

T cell reactivity to Collagen II as a possible prognostic marker in patients with rheumatoid arthritis

Nida Saleem,¹ Tahir Aziz Ahmed,² Mukarram Bashir,³ Muhammad Omair Riaz,⁴ Ali Hamid⁵

Abstract

Objective: To compare the frequency and the functional state of the collagen II reactive T cells with disease activity in rheumatoid arthritis patients and healthy controls.

Methods: This case-control cross-sectional study was carried out at the Department of Immunology; Armed Forces Institute of Pathology, Rawalpindi, Pakistan, from June to October 2014. Rheumatologist from Rehmat Noor Rheumatology Clinic, a private health facility of the city, was requested to send in patients with clinical diagnosis of rheumatoid arthritis. Samples were obtained and relevant investigations were carried out. Data were compared with a group of age and gender-matched healthy subjects. T cell proliferative response was assessed against bovine collagen II by measuring incorporation of bromodeoxyuridine into deoxyribonucleic acid of proliferating cells and by expression of CD25 on proliferating cells as percentage of CD3+/bromodeoxyuridine+ and CD3+/CD25+ T-cells, respectively.

Among the patients, the frequency of T cells with disease activity was compared. Patients were classified into groups of mild, moderate and severe disease and frequency of CD3+/bromodeoxyuridine+, frequency of CD3+/CD25+ cells, mean fluorescent intensity of bromodeoxyuridine-fluorescein isothiocyanate and mean fluorescent intensity of CD25-fluorescein isothiocyanate were compared in the groups.

Results: Of the 60 subjects, 30(50%) were patients and 30(50%) were controls. Of the patients, 5(16.66%) were males and 25(83.33%) were females with an overall mean age of 42±12 years. The mean age of the controls was 41±9.28 years. Mean disease duration of the patients was 10.5 ± 4.2 years.

Percentage of CD3+/CD25+ cells and CD3+/bromodeoxyuridine+ cells stimulated with collagen II, in patients was much higher than the controls ($p < 0.05$). Statistically significant differences were observed when frequency of CD3+/bromodeoxyuridine+ cells and CD3+/CD25+ cells was compared among the mild, moderate and severe patient groups ($p < 0.05$).

Conclusion: Collagen II was found to be an important auto antigen in joints of rheumatoid arthritis patients.

Keywords: Collagen II, BrdU, Collagen reactive T cells. (JPMA 68: 1222; 2018)

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that primarily affects synovial joints with cartilage destruction and subsequent bone erosion. RA is the most common cause of adult inflammatory arthritis and is associated with considerable disability and early mortality.¹ Although RA most often affects the joints, it is a disease of the entire body. It can affect many organs and body systems and can also affect lungs, heart, and kidneys.

It is most likely to strike people from 35 to 50 years of age, but it can occur in children, teenagers, and elderly people as well. On average, life expectancy is somewhat shorter for people with RA than for the general population. Like many autoimmune diseases, RA typically waxes and wanes.

RA is generally considered to be a T cell mediated autoimmune disease.² Facts that RA can develop in the absence of rheumatoid factor and that CD4 T cell derived cytokines form a major part of effect or mechanisms of RA suggest a role of CD4 T cells in pathogenesis of RA.³

Although the etiopathogenesis of RA remains unclear, there is convincing evidence that autoantigens presented by disease-associated human leukocyte antigen (HLA) initiate autoimmune response by activating auto-reactive T cells.

A number of autoantigens in RA have been identified such as collagen II (CII), human cartilage glycoprotein (HCgp-39) and binding immunoglobulin protein (BiP).³ Among these CII, a major component of hyaline cartilage, is an attractive candidate as a target antigen responsible for pathogenicity of RA.³⁻⁵

A study conducted in China found that the percentages of CII reactive T-cells (55%; range: 48-60%) in RA patients

.....
¹⁻⁴Armed Forces Institute of Pathology (AFIP), Rawalpindi, ⁵Military Hospital (MH), Rawalpindi.

Correspondence: Nida Saleem. Email: nidasaleem84@gmail.com

were much higher than those in the healthy controls (35%; range: 30-38%) ($p < 0.01$).⁶ Literature review has highlighted the fact that antigen-specific T cells play an important role in pathogenesis of RA.

