Immunocytochemical detection of some apoptosis regulating proteins (P53 and Bcl-2) in Peripheral Blood Lymphocytes of Rheumatoid arthritis patients

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Abstract

Background: There are several regulatory proteins involved in the control of lymphocyte apoptosis. Their impairment may play a role in the pathogenesis of several autoimmune diseases. Recent studies have reported impairment in the apoptosis of peripheral blood lymphocytes (PBLs) in patients with rheumatoid arthritis (RA).
Objective: The aim of this study is to evaluate the cellular expression of P53 and BcI-2 proteins in the PBLs and their roles in the apoptotic process, and correlate their cellular expressions with the percent of peripheral T cell population.
Methods: This study involved forty-six RA patients were examined and compared with 17 healthy control individuals of similar ages. Lymphocytes were separated from peripheral blood samples, the assessment of their cellular expression of CD3 and regulatory proteins p53 and Bcl-2 by immunocytochemistry staining method.
Results: The results showed abundant accumulation of CD3 T lymphocytes in the peripheral circulation of RA patients in comparison with controls. A highly significant increased percentage of Bcl-2 protein expression in RA PBLs, compared to healthy control ($p<0.001$) while there was no such statistical difference regarding P53 expression in PBLs from both groups ($p=0.278$). The results of linear regression showed a significant correlation between the increased peripheral blood T lymphocytes and cellular percentage of Bcl-2 protein expression ($p<0.001$), while there was no such correlation with the percentage of P53 expression ($p=0.587$).
Conclusion: in conclusion of these results, we found an increase in the peripheral blood T lymphocytes from patients with RA that could be resulted from the noticed up-regulation of cellular expression of Bcl-2 protein, rather than with changes in cellular expression of P53 protein.
Keyword: Rheumatoid arthritis, apoptosis, P53, Bcl-2, immunocytochemistry.

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Introduction

Apoptosis or programmed cell death is a physiologic, genetically encoded program that results in cell death. It plays an important role in the elimination of unwanted cells during development and also as a balancing factor in maintaining tissue homeostasis, including that of the immune system [(1)].

Apoptosis also has anti-autoimmune mechanism that deletes potentially pathogenic autoreactive lymphocytes, and limits tissue damage in autoimmune diseases, including Rheumatoid Arthritis (RA) [(2)].

The etiopathogenesis of RA is not fully understood. However, it has become increasingly clear that T cells play a crucial role in the induction and maintenance of RA lesions [(3)]. Meanwhile, it has been recently postulated that the increased number with abnormal differentiation pattern observed in peripheral lymphocytes in RA patients might be related to an abnormality in the apoptotic pathway [(4)]. This suggested being due to lymphocyte expression of apoptosis-related molecules leading to

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suppression of the apoptotic process; while suppression of apoptosis of T lymphocytes leads to their survival which relates to the chronic and relapsing characters of RA [5, 4].

P53 and Bcl-2 proteins are known to play a central role in the regulation of apoptosis. P53 is a tumor-suppressor gene that controls cellular proliferation. In its natural form (wild-type) P53 can bind to DNA and prevent cells from entering the S (synthetic) phase of the cell cycle so as to allow time for DNA repair. Alternatively, P53 dependent events can eliminate the cells by sending them down to an irreversible apoptotic pathway. Thus P53 allows the DNA either to be repaired or ultimately destroyed before replication renders the damage perminanent. On the other hand, the mitochondrial-mediated apoptosis is partially controlled by the family of Bcl-2 proteins, one of the biologically most relevant classes of apoptosis regulators [6]. The Bcl-2 protein was originally identified as the primary cause of some B-cell lymphoma (hence the designation Bcl), but was subsequently found to have strong anti-apoptotic activity in a variety of cell types, including lymphocytes [7]. Upregulation of Bcl-2 proteins may cause systemic autoimmune disease through accumulation of activated T lymphocytes [8, 9].

