Activation-induced cell death and Fas-induced apoptosis in patients with systemic or pauciarticular juvenile idiopathic arthritis

P. Pignatti, M. Massa[1], P. Travaglino, C. Meazza, A. Martini, F. De Benedetti

Clinica Pediatrica, and [1]Laboratorio di Biotecnologie e Tecnologie Biomediche, IRCCS Policlinico San Matteo, Pavia, Italy

Abstract

Objective

To investigate the functionality of the Fas-induced apoptotic pathway in peripheral blood mononuclear cells (PBMC) from patients with systemic or pauciarticular juvenile idiopathic arthritis (JIA).

Methods

PBMC from 12 patients with systemic and 6 with pauciarticular JIA were activated with anti-CD3 and rhIL-2 and then incubated in the presence or absence of the anti-Fas MoAb CH11 inducing activation of the Fas apoptotic pathway. Apoptosis was evaluated by flow cytometry and fluorescence microscopy.

Results

The percentage of apoptotic cells following triggering of Fas did not differ between patients with systemic JIA (12.5 ± 9.5%) or pauciarticular JIA (18.7 ± 8.9%) and controls (16.1 ± 6.8%). Evaluation of activation-induced cell death (AICD) in the absence of exogenous triggering of Fas showed that 44% (8/18) of the patients with JIA, compared to none of the controls (0/16), had a percentage of apoptotic cells higher than the mean + 2 SD of controls. The increased AICD was neutralized by the addition of an anti-TNF- antibody.

Conclusion

Patients with systemic or pauciarticular JIA do not show a defect in the Fas-dependent apoptotic pathway of T cells. The increased AICD present in some patients with JIA appears to be at least in part related to the inflammatory cytokine TNF- .

Key words

Apoptosis, Fas, juvenile idiopathic arthritis.

Clinica Pediatrica, and [1]Laboratorio di Biotecnologie e Tecnologie Biomediche, IRCCS Policlinico San Matteo, Pavia, Italy

This work was in part supported by IRCCS Policlinico San Matteo (grant 390RCR96/02) and by the Ministero dell'Università e della Ricerca Scientifica.

Patrizia Pignatti, Research Fellow, Ph.D; Margherita Massa, Dirigente Biologo, Ph.D; Paola Travaglino, Research Fellow, Cristina Meazza, Research Fellow; Alberto Martini, Professor of Pediatrics, MD; Fabrizio De Benedetti, Dirigente Medico Primo Livello, MD, Ph.D.

Please address correspondence and reprint requests to: Alberto Martini, MD, Clinica Pediatrica, IRCCS Policlinico San Matteo, Piazzale Golgi 2, 27100 Pavia, Italy. E-mail: amartini@smatteo.pv.it

Received on December 18, 2000; accepted in revised form on March 15, 2001.

Introduction

Apoptosis is an active, signal-dependent process leading to cell death with characteristic changes in the dying cells: cell shrinkage, blebbing of the plasma membrane, nuclear collapse and fragmentation of nuclear chromatin (1). In addition to representing a mechanism of negative selection employed by self-reactive thymocytes (2), apoptosis is also a key mechanism of immune homeostasis in the periphery, where activation-induced cell death (AICD) plays a crucial role in the extinction of the immune responses to foreign and self antigens (3). Although several mechanisms are involved in the induction of apoptosis, much interest has been focused on the role of the Fas/Fas ligand pathway in immune regulation. Mice with a mutation of the Fas gene (MRL/*lpr/lpr*) or of the Fas ligand gene (C3H/HeJ-*gld/gld*) present the spontaneous occurrence of autoimmunity (4). In humans mutations in the Fas, Fas ligand or caspase 10 genes (5-7), leading to impaired functionality of the Fas pathway, cause the recently identified disorder called autoimmune lymphoproliferative syndrome (ALPS) (8,9). This syndrome is characterized by a non-malignant lymphoadenopathy with expansion of double negative ($CD4^-CD8^-$) T cells and autoimmune features such as hemolytic anemia, thrombocytopenia, neutropenia (8, 9). Therefore, defects in the mechanisms of apoptosis induction could be responsible for autoimmune and/or inflammatory chronic diseases.