No single laboratory marker independently reflects disease activity in patients with RA. Many disease activity indices for RA like, Disease Activity Score 28 (DAS 28), Simplified Disease Activity Index (SDAI), Clinical Disease Activity Index (CDAI) and Health Assessment Questionnaire (HAQ) consider both clinical indices and laboratory biomarkers. The laboratory marker commonly used in these indices for measuring disease activity are RA factor, anti-cyclic citrullinated peptide (CCP) antibodies, Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), antibodies against interleukin-1 α (IL1 α) etc. Identifying a single laboratory parameter that independently and closely reflects disease activity in RA will allow physicians to initiate aggressive treatment early in disease process, thus minimising joint damage. No previous study, to the best of our knowledge, has so far determined the relationship of CII specific T cells with disease activity as a potential laboratory marker to assess the prognosis in patients with RA. The current study, therefore, was planned to see the response of T cells to CII in RA patients.

Patients and Methods

This case-control cross-sectional study was carried out at the Department of Immunology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan, from June to October 2014 after approval from the institutional review board. Rheumatologist from Rehmat Noor Rheumatology Clinic, a private health facility in the city, was requested to send in patients with clinical diagnosis of RA to AFIP where samples were obtained after informed consent, and relevant investigations were carried out.

Patients who fulfilled 1987 American College of Rheumatology criteria⁸ with disease duration of not more than 5 years and not taking any immunosuppressive therapy were included in the study. Age and gender-matched healthy donors of kidney and bone marrow transplant, who came to the immunology department for HLA-Typing, were selected as controls after taking informed written consent.

Further, 6-7ml venous blood was collected in heparinised tubes and diluted 1:1 by Roswell Park Memorial Institute (RPMI-1640) (Gibco-BRL, Grand Island, NY). Mononuclear cells were isolated by density-gradient centrifugation on Ficoll-Hypaque (Specific gravity 1.077g/ml) (Pharmacia LKB, Piscataway, PA), inside safety cabinet. Peripheral blood mononuclear cells (PBMCs) were isolated and

washed twice with RPMI-1640 and cell viability was >95% by Trypan blue exclusion assay. PBMCs were re-suspended in culture medium and cell count was adjusted to $1-2 \times 10^6$ /ml. Culture medium used consisted of RPMI-1640 supplemented with 5% foetal calf serum, penicillin (100U/ml), streptomycin (100 Ag/ml) and gentamicin (50 Ag/ml) (all from GibcoBRL Life Technologies, Rockville, MD, USA).

Lyophilised bovine CII Sigma-Aldrich (Saint Louis, MO, USA) was dissolved in 0.05N acetic acid at pH 3.0 (2 mg/ml) and sterilised by passing through a 0.2- μ m micropore filter paper. This CII was finally used as a cell culture substratum at 6-10 μ g/cm².

Cells were incubated with CII by adding 0.5ml of cell suspension to a CII coated sterilised 15ml falcon tubes. Two separate falcon tubes were labelled as positive and negative controls and 0.5ml of cell suspension was added to each tube. Next, 0.5ml of phytohemagglutinin (PHA) (at a final concentration of 5 μ g/ml) (GibcoBRL Life Technologies, Rockville, MD, USA) and 0.5ml of RPMI were added to the positive and negative control tubes, respectively. Tubes were then incubated for 72 hours in 5% carbon dioxide (CO₂) incubator at 37°C. In the last 6 hours of culture, stimulated cells were exposed to bromodeoxyuridine (BrdU) (BD Pharmingen™ - FITC BrdU Flow Kit).

Cells were harvested using 10mM ethylenediaminetetraacetic acid (EDTA) solution and shifted to two falcon polypropylene round-bottom tubes (BD Biosciences Immunocytometry Systems, San Jose, CA). In the first tube, cells were labelled using phycoerythrin (PE) conjugated monoclonal antibody (MoAb) for surface markers CD3 (BD Biosciences Immunocytometry Systems, San Jose, CA) and fluorescein isothiocyanate (FITC) conjugated antibody for CD25 (BD Biosciences Immunocytometry Systems, San Jose, CA). In the second tube, cells were labelled for surface marker CD3 with PE conjugated MoAb followed by deoxyribonucleic acid (DNA) digestion using deoxyribonuclease (DNase). Subsequently, cells were incubated with FITC labelled anti-BrdUMoAb (BD Pharmingen FITC BrdU Flow kit) for 45minutes. Finally cells in both falcon tubes were washed, re-suspended in PBS and analysed by two-colour flow cytometer.