The present study aims to evaluate P53 and Bcl-2 expression in PBLs isolated from RA patients regarding their role in the apoptotic process.

Subjects and methods

Patients and controls:

The study groups consisted of forty six Iraqi patients with RA fulfilled the American College of Rheumatology (ACR) classification criteria [10], were recruited from the out-patient clinic at the Department of Rheumatology and Rehabilitation, Al-Kadhumyia Teaching Hospital in Baghdad. Also 17 age-and sex-matched healthy controls were enrolled in the study. These controls were healthy blood donors.

The scoring system of present disease activity was done according to modified DAS28-3, that combines of both clinical and laboratory parameters. The clinical examination of joint swelling and tenderness was performed for 28 joints (include the same joints: shoulders, elbows, wrists, metacarpophalangeal joints, proximal interphalangeal joints and the knees [11]. While the general immunolaboratory assessments included erythrocyte sedimentation rate, C-reactive protein, and RF. Clinical and laboratory characteristics of the patients included in the study are summarized in (Table 1).

Blood samples and slides preparation:

A blood sample (Five ml venous blood) was aspirated from a suitable vein from all patients and unaffected controls. Blood was collected in pyrogen-free silicone-coated tubes with heparin. The blood samples were used for lymphocyte separation according to Isopaque-ficoll technique (originally described by Boyum in 1968) [12].

Heparinised peripheral blood was diluted 1/1 with phosphate buffered saline (PBS), and mononuclear cells were isolated by ficoll density gradient centrifugation at 2000 rpm for 20 minutes. Mononuclear cells were washed three times with PBS for 5 minutes, resuspended at 1 x 10^6 cells/ml, and fixed on poly-L-lysine-coated glass slides, wrapped, and kept at -20°C until assayed.

Measurements of T-cell population and apoptosis regulating proteins:

The percentage of PBLs reactivity was semi quantified by immunocytochemistry staining method.

Briefly, these precoated charged slides were removed from freezer, allowed to reach room temperature, unwrapped and then dipping the slides into PBS-filled jar for about 5 minutes and slides were placed on a flat level surface, then endogenous peroxidase was quenched by initial incubation of the smears by enough drops

of Peroxidase block for 5 minutes at room temperature then rinse with PBS from a wash bottle, slides then placed in PBS wash bath for 2 minutes and excess buffer were taped and wiped around smears. Then, enough power block reagent (1/10 diluted in PBS) were applied for 5 minutes and excess blocking reagent were taped but not washed to ovoid non-specific binding of antibodies. Then, the coated lymphocytes were covered by 20 µl of 1/30 diluted mouse monoclonal Ab (primary Ab) specific for human CD3, bcl2 and p53. Slides then incubated at 37°C for 1hr, then unreacted monoclonal Ab was removed by three cycle of washing with PBS each two minutes, then slides were washed wiped around the smear. After that enough solution of biotinylated secondary antibody (anti-mouse Ab) were applied to cover each smear, distributed evenly over the precoated slides then placed in humid chamber for 1 hour at 37°C and washed in buffer and bathed in PBS for 5 minutes then wiped around smear. Enough solution of streptavidin conjugated peroxidase were applied to cover the smear and slides were placed in humid chamber for 1 hour at 37°C then washed in buffer and bathed in PBS for 5 minutes then wiped around the wells. Then enough drops of freshly prepared DAB working solution were applied to cover the section at room temperature for 10 minutes or until the color is observed then the reaction terminated by rinsing gently with distilled water from a washing bottle. Slides then placed in bath of hematoxyline for 30 seconds at room temperature. Slides were rinsed gently with distilled water from a wash bottle then rinsed under gently running tap water for 5 minutes. A drop of mounting medium (DPX) was placed onto the wet smear and the spot quickly covered with a cover slip. Slides were let to dry.

