DOI: 10.5455/medarh.2012.66.336-339
Med Arh. 2012 Oct; 66(5): 336-339
Received: June 18th 2012
Accepted: August 27th 2012

CONFLICT OF INTEREST: NONE DECLARED

ORIGINAL PAPER

Monitoring of Disease Biomarkers Activity and Immunophenotyping as Important Factors in SLE Clinical Management

Djemo Subasic[1], Jasenko Karamehic[1], Marina Delic-Sarac[1], Mersija Kasumovic[2], Mevludin Mekic[3], Izet Eminovic[4], Nermina Hasanagic[1]
Institute of Clinical immunology, Clinical Centre University of Sarajevo, BiH[1], Cantonal Institute of Occupational Health Zenica, BiH[2], Clinic of Rheumatology, Clinical Centre University of Sarajevo, BiH[3], Faculty of Health Sciences University of Sarajevo, BiH[4]

The highly specific biomarkers for monitoring of SLE disease activity are not yet defined up to date, due to existing of different clinical SLE phenotypes caused by individual genetic variation. Basically, numerous clinical complications follow SLE patients such as nephritis, atherosclerosis and cardial, CNS, gastrointestinal and ophthalmological complications, as well. Their monitoring in clinical SLE management can be evaluated by analysing of specific biochemical parameters and require permanent clinical observation. The presence of ANAs and anti-ds-DNAs are usual diagnostic SLE autoimmunity parameters, while SLE disease activity biomarkers are C3 and C4 level, anticardiolipin antibodies, anti-Sm/RNPs and, recently level of CD4 and CD8 lymphocytes. However, the number of TCR molecules on the T-cells surface at SLE patients is lower then in normal condition, and otherwise for these receptors CD molecules make specific connection. On the other hand, the T lymphocytes can be also, therapeutical targets at SLE patients, beacause of their clear direct involving in SLE pathogenesis. The SLE phenotypes are characterized by double CD negativity (CD3+/-, CD4-) caused by abnormal level of IL-2 and IL-17. T-lymphocytes have usually alpha-beta and gamma-delta TCR receptors, but for SLE patients is characteristic lower number gama-delta TCR molecules, detected in the peripheral blood specimens. Taking into account all of the facts, we investigated the level of specific usual SLE activity biomarkers (anti-ds-DNAs, C3, C4, anticardiolipin antibodies (beta-2-IgG, beta-2-IgM, ACA-G, ACA-M, CD4 and CD8 level) in serum specimens of SLE patients who underwent to the corresponding chemotherapy in combination with other biochemical and clinical parameters. Once again proved to be, that SLE biomarker monitoring, could be useful aproach for SLE activity disease and prediction organ damage, as well. In our investigation we used the following methods: immunofluorescence microscopy (IFA-ANA), and nephelometry, Hycor ELISA system and Flow cytometry, for precisely quantitative measurements. We determined correlation between C3 and C4 complement components level, CD3 (T-Ly), CD3+/HLA-DR and total HLA-DR with regard to SLE disease activity. Also, CD4 (Th), CD4:CD8 ratio, beta-2-G, beta-2-M not proved to be useful biomarkers in this sense, despite some results specific for some special SLE phenotypes. Anti-Sm/RNPs proved to be better in SLE diagnostic process. **Key words: SLE, biomarkers, disease activity, IFA, ELISA, nephelometry, flow cytometry.**

Corresponding author: prof. Djemo Subasic, PhD. Istitute of Immunology. Clinical Centre University of Sarajevo. Bosnia and Herzegovina. E-mail: subasic.djemo@bih.net.ba

1. INTRODUCTION

The circulating immune complexes (CICs) formation, nephritis and arthritis are the most common features of chronic and progressive disease such as systemic lupus erythematosus (SLE). Other associated SLE complications are haemolytic anemia, trombocytopenia, renal failure and hypertension. In the normal conditions, CICs are formed permanently and in these conditions the antibodes level is low in the body. When complement system function normally, the CICs presence induced clasical way of complement activation in which the main role have C1q, C3 and C4 complement components. SLE as autoimmune disease is accompanied with presence of ANAs, and high level of C1q-CIC and C3 and C4 components in the active phase of the disease (1, 2). SLE is multigenic autoimmune disease. More than one thousand genes have changed expression at SLE patients. These genes are located on 6 chromosome (HLA locus) and these are mainly genes of HLA class I and HLA class II. Some information indicates the involving of other gene that does not belong to HLA locus such as IRF-5 gene (7 chromosome), Bank-1 gene (4 chromo-

some), TIMP-1 gene (X-chromosome) and MMP-1 gene (11 chromosome) as well. Also, SLE can be caused by drugs and most important of them are: procainamide, hydralazine, quinine, phenytoin. The incidence of SLE is 10 times common in women than in men, probably due hormone estrogen (3, 4).