Sample size was calculated using PS: Power and Sample Size Calculation version 3.0,⁷ 5% level of significance, power of test 90%, pooled SD 2.5, test value of population mean 35% and anticipated population mean 55%.⁶ The data was compiled and analysed using SPSS 20. Mean and standard deviation were calculated for numerical data like

age and T cell proliferative response in both groups. Independent sample t-test was used to compare the mean T cell responses in groups among the test (CII), positive control (PHA) and negative control (RPMI without any stimulant) groups. Results were considered significant at $p < 0.05$ level. Patient group was classified into subgroups of disease activity as per Simplified Disease Activity Index (SDAI)⁹ and frequency of CII specific T cells was compared among these groups using one-way analysis of variance (ANOVA).

Results

Of the 60 subjects, 30(50%) were RA patients and 30(50%) were healthy controls. Both groups had 5(16.66%) males and 25(83.33%) females. Mean age of the patients was 42 ± 12 years while that of the controls was 41 ± 9.28 years. Mean disease duration of the patients was 10.5 ± 4.2 years (range: 5.5-14 years).

Percentages of CD25+ cells and BrdU+ cells in CD3+ lymphocytes in PBMC from RA patients induced by CII were much higher ($45.22 \pm 7.9\%$ and $53.45 \pm 7.5\%$) than those in the control group ($33.51 \pm 6.8\%$ and $45.22 \pm 4.6\%$) ($p < 0.05$) (Figures-1-2).

The mild moderate and severe disease activity groups included 9(30%), 13(43%) and 8(27%) patients

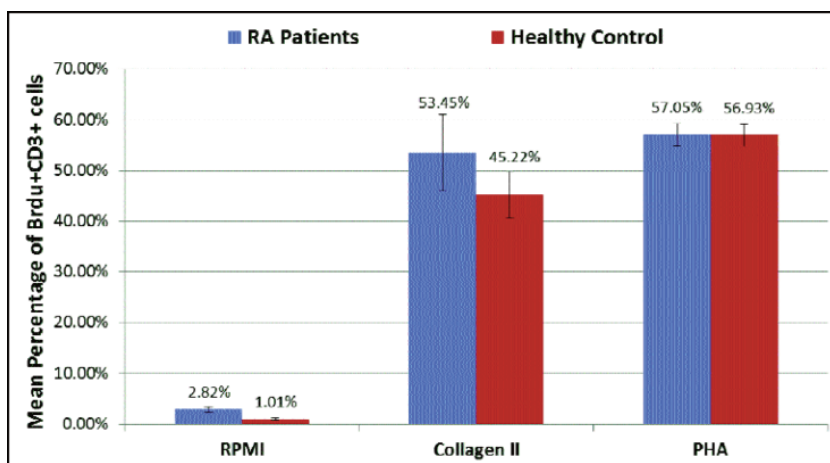


Figure-1: Difference between mean percentages of BrdU+CD3+ cells stimulated against Collagen II, PHA (+ve control) and RPMI (-ve control).

respectively.

Statistically significant differences were observed in percentages of BrdU+CD3+ cells and MFI of BrdU-FITC in comparison of mild to severe and moderate to severe groups, indicating a direct relationship of these parameters with disease activity (Table-1).

Similarly, statistically significant differences were observed between percentages of CD25+CD3+ cells and MFI of CD25-FITC when compared between the three patient groups ($p < 0.05$, Table-2).

Among females, mean proliferative response (BrdU

Table-1: Comparison of Collagen II stimulated BrdU + cells and Mean Fluorescent Intensity (MFI) of BrdU-FITC in mild, moderate and severe disease activity groups (statistical analysis by one-way-ANOVA).