Slides were examined under 400X-magnification power of light microscope (ZEISS). The dark brown (homogenous or membranous) staining identified positive labeled cells see (figure 1).

Statistical analysis

The percentage of each of the tested marker expression on PBLs was calculated by a simple calibration of percentage of reactivity as following formula: Percentage of expression= (No. of positive cells/ total No. of cells) ×100%.

Statistical differences were analyzed using Independent sample-test. P-values <0.01 were considered statistically significant. Simple linear regression was used to assess the relationship between studied variables.

Results

The study included forty-six RA patients (four men and forty two women), mean age (47.67 years) ranged in age from (25-66 year) with mean disease duration (6.5 years). Our patients were classified according to DAS into two main group the majority of them, 37 patients (80.4%) presented with high disease activity and the remainder were minimum disease group consist of 9 patients (19.6%).

Immunocytochemical examination:

The percentage of peripheral blood T cell population was indicated by CD3 reactivity. In our study we found significantly elevated percentage of CD3 positive cells in RA patients when compared with healthy controls ($p \leq 0.001$). Also, there was highly significant overexpression of Bcl-2 in RA patient than those from control group ($p \leq 0.001$), and no significant difference in the P53 expression between RA patients and control groups (p=0.278).

In RA patients and according to disease activity groups, we compare the percentage of expression of studied markers between and found that no significant statistical differences in CD3, Bcl-2 and P53 expressions (p=0.686, p=0.130 and p=0.823) respectively.

***Relation ship between CD3 with Bcl-2 and P53 PBL expression*:**

Our study reported that there was an increased T-cell population in the peripheral circulation, we try to investigate wether this increase related to anti apoptotic effect of Bcl-2 overexpression or due to P53 reduced expression. The results of linear regression found that there was highly significant linear relation between increased peripheral T cell population and the increased Bcl-2 expression ($p \leq 0.001$) (figure 4.A), but there was no relation between increased peripheral T cell population and P53 expression ($p=0.587$) (figure 4.B).

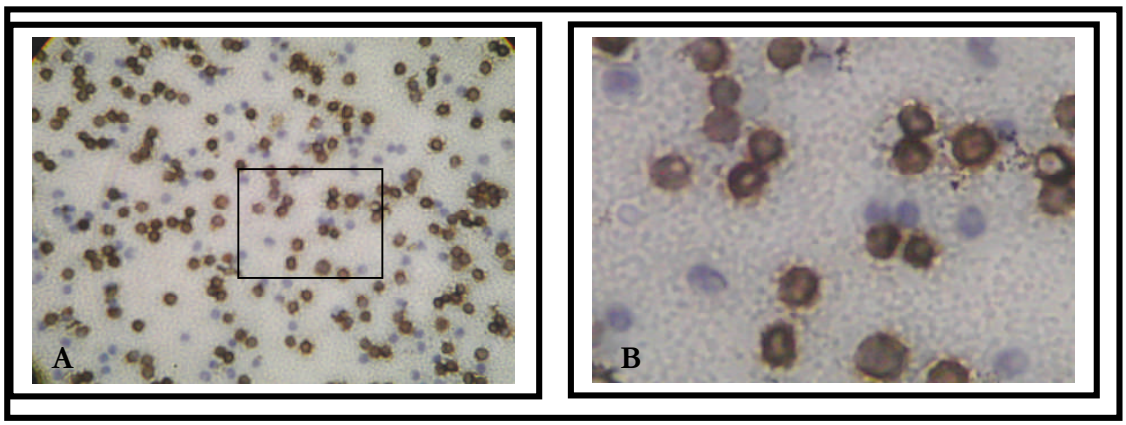

Figure 1. Immunocytochemical staining of PBL from RA patients stained with anti human Bcl-2 mAb, visualized by peroxidase/DAB (brown) and counter stained with hematoxylin. A: Low power magnifications of 400X. B: High power magnifications of 1000X.

Table 1. patients and control characterstics. data are presented as means (SD).