While a number of studies have evaluated the mechanisms leading to T cell apoptosis (in particular Fas-induced apoptosis) in patients with systemic lupus erythematosus or rheumatoid arthritis (10), to the best of our knowledge no such studies have been carried out in patients with juvenile idiopathic arthritis (JIA). Therefore, this study set out to investigate the functionality of the Fas-induced apoptotic pathway and of activation-induced cell death in patients with systemic or pauciarticular onset JIA, the two most characteristic forms of childhood chronic arthritis. We found that the induction of apoptosis by Fas triggering was comparable between patients and controls, demonstrating that systemic JIA (s-JIA) and pauciarticular JIA (p-JIA) are not associated with functional defects of Fas-triggered apoptosis. Evaluation of AICD did not show defective AICD in JIA patients compared to controls. On the contrary, increased AICD was found in a significant percentage of the JIA patients; this increase appears to be mediated by TNF- .

Materials and methods

Patients

Twelve patients (mean age 8.0 years, range 3-17) with s-JIA, and 6 with p-JIA who fulfilled the diagnostic criteria for JIA (11) were studied. All patients presented active disease, defined by the presence of synovitis upon examination. Table I shows the erythrocyte sedimentation rate (ESR) values, C-reactive protein concentrations, and the number of active joints. At the time of sampling, all patients were being treated with non-steroidal antiinflammatory drugs; 9/18 patients were receiving weekly methotrexate (MTX) (7 with s-JIA, and 2 with p-JIA and a

Table I. Erythrocyte sedimentation rate values, C-reactive protein concentrations and number of active joints in patients with systemic or pauciarticular JIA. Values are shown as mean ± standard deviation and range is indicated between parenthesis.

	Systemic JIA	Pauciarticular JIA
Erythrocyte sedimentation rate (mm/h)	45.9 ± 39.9	37.2 ± 26.9
	(10 - 116)	(6 - 73)
C-reactive protein (mg/L)	59.3 ± 48.2	33.0 ± 31.3
	(3-172)	(3 - 82)
Number of active joints	8.2 ± 5.4	1.8 ± 1.8
	(2-15)	(1-5)

polyarticular course). In addition, 8 patients with s-JIA were studied during treatment with prednisone (on an alternate day regimen in 5 of them). Sixteen healthy children (mean age 8.6 years, range 4-16), hospitalized for minor surgical procedures or bone marrow donation, were used as controls. Permission to draw extra blood during routine venipuncture was obtained from the parents of all children. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by standard Ficoll-Hypaque density centrifugation and resuspended in complete medium (RPMI 1640, supplemented with glutamine, gentamicine and 10% FCS).

Activation- and Fas-induced cell death, and apoptosis analysis

2.5×10^5 PBMC were cultured in 24-well plates in 1 ml of complete medium with 0.1 g/ml of anti-CD3 (Ortho, Raritan, NJ) and 20 U/ml of rhIL-2 (Hoffmann-La Roche, Nutley, NJ) for 5 days. To evaluate the induction of apoptosis triggered by Fas, 200 ng/ml of the activating MoAb CH11 to Fas (Upstate Biotechnology, Lake Placid, NY) were added during the last 24 hours of incubation, and then cells were harvested and analyzed for apoptosis. To evaluate activation-induced cell death (AICD), apoptosis was measured without the addition of the CH11 MoAb. In order to evaluate the relevance of the Fas or the TNF- pathway in AICD, activation of PBMC, as described above, was performed in the presence of 2 g/ml of a neutralizing anti-TNF- MoAb (R&D Systems, Minneapolis, MN), or of 1 g/ml of the Fas blocking MoAb ZB4 (Medical & Biological Laboratory Co., Nagoya, Japan).

