) stimulation determined a significant increase in IFN- $\gamma$  production in Control (+) and PG groups compared to the Control (-), but only the VitD3+PG association showed significantly decreased levels compared to Control (+). There was also a discrete spontaneous production of IL-17 in the Control (+), PG, and VitD3 groups. These three groups also produced this cytokine in response to stimulation with PG, but only the Control (+) and VitD3 groups produced significantly increased levels compared to Control (-). All arthritic groups also produced IL-6 spontaneously. However, the production was variable and only the Control (+) and PG groups showed significant levels compared to the Control (-) group. The VitD3+PG group produced significantly lower levels of this cytokine compared to Control (+). The same production profile was observed after stimulation with PG. The results concerning the production of IFN- $\gamma$ , IL-17 and IL-6 are illustrated in figure 3. Spontaneous production of IL-5 and IL-10 was also observed, but only the Control (+) and PG groups showed a significant increase compared to the Control (-) group. The production of these cytokines in cultures stimulated with PG was similar, i.e., the levels were higher in the Control (+), PG and VitD3 groups. We also observed a significant reduction of IL-5 and IL-10 in comparison to the Control (+) and VitD3+PG groups. The production of these cytokines is shown in figure 4.

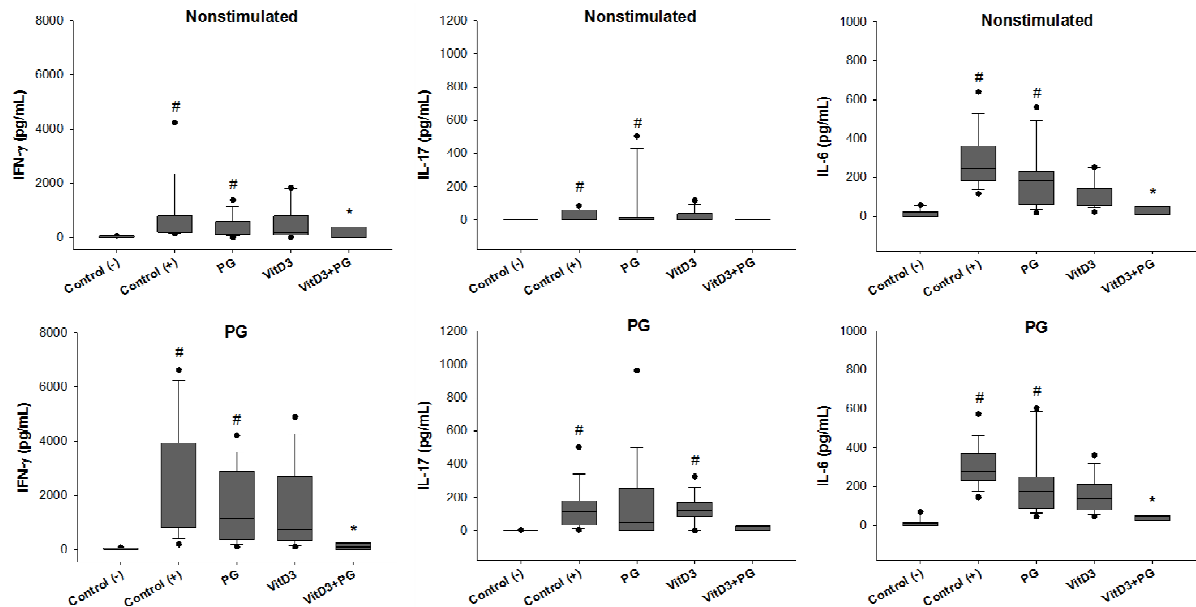


Figure 3: IFN- $\gamma$ , IL-17 and IL-6 production by arthritic BALB/c retired breeder mice: effect of early treatment with PG, VitD3 and PG+VitD3. Spleen cells were *in vitro* stimulated with PG and incubated for 48 hours. Cytokine levels were detected by ELISA. Control (-): not treated healthy group. Control (+): not treated arthritic group. \* $p \leq 0.05$  compared to the Control (+) group and # $p \leq 0.05$  compared to the Control (-) group.