Proliferation Parameters	Mild n=9	Moderate n=13	Severe n=8	p-value			
				Mild vs. Moderate	Mild vs. Severe	Moderate vs. Severe	ANOVA-Test
% BrdU Incorporation	44±3.6	53±4.8	62±3.2	0.034	0.001	0.002	<0.001
BrdU-FITC MFI	325±32	411±52	534±42	0.027	<0.001	0.002	<0.001

BrdU: Bromodeoxyuridine

BrdU-FITC: Bromodeoxyuridine- fluorescein isothiocyanate

MFI: Mean fluorescent intensity

ANOVA: Analysis of variance.

Table-2: Comparison of Collagen II stimulated CD25 + cells and Mean Fluorescent Intensity (MFI) of CD25 in mild, moderate and severe disease activity groups (statistical analysis by one-way-ANOVA).

Proliferation Parameters	Mild n=9	Moderate n=13	Severe n=8	p-value			
				Mild vs. Moderate	Mild vs. Severe	Moderate vs. Severe	ANOVA-Test
% CD25 Expression	28±5.6	44±6.3	49±5.6	0.006	0.0001	0.005	<0.001
CD25-FITC MFI unit	222±13	302±66	412±38	0.001	0.0001	0.001	<0.001

MFI: Mean fluorescent intensity

ANOVA: Analysis of variance.

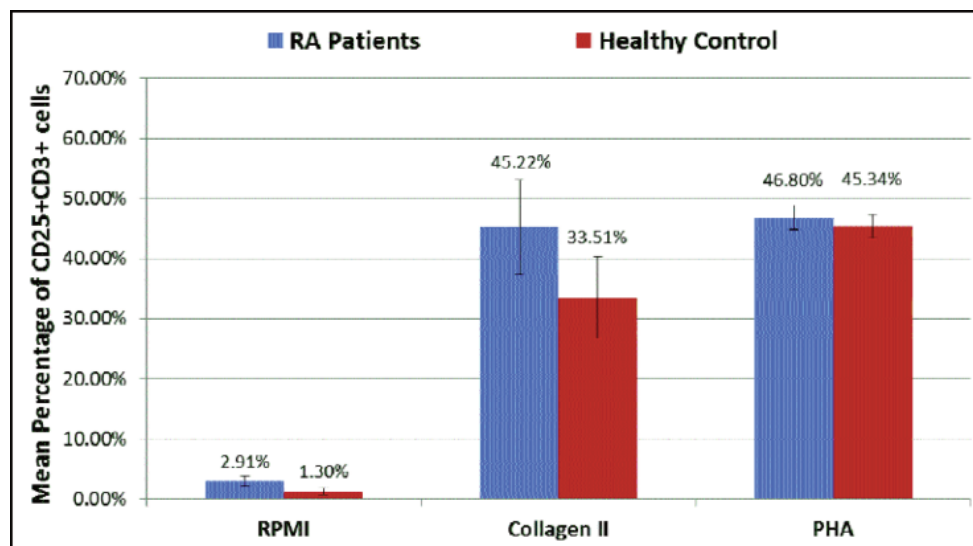


Figure-2: Difference between mean percentages of CD25+CD3+ cells stimulated against Collagen II, PHA (+ve control) and RPMI (-ve control).

+CD3+ Cells) against CII was 52.55% whereas in males, proliferative response was 54.33% ($p > 0.05$).

Discussion

The hypothesis that RA is a T cell mediated autoimmune disease has been widely supported by data in a number of investigations.¹⁰ One obstacle to confirming the central role of T cells has been the identification of auto antigens specific to the joint, where immune mediated inflammation and injury is most intense. CII is a strong candidate autoantigen relevant to RA, because of its abundance in cartilage and because of its ability to trigger immune mediated destructive polyarthritis in rodents and primates.¹¹ There have been few studies on T cell immunity to CII in RA^{12,13} but the findings are variable. Researchers reported that rheumatoid PBMC cultured with CII produced increase amount of chemotactic factor in the majority of RA patients.¹⁴⁻¹⁶ Similar findings were recorded by other investigators using a variety of assay systems.¹⁷⁻¹⁹ In contrast, another researcher reported that increased T cell responses to CII were present in only a minority of patients with RA.²⁰ This difference may reflect variability in sensitivity or specificity of the assay system used.