In clinical SLE management, the diagnostic SLE criteria are the presence of ANAs, anti-ds-DNAs, anti-Sm, anti-Sm/RNPs. The parameters of SLE disease activity depends on SLE phenotype and most important are C3 and C4 components complement level, anticardiolipin antibodies, and number of CD4 and CD8 T-cells. It is clear that clinical importance of these SLE activity parameters depends on clinical SLE phenotype and that is the reason why this aspect of investigations is always very interesting (5, 6). Because of clear involving of C3, C4 complement components, anticardiolipin antibodies, CD4 and CD8 T-cells, in pathoetiology of SLE and SLE disease activity, we investigated the level of specific usual SLE activity biomarkers (anti-ds-DNAs, C3, C4, anticardiolipin antibodies beta-2-IgG, beta-2-IgM, ACA-G, ACA-M, CD4 and CD8 level) in serum specimens of SLE patients, who underwent the corresponding chemotherapy, in combination with other biochemical and clinical parameters, considering the individual genetic variation and existence of specific clinical SLE phenotypes (7,8).

2. MATERIAL AND METHODS

IFA-anti-ds-DNA test

NOVA LITE-TM-ds-DNA-Crithidia Lucilliae is indirect imunophluorescence method for anti-ds-DNA screening and semiquantitative determination in the serum specimens of patients suspected to autoimmune diseases such as SLE. These results must be taken into consideration together with other clinical, serological, biochemical and molecular findings during very complex process in final SLE diagnosis. The slides contains haemophlagelate protozoa Crithidia Luciliae as specific substrate, because of their very aboundant circular ds-DNA molecules content of kinetoplasts, which can bind the specific anti-ds-DNA autoantibodies. These DNA molecules are without histones and others nuclear proteins. Almost always, the anti-ds-DNA autoantibodies are present at serum specimens of SLE patients and represents a mandatory criterion according to ARA (Arthritis and Rheumatism Association) from 1982.

Before use, all reagents must be kept at room temperature at least half an hour. It is necessary to make appropriate dilutions of 40x concetrated PBS buffer (1:40) with destiled water and serum specimens in the ratio 1:10 with 1 x PBS buffer. Drop (20-25 µl) of serum specimens and positive and negative control is enough per analyzis. After the process of incubation and washing according to manufacturer instructions, adding of labeled fluorescein conjugate, mounting medium, the slides were analysed under fluorescence microscope with immersion objective (10X magnification). All specimens with fluorescing kinetoplasts by same or more intensity as positive controls, are positive to presence of anti-ds-DNA antibodies (9).

Nephelometry

Nephelometric measurements were performed by Nefelometer-Siemens-BN-II, for quantitative determination of C3 and C4 complement components level. The principle of this method is based on circulate immune complexes (CIC) light dispersion which pased through serum specimens. The intensity of dispered light is proportional to C3 or C4 component concentration and using specific standards of known concentrations, in the process of evaluation of obtained results. The reference values are: 0,1 – 0,4 mg/l for C4 and 0,9 – 1,8 mg/l for C3 (10).

ELISA-TMII-anticardiolipin antibodies and anti-ds-DNAs

The concentration of specific anticardiolipin antibodies and anti-ds-DNA antibodies in serum specimens, were detected by using of automated ELISA-HYTEC system. Preparation of all reagents (controls and specimens) is performed automatically in Hytec engine (dilution ratio 1:5 or 50 µl in 200 µl controls or serum specimens). Conjugate, substrate and stop solution are ready for use. We performed the several steps of incubating and washing according the manufacturer procedure and instructions.The final yellow color is proportional to the anticardiolipin and anti-ds-DNAs antibodies concentrations detected in serum specimens. The reference values are: ACA-G, ACA-M neg<10 low positive 10-20, middle positive 20-40, highly positive >40. Anti β2GPIG, Anti β2GPIM negative <10, borderline 10-14, positive >14 (11).