	Controls	RA patients	RA patients	
			High disease activity group	Minimum disease activity group
Women/men	**15/2**	**42/4**	**34/3**	**8/1**
Age	**48.6 (10)**	**47.67(12.09)**	**48.06(11.96)**	**46.45(12.97)**
Disease duration (months)	**----**	**88.61(72.88)**	**92.34(68.28)**	**76.73(92.67)**
ESR (mm/1st h	**12.50(3.31)**	**67.43(20.26)**	**70.94(19.54)**	**53(17.33)**
CRP (mg/l)	**10.20(15.24)**	**43.956(55.078)**	**49.78(59.53)**	**20(17.75)**
Tender joints	**-----**	**10.58(5.42)**	**12.54(4.62)**	**4.77(3.19)**
Swollen joints	**-----**	**7.35(4.52)**	**8.63(4.36)**	**3.66(1.80)**
DAS-28 (3)	**-----**	**5.77(0.83)**	**6.11(0.63)**	**4.844(0.24)**
RF sero-positive (No. (%))	**3(21.4%)**	**34 (73.9%)**	**27(72.9%)**	**6(63.54%)**
Duration of morning stiffness (minutes)	**-----**	**76.41(41.30)**	**84(41.72)**	**52.27(30.28)**

ESR=erythrocytes sedimentation rate, CRP= C reactive protein, DAS= disease activity score, RF=rheumatoid factor.

Table 2. Mean (standard deviation) of PBL cellular expression of CD3 and apoptosis regulating proteins (Bcl-2 and P53) in RA patients and control group.

	Controls	RA patients	RA patients	
			High disease activity group	Minimum disease activity group
CD3	72.92(0.44)	79.21(1.42)	79.26(1.33)	79.04(1.8)
Bcl-2	20.5(3.55)	34.17(6.5)	35(5.48)	31.55(9.1)
P53	2.17(0.68)	3.26(0.32)	3.2(3.1)	3.45(3.78)

Table 3. Results of comparison among different study groups using independent sample t-test.

	RA patients *vs.* Controls	High disease activity group *vs.* Minimum disease activity group
CD3	p=0.000**	P=0.686
Bcl-2	P=0.000**	P=0.130
P53	P=0.278	P=0.823

(*) significant($p \leq 0.05$),
(**) highly significant ($p \leq 0.005$)