It is also well documented that autoantibodies to CII play an important role in development of collagen-induced arthritis (CIA).²¹

Lymphocyte proliferation assays in vitro have remained important tools for measuring cellular immune status in immune compromised patients.²² Antigen specific T cell

responsiveness is commonly assayed in vitro by measuring the proliferative response of primed T lymphocyte to an antigen challenge. Traditional measurement of DNA synthesis as a parameter of lymphocyte activation and proliferation has a significant disadvantage, in that it relies on bulk culture nucleic acid incorporation (e.g. 3H-thymidine).²³ Therefore, it is difficult to accurately identify the relative frequency of different cellular phenotypes responding to a specific antigen. In our study we reported a simultaneous multiparametric (mp)

analysis of cell proliferation, measuring DNA synthesis by BrdU (a thymidine analogue) incorporation and expression of CD25 antigen as T cell activation marker.²⁴ Additional benefits of the technique are to identify various cells in one assay, including T cells and their subsets, B cells, natural killer (NK) cells and monocytes/macrophages.^{25,26} It is also possible to study cytokine profile in response to a specific antigen-induced T cell proliferation to analyse Thelper1 (Th1) and Thelper2 (Th2) (interferon [IFN]- γ /interleukin 4 [IL-4]) disequilibrium and to gain more insight into disease pathology.²⁷

A salient feature of this method is that it allows for immunofluorescent BrdU staining concurrently with the use of antibodies specific for cell surface molecules, including surface antigen (e.g., CD69, CD25) or intracellular proteins (e.g. cytokines). BrdU flow (BD Biosciences, San Diego) staining protocol avoids DNA denaturing agents, such as acid, ethanol that can alter and limit the recognition of cellular antigens by fluorescent antibodies.²⁸

By this method we found that CII in vitro could stimulate stronger T cell responses in terms of DNA synthesis (BrdU incorporation) and expression of activation marker (CD25) in RA patients compared to those in healthy controls.

Our results highlight a discrepancy in percentages of CD25+ CD3 cells and percentages of BrdU+ CD3 cells in individual patient sample stimulated against Collagen. A

likely explanation is that CD25 antigen is a late activation marker and is expressed by T cells somewhat later in response to these stimuli.²⁹

Another interesting observation was significantly increased baseline stimulation (against RPMI) in RA patients when compared with that of healthy controls. Possible explanation is presence of previously activated disease specific auto reactive T cells in patient samples ($p < 0.0001$ when BrdU incorporation and CD25 expression was compared against negative control between patient and control group).

PHA is a polyclonal stimulator of T cells and triggers T lymphocyte cell division following T-cell receptor (TCR)-CD3 mediated activation of tyrosine phosphatases.³⁰ Percentages of BrdU+CD3+PHA stimulated cells are not significantly different in patient group and control group, following subtraction of BrdU+CD3+cells in unstimulated population ($p = 0.823$).

Although CII stimulated significantly increased number of T cell in patient group ($p < 0.05$). However, T cell reactivity against CII was also observed in healthy individuals. This highlights the fact that deletion of T cells by thymic selection is not 100% and that autoreactive T cells escape the selection process. In peripheral blood, reactivity and expansion of these autoreactive T cells is suppressed by CD4+CD25+ Tregs (Regulatory T-cells).³¹

In our study frequency of collagen specific T cells was found to be directly related with RA disease activity groups segregated on laboratory criteria, as their values increased from mild to moderate to severe disease activity groups. Since the collagen specific auto reactive T cells are responsible for joint inflammation and erosion in RA. It is therefore suggested that percentage of BrdU+ cells stimulated against collagen, in addition to clinical and laboratory criteria, may be more beneficial for assessment of disease activity or monitoring of immunotherapy in RA.

Our observations together with previous findings provide strong evidence that CII is likely to be useful as an inducing antigen in treatment strategies for immunotherapy of RA, with benefits possibly being derived from mechanisms of antigen-driven active suppression of auto reactive T cell responses to CII.

Study of CII reactive T lymphocytes is likely to yield specific targets in the form of T cell associated products, receptors and their ligands. The assessment of CII reactive T lymphocytes may be used as a specific prognostic laboratory marker for

assessment of specific immunotherapies designed for treatment of patients with RA.