Flow cytometry

Immunophenotyping was performed on flow cytometer BD-FACSCANTO II. The princip of flow cytometry is based on laser light dispersion, sensibilisation and their detection from fluorochrome molecules. The multiparametar obtained data for particles with diametar 0,2-150 µm are analysed with software. Flow cytometry is based on light scattering, light excitation, and emission of fluorochrome molecules in the process of specific multiparameter generation. The cells sized from 0.5 µm to 40 µm in diameter are analised. Flow cytometry uses lasers as a light source. Detection of dispersed laser light is possible by flow cytometric detectors and obtained data are informatically processed by using of corresponding software.

It is possible to analyze by BD FACSCANTO-II several thousands cells in one second and in real-time as well. In our investigation we used 100 µl of whole bood specimens, 10µl of four-color designed monoclonal antibodies for analysis as followed scheme:

- CD3-FITC- fluorescin isotiocyanat
- CD8-APC- alofikocijanin
- CD16+56 – PE- fikoeritrin
- CD45 – PerCP- peridinin chlorofil protein
- CD4-FITC,–fluorescin isotiocyanat
- CD19 -APC-alofikocijanin
- HLA-DR-FITC- fluorescin isotiocyanat
- CD-25 – PE fikoeritrin

After appropriate preparation of lymphocytes according to manufacturer instructions, the specimens were analysed by flow cytometer (12,13,14).

3. RESULTS

We analysed total 300 serum specimens taken from patients suspected to autoimmunity. Among them, 69 or 23% showed specific SLE pathological,

Specimens	C3 Level	C4 Level
2	0.757	0.076
5	0.866	0.090
6	1.280	0.291
8	1.300	0.248
9	1.110	0.298
11	0.962	0.182
12	1.460	0.159
13	0.974	0.139
15	1.370	0.176
17	1.580	0.437
20	0.897	0.180
24	1.410	0.221
30	1.270	0.179
32	1.100	0.200
34	1.360	0.311
35	1.010	0.274
37	1.190	0.160
39	0.863	0.108
52	1.840	0.343
54	1.120	0.190
61	1.230	0.124
62	1.050	0.321
63	0.718	0.018
68	0.486	0.090

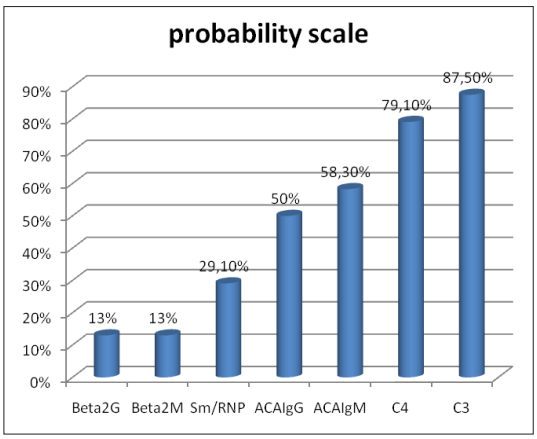
Table 1. The C3 and C4 level determined by nephelometry in serum specimens of SLE patients under specific chemotherapy.

and clinical parameters but only 24 patients had final SLE diagnosis. These patients were hospitalised in Clinical Centre University of Sarajevo and subjected to appropriate therapeutical treatement and monitored to disease activity by quantitative determination of SLE activity specific biomarkers by nephelometry (C3 and C4 complement components level (Table-1), ELISA Hycor (ACA-G, ACA-M, Beta-2-G, Beta-2-M, Sm/RNPs-Table -2) and Flow cytometry (CD3-T, CD4-Th, CD8-CTL, CD19-B, CD16+56-NK, CD4:CD8 ratio, CD3+/HLA DR-activated T lymphocytes, total HLA-DR expressed on lymphocytes, CD4+CD25+, CD4+CD25-).