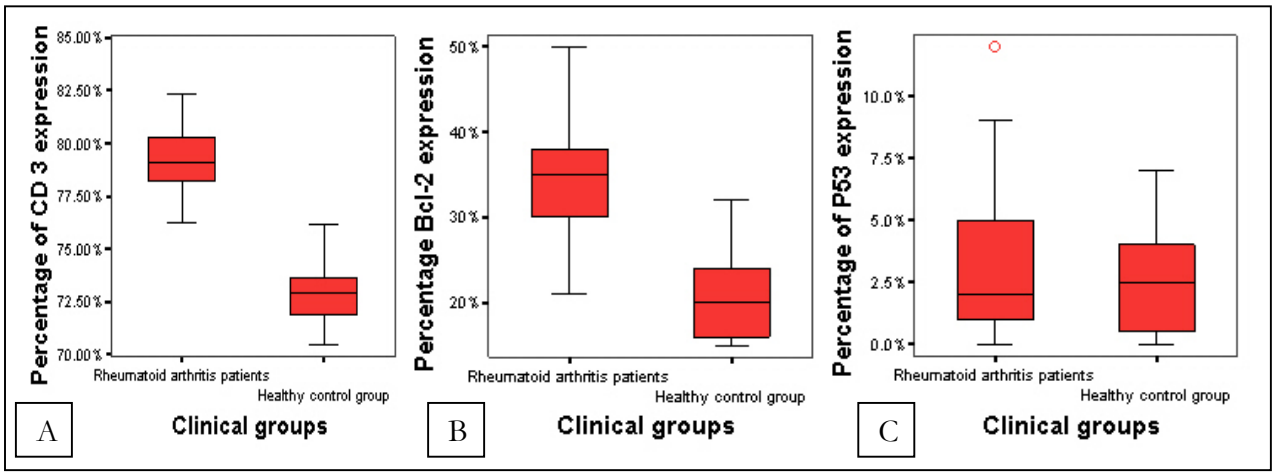

Figure 2. Cellular expression of CD3 (A), Bcl-2 (B) and P53 (C) in peripheral blood lymphocytes from RA patients and control group measured by immunoytochemistry method. Box plots represent median (line), 25th and 75th centiles (box), and whiskers indicate the 10th and 90th centiles.

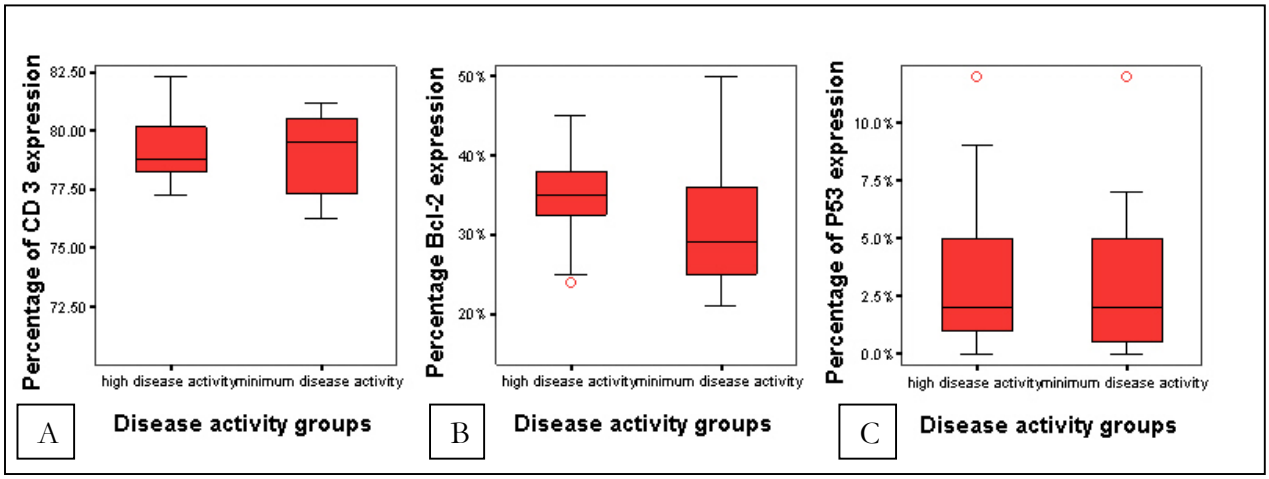

Figure 3. Cellular expression of CD3 (A), Bcl-2 (B) and P53 (C) in peripheral blood lymphocytes from RA patients suffering from high disease activity and minimum disease activity measured by immunoytochemistry method. Box plots represent median (line), 25th and 75th centiles (box), and whiskers indicates the 10th and 90th centiles