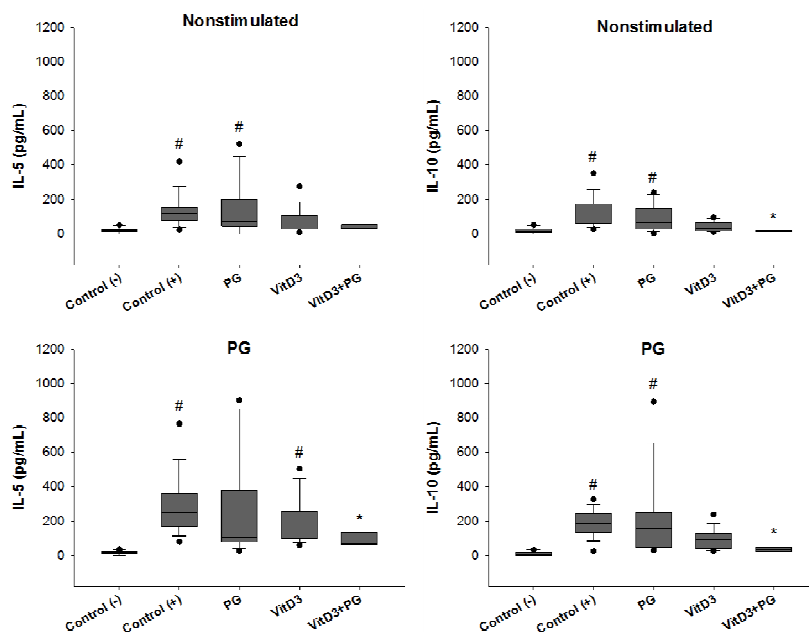


Figure 4: IL-5 and IL-10 production by arthritic BALB/c retired breeder mice: effect of early treatment with PG, VitD3 and PG+VitD3. Spleen cells were *in vitro* stimulated with PG and incubated for 48 hours. Cytokine levels were detected by ELISA. Control (-): not treated healthy group. Control

(+): not treated arthritic group. \* $p \leq 0.05$  compared to the Control (+) group and # $p \leq 0.05$  compared to the Control (-) group.

### Flow cytometry analysis

Early treatment with VitD3 associated or not with the specific antigen increased DCs frequency in mice spleen compared to Control (-) group. However, only in the VitD3+PG group we observed a significantly higher frequency of mature DCs in comparison to Control (+) group. Moreover, VitD3 treatment did not increase Tregs frequency. Interestingly, the frequency of these cells was significantly lower in the VitD3 group compared to Control (+) group.