Two different methods were used for apoptosis analysis: (i) PBMC were stained as described by Nicoletti *et al.* (12) with 1.5 ml of a hypotonic fluorochrome solution (PI 50 m/ml in 0.1% sodium citrate plus 0.1% Triton X-100, Sigma, St. Louis, MO) for 18 h at 4°C before flow cytometric analysis; and (ii) PBMC were stained with 15 ng/ml of acridine orange and 50 ng/ml of ethidium bromide and analyzed for the presence of condensed and fragmented nuclei by fluorescence microscopy (13). At least 200 cells were read per sample by a blinded observer. A very high correlation in the percentage of apoptotic cells measured using the two methods was found ($r = 0.81$). All data shown in the figures were obtained using fluorescence microscopy.

Statistical analysis

Data were analyzed using the Mann-Whitney U test for unpaired samples, the Wilcoxon test for paired samples, and Fisher's exact test, as appropriate. Correlations were evaluated with the Spearman rank test. A p value < 0.05 was considered statistically significant.

Results

Apoptosis induced by anti-Fas antibody in PBMC

In order to evaluate whether patients with JIA had a defect in Fas-induced apoptosis, we measured the percentage of apoptotic cells induced by triggering Fas with the anti-Fas MoAb CH11 following PBMC activation with anti-CD3 and IL-2. The percentage of apoptotic cells was comparable between JIA patients and controls (Fig. 1). When the patients were divided according to the onset type of the disease, the percentage of apoptotic cells induced by the anti-Fas antibody tended to be lower in patients with s-JIA (12.5 ± 9.5%) than in patients with p-JIA (18.7 ± 8.9%) or in controls (16.1 ± 6.8%), but the difference was not statistically significant ($p > 0.1$) (Fig. 1).

We then evaluated whether the modest decrease in anti-Fas induced apoptosis in patients with s-JIA could be secondary to a reduced expression of the Fas molecule on the cell surface. Evaluation of the percentage of CD3+ cells expressing Fas did not show significant differences between patients with s-JIA and controls (CD3+ cells expressing Fas 33.0 ± 15.5% and 27.1 ± 7.8%, respectively). Moreover, after activation of PBMC with anti-CD3 and IL-2 for 5 days, the expression of Fas on T cells was higher than 90% both in patients with s-JIA and in controls. We did not find a significant correlation of the percentage of Fas-induced apoptosis with different treatments or with clinical and laboratory parameters of disease activity, such as the number of active joints, serum C-reactive protein concentration, and the ESR (data not shown). These data show that patients with JIA do not present a defect in the Fas-triggered pathway of apoptosis.

Activation-induced cell death (AICD) in patients with JIA

Activation of T cells results in programmed cell death in the absence of any exogenous triggering of the Fas molecule. Therefore, we evaluated the percentage of apoptotic cells after the activation of PBMC with anti-CD3 and IL-2, in the absence of the anti-Fas MoAb. Results expressed as a percentage of apoptotic cells are shown in Figure 2. None of the patients with s-JIA or p-JIA showed a percentage of apoptotic cells lower than the mean - 2 SD of the controls, thus ruling out a possible defect of

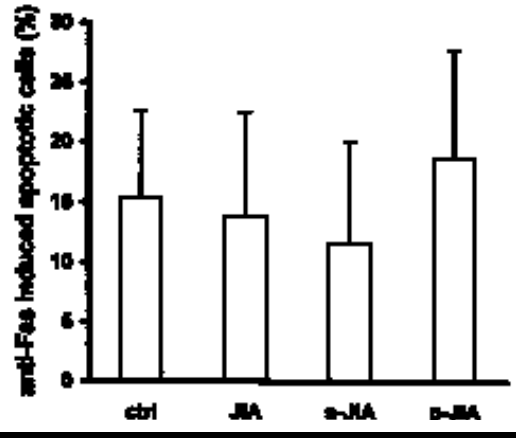

Fig. 1. Apoptosis induced by triggering Fas with the anti-Fas MoAb CH11 following T cell activation in healthy controls (ctrl), in patients with JIA, and in patients divided according to the onset type of the disease in systemic-JIA (s-JIA) and pauciarticular-JIA (p-JIA). Results, expressed as percentage of apoptotic cells, are shown as mean + standard deviation.