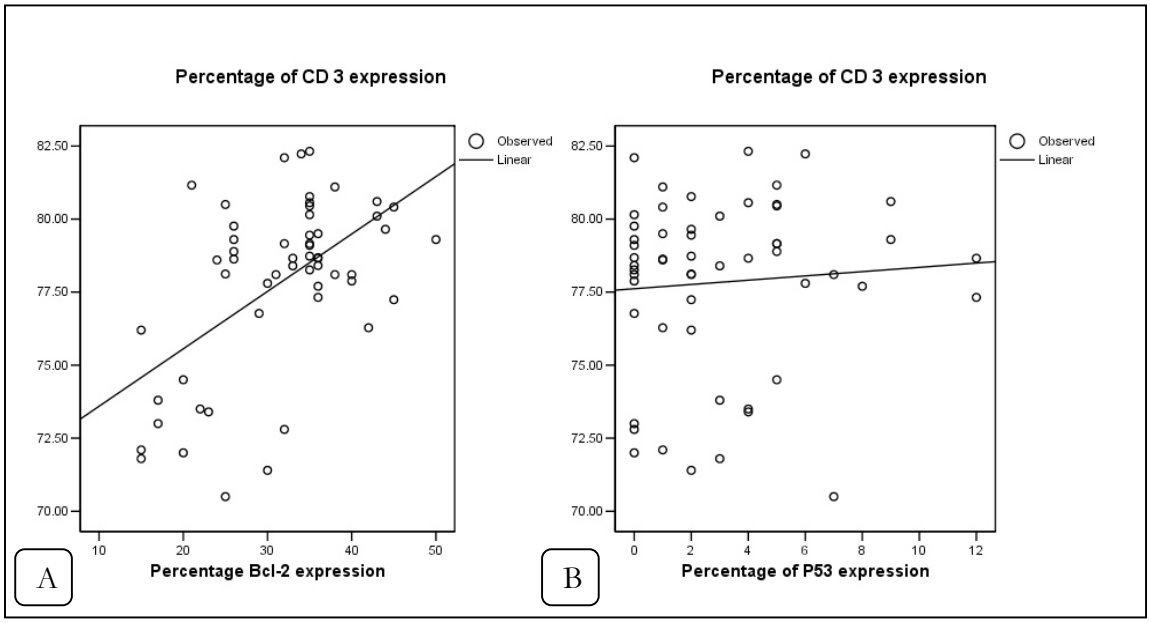

Figure 4. Relation between PBL expression of CD3 and Bcl-2 (A), and P53 (B) from RA patients. Points represent individual peripheral blood samples. Data show line of linear regression ($p \leq 0.001$) and ($p = 0.587$) for (A) and (B) respectively.

Discussion

Rheumatoid arthritis (RA) is a common autoimmune disease in which patients suffer from chronic inflammatory synovitis that is dominated by the presence of macrophages, neutrophils, lymphocytes and synovial fibroblasts. Synovial macrophages are highly resistant to apoptotic stimuli [13].

Several lines of evidence have shown the importance of T lymphocytes in the pathogenesis of rheumatoid arthritis using animal models of arthritis [16–21]. The data also suggest that both regulatory and pathogenic T lymphocytes might be involved [22–26]. In human rheumatoid arthritis (RA), clonal expansion of T cells in both peripheral blood and local synovium has been described repeatedly in the past 10 years. The clonally expanded T lymphocytes appear to be autoreactive [27, 28].

We recorded abundant accumulation of CD3+ve T-cells in the peripheral circulation with highly statistical significant difference in the mean percentage of expression than those of controls. In RA, workers proposed that thymic output is prematurely compromised in RA patients and a compensatory expansion of peripheral T cells results in a contracted and distorted repertoire. However, a shift of a contracted repertoire towards autoreactivity may result in increasing the size of individual clones and probably increase the risk of self-recognition. This could amplify their potential to cause ongoing autoimmune disease [14].

Lymphocytes are critical player in the pathogenesis of RA, in which apoptosis may play divergent roles. The study of lymphocyte development and survival, programmed cell death and the genes regulating this process has become a focus of interest and defects in lymphocyte apoptosis are hypothesized to contribute to development of autoimmunity in RA [15].

These studies suggest that pathogenic T lymphocytes accumulate *in vivo* for unknown reasons and cause autoimmune arthritis in RA patients. The mechanisms for the accumulation of those pathogenic T lymphocytes are still unknown, while a failure of apoptosis has been proposed to be an important mechanism [29-38].