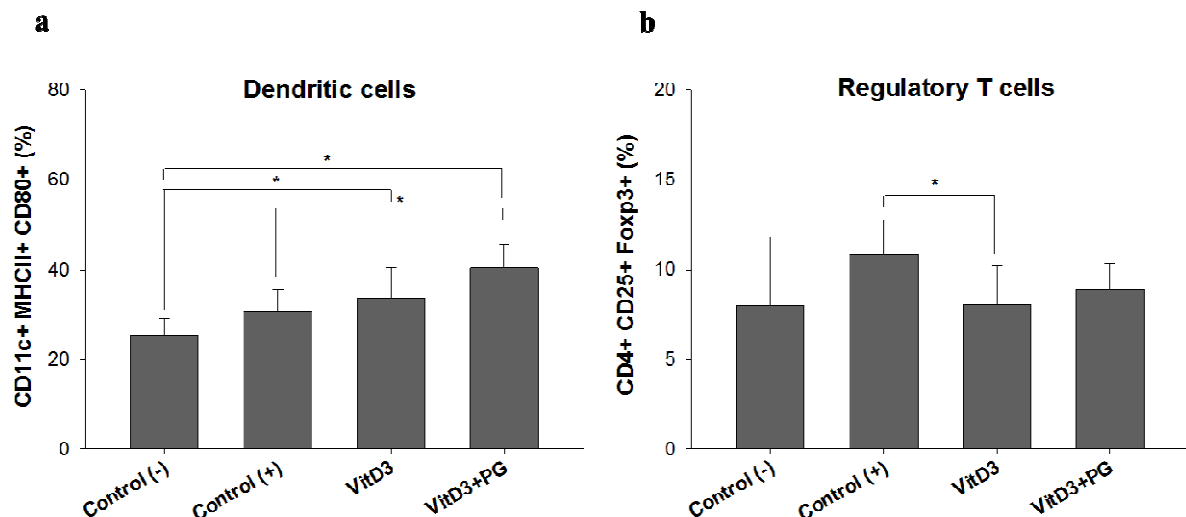


Figure 5: Splenic dendritic and regulatory T cells from BALB/c retired breeder mice: effect of early treatment with VitD3 and VitD3+PG. Immunophenotyping was performed by flow cytometry. Control (-): not treated healthy group. Control (+): not treated arthritic group. Frequency of MHCII+ CD80+ cells in total CD11+ cells (a). Frequency of CD25+ Foxp3+ cells in total CD4+ cells (b). \* $p \leq 0.05$  compared to Control (-) group.

### Discussion

It is now well established that active vitamin D has numerous activities other than the ones involved in calcium homeostasis and bone metabolism. In addition to the effects over the innate immunity that booster the defense against several infectious agents it also modulates the specific immunity at various levels. Briefly, it is inhibitory for Th1 and Th17 subsets whereas it is generally stimulatory for Th2 and regulatory T cells (Yang et al., 2013). A large

body of evidence suggests that VitD3 may be beneficial in autoimmune and allergic pathologies (Szodoray et al., 2008; Muehleisen & Gallo, 2013). In this context, the main objective of this study was to evaluate whether the association of VitD3 and a specific cartilage antigen has a therapeutic effect in experimental arthritis. To evaluate this possibility, we induced arthritis in BALB/c retired breeder mice by immunization with bovine PG and the DDA adjuvant. One day after the first immunization, the animals were treated with VitD3 every other day during 15 days and PG was co-administered on the second and on the eleventh day of VitD3 treatment. This combined treatment was clearly effective as it significantly decreased the severity of the disease. The histopathological analysis confirmed the efficiency of this therapeutic association, showing that a large percentage of joint paws remained preserved, with no structural alterations as inflammation, *pannus* and cartilage destruction.

Some aspects of this therapeutic effect will be highlighted due to a possible future translation of these experimental findings to patients. First of all, this treatment began during an early phase of the experimental disease. Nowadays RA therapy is postponed until the patient shows clinical symptoms (Colmegna et al., 2012). However, we could envision that future therapies for these pathologies will be adopted during the pre-clinical phase because some pre-clinical markers such as antibodies against citrullinated proteins are already available (Uysal et al., 2010; Song & Kang, 2010). In addition, we also observed (results not shown) that VitD3 administration in larger intervals, i.e., every 48 hours, lost the efficacy. As active vitamin D has a half-life of 15 hours (Jones, 2008), we interpreted this finding as an indicative that the protective effect depends upon certain critical levels.

For the best of our knowledge, this is the first investigation to be done with this type of combination of antigen and VitD3. The employment of an arthritis model induced by PG is also original. These facts make it difficult to directly compare this data with the literature. However, earlier data already demonstrated that dietary supplementation with 1,25-dihydroxy cholecalciferol given to mice with early symptoms of collagen induced arthritis prevented the progression to severe disease (Cantorna et al., 1998). Some indirect data from humans also suggest that this vitamin can be useful in arthritis. For example, in the study performed by Haga et al., 2013, deficiency of VitD3 was detected in 33,4% of the RA patients. Interestingly, these authors also found that a subset of patients with the higher disease activity and requiring treatment with at least three disease-modifying anti-rheumatic drugs (DMARDs) were, coincidentally, the ones with the most accentuated vitamin D deficiency.

Direct data that could convincingly establish the therapeutic effect of VitD3 on RA is still missing.