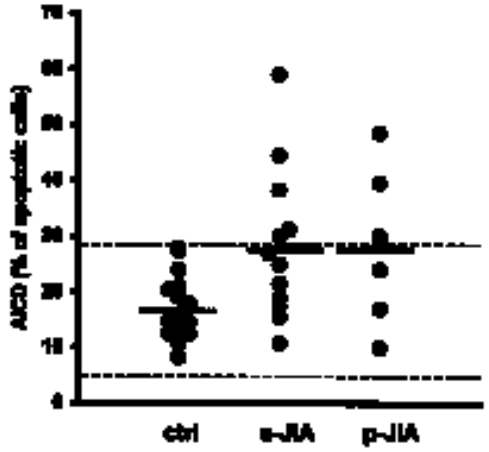

Fig. 2. Apoptosis induced by T cell activation (AICD) in healthy controls (ctrl) and in patients with systemic-JIA(s-JIA) or pauciarticular-JIA (p-JIA). The mean value of each group is represented by the continuous lines. The upper and lower limits of normal (means ± 2 SD of controls) are shown by the dotted lines.

Table II. Apoptosis following T cell activation (AICD) in patients with JIA divided according to the presence or absence of prednisone (PDN) or methotrexate (MTX) treatment at time of sampling. Results, expressed as percentage of apoptotic cells, are shown as mean ± standard deviation.

	PDN		MTX	
	Yes	No	Yes	No
AICD (% of apoptotic cells)	24.0 ± 11.5 (*) (n = 8)	30.7 ± 14.5 (n = 8)	30.8 ± 17.0 (*) (n = 9)	24.6 ± 9.0 (n = 7)

(*) p > 0.1 versus corresponding patients not treated with PDN or MTX.

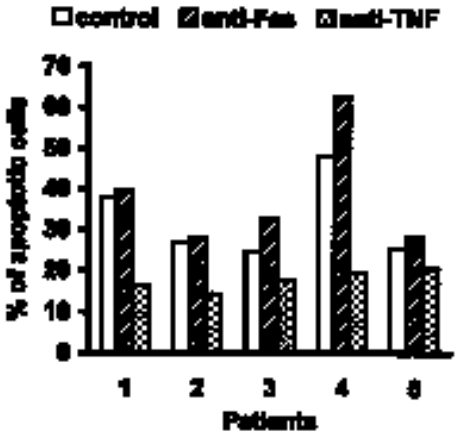

Fig. 3. Increased activation induced cell death (AICD) in patients with JIA is corrected by the neutralization of TNF- , but not of Fas. PBMC were activated as described in the method section,and cultured in absence (white bars) or in the presence of a neutralizing antibody to Fas (shaded bars) or to TNF- (dotted bars),as described in the method section.

AICD in patients with JIA. On the contrary, 44% (8/18) of the patients with JIA (5/12 with s-JIA and 3/6 with p-JIA), compared to none of the controls (0/16) showed a percentage of apoptotic cells higher than the mean + 2SD of the controls ($p < 0.005$, by Fisher's exact test). When patients were divided according to current treatment, we did not find any significant difference in the percentage of apoptotic cells in patients receiving MTX or corticosteroid treatment (Table II). In addition, the percentage of apoptotic cells was comparable between patients receiving daily glucocorticoids compared to those on an alternate day regimen, and no relation was found with the glucocorticoid dose (data not shown). These results rule out a direct effect of MTX or corticosteroid treatment on *in vitro* AICD (Table II). No correlation of the levels of AICD with clinical and laboratory parameters of disease activity, such as the number of active joints, serum C-reactive protein concentration, and the ESR was found (data not shown).