Conclusion

CII was found to be an important autoantigen in joints of RA patients with CII reactive T cells playing a critical role in pathogenesis of RA. Flow cytometry can be used to detect antigen specific T cell responses at a single cell level by simultaneously analysing cell surface markers and BrdU incorporation (DNA synthesis). By combining all these parameters, this method can be used to study immune responses at molecular and cellular level and to further study the immunopathology and immunotherapy of many diseases.

Disclaimer: None.

Conflict of Interest: None.

Source of Funding: Immunology Laboratory, AFIP, funded the project.

References

1. Remmers EF, Plenge RM, Lee AT, Graham RR, Hom G, Behrens TW, et al. STAT4 and the Risk of Rheumatoid Arthritis and Systemic Lupus Erythematosus. *N Engl J Med* 2007; 357: 977-86.
2. Ohnishi Y, Tsutsumi A, Matsumoto I, Goto D, Ito S, Kuwana M, et al. Altered peptide ligands control type II collagen-reactive T cells from rheumatoid arthritis patients. *Mod Rheumatol* 2006; 16: 226-8.
3. Cope AP. T cells in rheumatoid arthritis, *Arthritis Res Ther*. 2008; 10: S1.
4. Latham KA, Whittington KB, Zhou R, Qian Z, Rosloniec EF. Ex Vivo Characterisation of the Autoimmune T Cell Response in the HLA-DR1 Mouse Model of Collagen-Induced Arthritis Reveals Long-Term Activation of Type II Collagen-Specific Cells and Their Presence in Arthritic Joints. *J Immunol* 2005; 174: 3978-85.
5. Choi JJ, Yoo SA, Park SJ, Kang YJ. Mesenchymal stem cells overexpressing interleukin-10 attenuate collagen-induced arthritis in mice. *Clin Exp Immunol* 2008; 153: 269-76.
6. Zhu P, Li XY, Wang HK, Jia JF, Zheng ZH, Ding J, et al. Oral administration of type-II collagen peptide 250-270 suppresses specific cellular and humoral immune response in collagen-induced arthritis. *Clin Immunol* 2007; 122: 75-84.
7. Dupont WD, Plummer WD. Power and Sample Size Calculations for Studies Involving Linear Regression. *Control Clin Trials* 1998; 19: 589-601.
8. American College of Rheumatology: ACR-endorsed Criteria for Rheumatic Diseases [Online] 2017 [Cited 2017 Sept 14]. Available from URL: <https://www.rheumatology.org/Practice-Quality/Clinical-Support/Criteria/ACR-Endorsed-Criteria>.
9. Aletaha D, Smolen J. The Simplified Disease Activity Index (SDAI) and the Clinical Disease Activity Index (CDAI): a review of their usefulness and validity in rheumatoid arthritis. *Clin Exp Rheumatol* 2005; 23: S100-8.
10. Min SY, Yan M, Du Y, Wu T, Khobahy E, Kwon SR, et al. Intra-articular nuclear factor- κ B blockade ameliorates collagen-induced arthritis in mice by eliciting regulatory T cells and macrophages. *Clin Exp Immunol* 2013; 172: 217-27.
11. Terato K, Harper DS, Griffiths MM, Hasty DL, Ye XJ, Cremer MA, et al. Collagen-induced arthritis in mice: synergistic effect of E. coli lipopolysaccharide bypasses epitope specificity in the induction