To disclose the mechanism by which the association of VitD3 and PG was being therapeutical in this disease, we evaluated the main molecular and cellular targets of VitD3, that are cytokines, dendritic cells and regulatory T cells. The effect of VitD3+PG treatment on pro-inflammatory cytokine production by spleen cells was impressive. Both levels, spontaneous and PG induced, of IL-6, IFN- $\gamma$  and IL-17 were clearly downmodulated in animals treated with this association. Analysis of cytokine production by the experimental groups treated with PG or VitD3 alone, showed that VitD3 by itself was already decreasing the production of these cytokines. As this experimental group also presented a significant reduction in arthritis severity, we believe that VitD3 plays a relevant role in the therapeutic effect observed in the VitD3+PG group. However, there is also an important contribution of PG. It is important to highlight that PG alone did not usually modified cytokine production, however, pro-inflammatory cytokine production was significantly decreased in animals treated with the VitD3+PG combination. This finding suggests that VitD3 is acting as a tolerogenic adjuvant. The concept of tolerogenic adjuvants is based on the ability of certain substances, mainly immunosuppressants and immunoregulators, to down-regulate unwanted immune responses and induce immune tolerance (Kang et al., 2008). In this scenario, we could think that PG, in the presence of VitD3, is inducing a state of tolerance towards itself. This tolerance condition would result in a decreased production of cytokines detected by an *in vitro* recall response. The fact that cytokine production induced by ConA was similar in all experimental groups (not shown), strengthens the interpretation of a specific tolerance induction. The efficacy of this concept was already demonstrated in other autoimmune experimental diseases. Kang et al., 2009, for example, demonstrated that immunization of mice with a myelin oligodendrocyte glycoprotein-derived peptide associated with FK506 (tacrolimus), that is a widely used immunosuppressant, induced antigen-specific tolerance and prevented the development of experimental autoimmune encephalomyelitis (EAE). In another EAE model, it was described that IFN- $\beta$  can also behave as a tolerogenic adjuvant for induction of neuroantigen-associated tolerance (Mannie et al., 2009). Even though the therapeutic effect of VitD3 is well documented in experimental arthritis (Moghaddami et al., 2012; Bansal et al., 2012), our results constitute the first direct experimental *in vivo* assessment of its tolerogenic potential as an adjuvant. On the other hand, VitD3 is widely

characterized as able to modify the *in vitro* differentiation of DCs into a phenotype that resembles semi-mature DCs, with reduced T cell co-stimulatory molecules and decreased IL-12 production. These VitD3 modified DCs are able to induce an *in vitro* state of tolerance that is associated with T cell anergy, decreased Th1 polarization and recruiting and differentiating regulatory T cells (Pedersen et al., 2011). This *in vitro* procedure is being considered one of the most promising approaches to the treatment of autoimmune pathologies. To evaluate if VitD3 or VitD3+PG association were affecting the differentiation of DCs in this *in vivo* model, we characterized the phenotype of DCs present in the spleen in the end of the experiment (day 70), when the therapeutic effect was still very clear. Unexpectedly, the group treated with VitD3+PG presented a significantly higher expression of mature DCs, characterized by a higher expression of MHCII and CD80 than the Control (+) group. According to the literature (Larsen et al., 1994; Inaba et al., 2000), the presence of these molecules means that these are mature cells able to induce a Th1 polarized immune response. A more encompassing analysis of these cells will be needed to understand their possible role in the efficacy of arthritis control by VitD3+PG. In this sense, it would be relevant to evaluate their relative production of IL-12 and IL-10 that have been correlated with its immunopotentiating and immunosuppressing ability, respectively. Ferreira et al., 2011, demonstrated that *in vitro* treatment with 1,25(OH)<sub>2</sub>D3 alters the behavior of murine bone marrow-derived DCs leading to a tolerogenic profile characterized by increased production of IL-10 and TGF- $\beta$  and reduction of T-cell responsiveness (lower IL-12 production). Other aspects that deserve attention are the time points and microenvironments to look for DCs. Considering that VitD3 and PG were both injected inside the peritoneal cavity and that the peritoneal space drains, in rodents, to celiac superior mesenteric and periportal lymph node groups (Parungo et al., 2007), it is conceivable that VitD3+PG effect would be more adequately evaluated in these lymphoid organs.