Among the various pathways capable of inducing AICD, a vast body of evidence points to the role of activation of the Fas/FasL pathway (3). Therefore, we evaluated whether in 5 patients with increased AICD, the addition of an anti-Fas neutralizing antibody inhibited AICD. As shown in Figure 3, addition of the anti-Fas antibody ZB4 did not result in any significant decrease in AICD ($p > 0.1$ by the Wilcoxon test for paired samples). Several studies have shown that TNF- can also cause AICD (14-16). Since increased production of TNF- is well documented in patients with JIA (17-19), we evaluated the effect of the addition of an anti-TNF- neutralizing antibody in the patients with increased AICD. As shown in Figure 3, addition of the neutralizing anti-TNF- antibody resulted in a significant decrease ($p = 0.03$ by Wilcoxon test for paired samples) in AICD to levels similar to those of controls.

Taken together these data show that patients with s-JIA or p-JIA do not have a defect in AICD, but rather that increased AICD occurs in some patients. This increased AICD appears to be at least in part related to the inflammatory cytokine TNF- .

Discussion

In this study we show that patients with s-JIA or p-JIA do not have a defect in the induction of apoptosis following T cell activation (AICD). This suggests that no major defects in the mechanisms leading to T cell apoptosis are present in patients with these onset forms of JIA. Apoptosis induced in T cells after continuous TCR stimulation is in great part determined by activation of the Fas dependent cascade (3). In this study we also found that patients with s-JIA or p-JIA do not show a defect in apoptosis induced by the triggering of the Fas molecule. Consistent with these functional data, we found that the cell surface expression of Fas on T lymphocytes was comparable between patients and controls. Adding further evidence to the presence of normal Fas expression, Knipping *et al.* have reported normal levels of soluble Fas in patients with JIA (20).

As mentioned above, a marked increase in double negative ($CD4^-$ $CD8^-$) T cells is a characteristic feature of ALPS (8, 9). We have previously reported that patients with JIA, particularly s-JIA, also show an increase in double negative T cells, albeit at an apparently lower level than patients with ALPS (21). Incidentally, this finding was confirmed in the present study (data not shown). In ALPS the increase in double negative T cells is believed to be secondary to defective Fas-induced apoptosis, leading to the accumulation of T cells at a late stage of activation in lymphoid organs and in the periphery (8, 9). Since we excluded a defect in Fas-induced apoptosis in JIA, the same mechanism does not appear to be involved in this disease. The mechanism leading to the increased number of double negative T cells in JIA remains to be established. Recent observations showed that double negative T cells may represent a T cell population with regulatory and/or immunosuppressive functions (22, 23).

In this study we also found that in a significant percentage of patients the AICD was higher than that of controls. It is noteworthy that MTX (0.1 - 10 M) induces the apoptosis of *in vitro* activated T cells from human peripheral blood (24). A significant increase in the apoptosis of *in vitro* activated T cells from patients with RA treated with MTX was found only at 8 hours, with a subsequent decrease following MTX injection (24). However, all of our patients were studied at least 24 hours from the last MTX administration, and we did not find a correlation between increased AICD and treatment with MTX, nor with PDN, at the time of sampling. Increased AICD might be secondary to a pre-existing *in vivo* cellular activation state of the PBMC of patients, with the *in vitro* stimulation actually representing a re-stimulation, therefore leading to increased apoptosis. However, although several reports have demonstrated that synovial T cells are activated in JIA, the available evidence, albeit limited to a few and not very recent papers, does not suggest an activation state of peripheral blood T cells in JIA. For example, it has been reported that the percentage of HLA-DR-positive T cells in JIA patients was low and not different from that of controls (25).

As previously mentioned, activation of the Fas-dependent cascade plays a major role in activated T cell apoptosis. However, the increased AICD in patients with JIA was not inhibited by the addition of a neutralizing anti-Fas MoAb, thus ruling out a role for the Fas-dependent cascade in this phenomenon. On the contrary, we found that the addition of a neutralizing anti-TNF- MoAb markedly decreased AICD to levels similar to those of controls. TNF- levels are increased *in vivo* in patients with JIA (17-19), and increased TNF- production plays a role in the pathogenesis of the disease, as demonstrated by the therapeutic efficacy of treatment with a TNF- antagonist (26). Further supporting a relationship between increased AICD and TNF- , we found a positive correlation (Rs = 0.540) between serum TNF- levels, measured in 10 of the patients studied, and AICD (data not shown).