- of arthritis with monoclonal antibodies to type II collagen. *Autoimmunity* 1995; 22: 137-47.
12. He X, Kang AH, Stuart JM. Accumulation of T cells reactive to type II collagen in synovial fluid of patients with rheumatoid arthritis. *J Rheumatol* 2000; 27: 589-93.
13. Nissim A, Winyard PG, Corrigan V, Fatah R, Perrett D, Panayi G, et al. Generation of neoantigenic epitopes after posttranslational modification of type II collagen by factors present within the inflamed joint. *Arthritis Rheum* 2005; 52: 3829-38.
14. Trentham DE, Dynesius RA, Rocklin RE, David JR. Cellular sensitivity to collagen in rheumatoid arthritis. *Trans Assoc Am Physicians* 1978; 91: 118-28.
15. Trentham DE, Dynesius-Trentham RA, Orav EJ, Combitchi D, Lorenzo C, Sewell KL, et al. Effects of oral administration of type II collagen on rheumatoid arthritis. *Science* 1993; 261: 1727-30.
16. Trentham DE, Townes AS, Kang AH. Autoimmunity to type II collagen an experimental model of arthritis. *J Exp Med* 1977; 146: 857-68.
17. Stuart JM, Postlethwaite AE, Townes AS, Kang AH. Cell-mediated immunity to collagen and collagen alpha chains in rheumatoid arthritis and other rheumatic diseases. *Am J Med* 1980; 69: 13-8.
18. Londei M, Savill CM, Verhoef A, Brennan F, Leech ZA, Duance V, et al. Persistence of collagen type II-specific T-cell clones in the synovial membrane of a patient with rheumatoid arthritis. *Proc Natl Acad Sci USA* 1989; 86: 636-40.
19. Desai BV, Dixit S, Pope RM. Limited proliferative response to type II collagen in rheumatoid arthritis. *J Rheumatol* 1989; 16: 1310-4.
20. Snowden N, Reynolds I, Morgan K, Holt L. T cell responses to human type II collagen in patients with rheumatoid arthritis and healthy controls. *Arthritis Rheum* 1997; 40: 1210-8.
21. Holmdahl R, Andersson ME, Goldschmidt TJ, Jansson L, Karlsson M, Malmström V, et al. Collagen induced arthritis as an experimental model for rheumatoid arthritis. *Immunogenetics, pathogenesis and autoimmunity. APMIS* 1989; 97: 575-84.
22. Maino VC, Suni MA, Ruitenber JJ. Rapid flow cytometric method for measuring lymphocyte subset activation. *Cytometry* 1995; 20: 127-33.
23. Kubbies M, Schindler D, Hoehn H, Rabinovitch PS. BrdU-Hoechst flow cytometry reveals regulation of human lymphocyte growth by donor-age-related growth fraction and transition rate. *J Cell Physiol* 1985; 125: 229-34.
24. Zhu P, Li XY, Wang HK, Jia JF, Zheng ZH, Ding J, et al. Oral administration of type-II collagen peptide 250-270 suppresses specific cellular and humoral immune response in collagen-induced arthritis. *Clin Immunol* 2007; 122: 75-84.
25. Von Baehr V, Mayer W, Liebenthal C, von Baehr R, Bieger W, Volk HD. Improving the in vitro antigen specific T cell proliferation assay: the use of interferon-alpha to elicit antigen specific stimulation and decrease bystander proliferation. *J Immunol Methods* 2001; 251: 63-71.
26. Zhu P, Li X, Ding J, Fan C, Wang Y, Wang H, et al. Simultaneous detection of DNA synthesis, activation and cytokine secretion in collagen II (250-270)-activated T lymphocytes by flow cytometry. *Cell Biol Int* 2004; 28: 765-72.
27. Van der Graaff WL, Prins AP, Dijkmans BA, van Lier RA. Prognostic value of Th1/Th2 ratio in rheumatoid arthritis. *Lancet* 1998; 351: 1931.
28. Thiele A, Bang R, Gütschow M, Rossol M, Loos S, Eger K, et al. Cytokine modulation and suppression of liver injury by a novel analogue of thalidomide. *Eur J Pharmacol* 2002; 453: 325-34.
29. Caruso A, Licenziati S, Corulli M, Canaris AD, De Francesco MA, Fiorentini S, et al. Flow cytometric analysis of activation markers on stimulated T cells and their correlation with cell proliferation. *Cytometry* 1997; 27: 71-6.
30. Nguyen XD, Eichler H, Dugrillon A, Piechaczek C, Braun M, Klüter H. Flow cytometric analysis of T cell proliferation in a mixed lymphocyte reaction with dendritic cells. *J Immunol Methods* 2003; 275: 57-68.
31. Chilson OP, Kelly-Chilson AE. Mitogenic lectins bind to the antigen receptor on human lymphocytes. *Eur J Immunol* 1989; 19: 389-96.
32. Danke NA, Koelle DM, Yee C, Beheray S, Kwok WW. Autoreactive T cells in healthy individuals. *J Immunol* 2004; 172: 5967-70.