Otherwise, these findings could be also analyzed from a different perspective. A few publications have indicated that mature DCs are required for tolerance induction. According to Albert et al., 2001, the critical checkpoint in tolerance establishment is not DCs maturation but instead, the presence of an additional signal which takes place at the CD-CD4<sup>+</sup> T cell interface. The contribution of mature DCs to protection against autoimmunity was clearly demonstrated by Menges et al., 2002. These authors described that injection of DCs matured with TNF- $\alpha$  were able to protect mice from EAE development. Interestingly, these TNF- $\alpha$

matured DCs expressed high levels of MHC class II and co-stimulatory molecules but released very low concentrations of pro-inflammatory cytokines.

Currently, the most commonly known regulatory T cell subset is identified as being CD4<sup>+</sup>CD25<sup>high</sup>Foxp3<sup>+</sup>. As vitamin D availability is being positively correlated with these regulatory T cells (Jeffery et al., 2012) and with reduced severity of autoimmune experimental diseases (Farias et al., 2013), we expected a higher proportion of these cells in mice treated with VitD3 or with VitD3+PG. This expectation was also not fulfilled. Our most immediate explanation for this finding is that these cells migrated to the lesion sites to interrupt an initial inflammatory process as has been characterized in a series of functional studies (Huehn & Hamann, 2005; Bin Dhuban et al., 2014). It is important to also highlight that these cells could be found in lymph nodes. Current studies suggest that Tregs migrate in a synchronized form between peripheral organs and draining lymph nodes to be endowed with more efficient immunoregulatory abilities (Ding & Bromberg, 2012).

Our study indicates that the association between VitD3 and the specific antigen, PG in this case, was highly effective to control arthritis development. The results also suggest that VitD3 functioned as a tolerogenic adjuvant significantly decreasing cytokine production. Further experiments will be necessary to determinate if Tregs are present at the arthritic lesions. In the context of RA treatment, which is mainly based on combined therapies, our expectation is to further test the effectiveness of VitD3 with drugs that are already in use, as methotrexate. Although the PG have increased the therapeutic effect of VitD3, it is difficult to translate this finding to patients' treatment since the etiology of RA remains still extremely complex (Imboden, 2009). Regarding this, many other self-antigens such as IgG, collagen, citrulline, calpastatin, calreticulin, fibrin and fibrinogen are possibly involved in the autoimmune process (Bläss et al., 2001). In this scenario, we hope that the combination of any joint specific antigen with VitD3 could determine a state of tolerance that would protect the joint.

#### *Acknowledgments*

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## *Capítulo 5*

### *Conclusões*

## CONCLUSÕES

- O modelo experimental de artrite reumatoide estabelecido durante o desenvolvimento deste projeto possui características clínicas, histopatológicas e imunológicas que se assemelham muito com a doença humana. O fato do mesmo ser induzido por proteoglicano de origem bovina é inovador dentro desta área de pesquisa, pois diferentemente do proteoglicano de origem humana, que é normalmente utilizado, o proteoglicano bovino está disponível comercialmente podendo ser, portanto, facilmente adquirido.
- A administração sistêmica de proteoglicano, que é um antígeno específico da cartilagem, desencadeou uma tolerância antígeno-específica no modelo experimental de artrite induzida por proteoglicano. O efeito profilático se caracterizou principalmente pela diminuição da incidência e do escore clínico da doença. O provável mecanismo imunológico envolvido na proteção é a diminuição das citocinas pró-inflamatórias (IFN- $\gamma$  e IL-17) e o aumento das citocinas anti-inflamatórias (IL-5 e IL-10) detectadas no baço dos animais que receberam o antígeno antes da indução da artrite.
- O tratamento com a vitamina D ativa associada ao proteoglicano determinou um efeito terapêutico na artrite autoimune experimental caracterizado principalmente por diminuição da gravidade da doença. Embora o possível mecanismo imunológico envolvido ainda permaneça desconhecido, nosso estudo demonstrou que a vitamina D pode ser utilizada como adjuvante tolerogênico, ou seja, para desencadear tolerância específica a um antígeno com o qual esteja associada.