Although our data point to the TNF pathway as being responsible for the increased AICD in patients with JIA, it remains to be established whether this finding is secondary to the increased production of TNF- in the culture system or to increased sensitivity to the pro-apoptotic effects of TNF- by T cells from patients with JIA. Indeed, the role of TNF- in mediating pro-apoptotic effects in T cells has been a matter of debate. While some authors have shown that TNF- induced apoptosis in mature T lymphocytes (14-16), other studies showed a protective effect of TNF- on T cell apoptosis, with the protective effect being mediated by the TNF-induced activation of NF- B (27). Interestingly, both salicylates and glucocorticoids have been shown to inhibit the activation of NF- B (28,29). It is therefore conceivable to speculate that both increased production of TNF- and reduced activation of NF- B, secondary to treatment, may be involved in the increased AICD in JIA patients.

In conclusion, this study shows that patients with s-JIA or p-JIA do not have a defect in AICD or in the Fas-dependent apoptotic pathway, thus ruling out an association between JIA and abnormalities in the Fas-dependent mechanisms leading to T cell apoptosis. The increased AICD observed in a significant percentage of the patients appears to be related to TNF- .

References

1. CHOEN JJ: Apoptosis. *Immunol Today* 1993; 14: 126-30.
2. STRASSER A: Life and death during lymphocyte development and function: Evidence for two distinct killing mechanisms. *Curr Opin Immunol* 1995; 7: 228-34.
3. AKBAR AN, SALMON M: Cellular environments and apoptosis: tissues microenvironments control activated T-cell death. *Immunol Today* 1997; 18: 72-6.
4. NAGATA S, SUDA T: Fas and Fas ligand: lpr and gld mutations. *Immunol Today* 1995; 16: 39-43.
5. FISHER GN, ROSENBERG FJ, STRAUS SE *et al*.: Dominant interfering Fas gene mutations impair apoptosis in human lymphoproliferative syndrome. *Cell* 1995; 81: 935-946.
6. WU J, WILSON J, HE J, XIANG L, SCHUR PH, MOUNTZ JD: Fas Ligand mutation in a patient with systemic lupus erythematosus and lymphoproliferative disease. *J Clin Invest* 1996; 98: 1107-13.
7. WANG J, ZHENG L,LOBITO A, et al.:Inherited human caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome Type II. *Cell* 1999; 98: 47-58.
8. DIANZIANI U, BRAGARDO M, DIFRANCO D, *et al*.: Deficiency of the Fas apoptosis pathway without Fas gene mutation in pediatric patients with autoimmunity/lymphoproliph-eration. *Blood* 1997; 89: 2871-9.
9. DRAPPA J, VAISHNAW AK, SULLIVAN KE, CHU JL,ELKON KB: Fas gene mutations in the Canale-Smith syndrome, an inherited lymphoprolipherative disorder associated with autoimmunity. *N Engl J Med* 1996; 335: 1643-9.
10. EMLEN W, NIEBUR JA,KADERA R: Accelerated *in vitro* apoptosis of lymphocytes from patients with systemic lupus erythematosus. *J Immunol* 1994; 152: 3685-92.
11. PETTY RE, SOUTHWOOD TR, BAUM J *et al*.: Revision of the proposed classification criteria for juvenile idiopathic arthritis: Durban 1997. *J Rheumatol* 1998;25:1991-4.
12. NICOLETTI I, MIGLIORATI G, PAGLIACCI MC, GRIGNANI F, RICCARDI C: A rapid and simple method for measuring thymocytes apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 1991; 139: 271-9.
13. LECOEUR H, GOUGEON ML: Comparative analysis of flow cytometric methods for apoptosis quantitation in murine thymocytes and human peripheral lymphocytes from controls and HIV-infected persons. Evidence for interference by granulocytes and erytrocytes. *Immunol Methods* 1996; 198: 87-99.
14. ZHENG L,FISHER G, MILLER RE,PESCHON J,