However, the mechanisms of accumulation and expansion of autoreactive T lymphocytes in RA patients are still unknown. There may be three possibilities for accumulation and expansion of autoreactive lymphocytes in RA patients: one is a continual input of autoreactive lymphocytes into the peripheral lymphocyte pool, certain genetic backgrounds may predispose an individual to accumulate autoreactive T cells *in vivo*; [39, 40] the second is a failure to suppress autoreactive lymphocytes via anergy; [41, 42, 43] the third possibility is a failure to remove autoreactive lymphocytes from the peripheral lymphocyte pool by apoptosis [44]. While limited data are available regarding functional relationships among different T lymphocytes in RA, the positive effect of inducing T-cell tolerance in treating RA in both animal models and human clinical trials implicates that breakdown of peripheral tolerance plays an important role in the pathogenesis of RA [45, 46].

Activation-induced cell death (AICD) is an important mechanism by which the immune system can eliminate peripherally activated lymphocytes, maintain homeostasis and maintain peripheral tolerance in the immune system [31–33]. Previous data support that a failure of T-lymphocyte apoptosis is involved in the pathogenesis of RA [32, 33]. Most available studies were performed in RA synovium [34]. The data suggests that T-cell apoptosis is suppressed [35]. It could be due to activated CD14+ cells that secrete a soluble survival signal (CD14 cocktail) that protects activated lymphocytes from undergoing AICD [45].

Apoptosis is controlled genetically where P53 and Bcl-2 play a central role in its regulation [46, 47]. Actively proliferating

cells typically express Bcl-2 that protects them against apoptotic stimuli while terminally differentiated cells lose Bcl-2 expression, found that Bcl-2 protein in PBLs overexpressed and proposed a defect in the mechanism of deletion of over-produced lymphocytes that probably play role in the pathogenesis of RA [34].

Our results clearly demonstrate that there is uniform underexpression of p53 in PBLs from patients with RA. Other investigations have addressed the role of p53 in RA. The majority of these studies have focused on the synovium. It has been proposed that high levels of oxidative stress in rheumatoid synovium may cause somatic mutations in the TP53 gene [48]. Mutations in synovial p53 may allow pathologic proliferation of synovial cells that may lead to joint destruction and other clinical manifestations of RA. Alternatively, it has been proposed that the cytokine, macrophage migratory inhibitory factor (MIF), may cause decreased cellular p53 levels [49, 50]. Elevated MIF levels may contribute to the underexpression of p53 in PBMCs from RA patients. However, our results clearly show that T lymphocytes, representing 80% of our PBMC preparations, are defective in p53-mediated apoptosis, and T lymphocytes are not known to respond to MIF. There is also evidence that p53 maintains tolerance in lymphocytes by regulating cell cycle progression. Human T lymphocytes from peripheral blood or intestinal lamina propria show an inverse relationship between p53 levels and the rate of progression through the cell cycle [51]. Cell cycle delays mediated by elevated levels of p53 in lamina propria T lymphocytes may be a mechanism that maintains tolerance against environmental antigens. Preliminary studies by Leech et al, using an antigen-induced arthritis model on a p53_/_ background, revealed that T lymphocytes proliferate more readily and produce more IFN_ in the absence of p53 [52]. However, our results agreed by Mass et al. when found that P53 protein was reduced in lymphocytes of patients with RA, making the cells less likely to under go apoptosis [53]. Similar results in models of collagen-induced arthritis [48] suggest that inflammatory responses may be exacerbated in the absence of p53.

These data show that the highly differentiated and apparently unstable state PBLs in RA may result in part from active inhibition of T cell apoptosis by environmental factors associated with the inflammation itself.

In conclusion defective lymphocyte apoptosis is playing an important role in RA inflammation. It could most probably mediate through other mechanism rather than the P53 pathway (P53 independent). Mean while, overe-xpression of Bcl-2 protein by PBLs could protect them from apoptosis, leading to their persistence and chronic characters of the disease.

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