LYNCH DH, LENARDO MJ: Induction of apoptosis in mature T cells by Tumor Necrosis Factor. *Nature* 1995; 377: 348-51.
15. PIMENTEL-MUINOS FX, SEED B: Regulated commitment of TNF receptor signaling: A molecular switch for death or activation. *Immunity* 1999; 11: 783-93.
16. GRELL M, ZIMMERMANN G, GOTTFRIED E, et al.:Induction of cell death by tumor necrosis factor (TNF) receptor 2,CD40 and CD30: a role for TNF-R1 activation by endogenous membrane-anchored TNF. *EMBO J* 1999; 11: 3034-43.
17. DE BENEDETTI F, PIGNATTI P, MASSA M *et al*: Soluble tumor necrosis factor receptor levels reflect coagulation abnormalities in systemic juvenile chronic arthritis. *Br J Rheumatol* 1997; 36: 581-8.
18. MANGGE H, KENZIAN H, GALLISTL S *et al.*: Serum cytokines in juvenile rheumatoid arthritis. Correlation with conventional inflammation parameters and clinical subtypes. *Arthritis Rheum* 1995;38:211-20.
19. ROONEY M, DAVID J, SYMONS J, DI GIOVINE F, VARSANI H, WOO P: Inflammatory cytokine responses in juvenile chronic arthritis. *Br J Rheumatol* 1995; 34: 454-60.
20. KNIPPING E, KRAMMER PH, ONEL KB, LEHMAN TJA,MYSLER E,ELKON KB: Levels of soluble Fas/APO-1/CD95 in systemic lupus erythematosus and juvenile rheumatoid arthritis. *Arthritis Rheum* 1995; 36: 1735-7.
21. MASSA M, DE BENEDETTI F, ROBBIONI P, RAMENGHI B, ALBANI S, MARTINI A: Association of methotrexate treatment with a decrease of double negative (CD4-CD8-) and T cell levels in patients with juvenile rheumatoid arthritis. *J Rheumatol* 1993; 20: 1944-8.
22. NIEHUES T, EICHLBAUER D, SCHNELDER EM: Functional characteristics of human peripheral blood /TCR+, CD4- and CD8- double-negative (DN) T cells. *Microbiol Immunol* 1999; 43 (2): 153-9.
23. ZHANG ZX, YANG L, YOUNG KJ, DU TEMPLE B, ZHANG L: Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. *Nat Med* 2000; 6:782-9.
24. GENESTIER L, PAILLOT R, FOURNEL S, FERRARO C,MIOSSEC P, REVILLARD JP: Immunosuppressive properties of methotrexate: apoptosis and clonal deletion of activated peripheral T cells. *J Clin Invest* 1998; 102: 322-8.
25. THOEN J, FØRRE Ø, WAALEN K, KÅSS E: Phenotypes of T lymphocytes from peripheral blood and synovial fluid of patients with rheumatoid arthritis and juvenile rheumatoid arthritis. *Scand J Rheumatol* 1987;16:247-56.
26. LOVELL DJ, GIANNINI ED, REIFF A, *et al.*: Etanercept in children with polyarticular juvenile rheumatoid arthritis. *N Engl Med* 2000; 342: 763-9.
27. VAN ANTWERP DJ, MARTIN SJ, KAFRI T, GREEN DR,VERMA IM: Suppression of TNF-induced apoptosis by NF- B. *Science* 1996; 274:787-9.
28. SCHEINMAN RI, COGWELL PC, LOFQUIST AK, BALDWIN AS JR: Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science* 1995; 270: 283-6.
29. KOPP E, GHOSH S: Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* 1994; 265: 956-9.