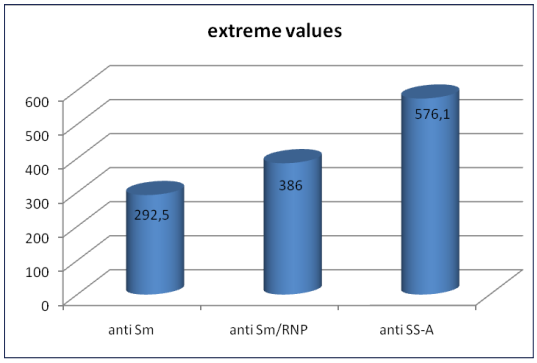
According to obtained results we designed the probability scale of analysed biomarkers reliability (Diagram 1.) and diagram presentation of biomarker extreme values (Diagram 2.)

The obtained results can be summarized in the following:

- Manly low values of C3 and C4 and extreme rarely low C4 level (0,018-0090)
- There is no detected extremely low C3 values.
- The high level of ACA-G and ACA-M (41,6%).
- The large number of specimens (83%) showed normal level of beta-2-G and beta-2- M biomarkers.
- Positive corelation between Sm/RNPs and ACA-G and ACA-M values.
- Increased level of CD3 (T-Ly), CD3+/HLA-DR and total HLA-DR.
- Stable level of CD4 (Th), CD4:CD8 ratio.



Graph 1. Graphic presentation of analysed biomarkers reliability in the SLE activity disease monitoring.



Graph 2. Some specific extreme values of SLE biomarkers.

4. DISCUSSION

Systemic lupus erythematosus (SLE) is autoimmune disease defined by specific clinical characteristics and diagnostic parameters. Basically, the presence of ANAs (antinuclear antibodies) against nuclear antigenes such as Sm, Sm/RNPs, SS-A is the the main feature for all SLE clinical phenotypes. Often, SLE patients have some clinical complications as rheumatoid arthritis, scleroderma, poliarthritis, pericarditis, trombocytopenia. Early SLE symptoms are depresia, chronic fatigue, anxiety and in some cases are common renal and CNS disorders (15).

Sample	Anti-ds-DNA	ACA-G	ACA-M	Beta-2-G	Beta-2-M	Sm/RNPs
2	+	27.50(P)	3.69	3.69	5.25	73.87 (P)
5	+	5.33	3.49	2.65	1.57	142.2(P)
6	+	59.14(P)	15.44(P)	3.81	5.14	5.57
8	+	14.11(P)	10.0(P)	1.99	0.74	2.11
9	+	10.52(P)	4.26	3.43	0.46	2.41
11	+	2.57	1.54	1.70	0.85	2.49
12	+	7.41	29.15(P)	4.51	0.82	47.03(P)
13	+	8.83	27.61	4.73	1.64	119.2(P)
15	+	28.60(P)	11.25(P)	2.77	2.48	151.9(P)
17	+	3.83	1.89	2.77	0.84	9.52
20	+	6.08	3.43	1.80	2.70	5.43
24	+	7.06	6.71	1.78	1.63	62.21
30	+	11.35(P)	17.65(P)	7.74	8.19	2.22
32	+	13.33(P)	21.73(P)	3.86	1.41	119.3(P)
34	+	8.04	6.55	4.39	1.64	1.32
35	+	32.97(P)	29.86(P)	>100(P)	29.49(P)	1.19
37	+	1.54	11.74(P)	2.29	0.58	1.08
39	+	>100(P)	>60(P)	1.77	0.82	1.08
52	+	16.12(P)	16.20(P)	2.40	3.70	2.40
54	+	58.64(P)	>60(P)	10.44(P)	12.10(P)	1.18
61	+	3.77	12.66(P)	1.09	0.58	0.72
62	+	12.98(P)	14.17(P)	2.35	1.67	1.45
63	+	4.46	3.75	1.75	5.87	1.39
68	+	>100(P)	>60(P)	11.66(P)	3.63	24.21(P)

Table 2. Quantitative values of SLE activity disease biomarkers (ACA-G, ACA-M, Beta-2-G, Beta-2-M, Sm/RNPs) determined by ELISA Hycor system. Characteristic abnormal values are bold.

SLE diagnostic biomarkers of disease activity are serum level of C3, C4, anti-ds-DNAs, anticardiolipin antibodies, anti-SS-A (Ro), anti-Sm, anti-Sm/RNPs and number of T, B lymphocytes and NK cells (CD3, CD4, CD8, CD16+CD56+ NK). Anti-T-lymphocyte antibodies, in fact the presence of autoantibodies to their surface membrane molecules is common appearance at SLE patients. Their role in SLE pathoetiology is still unclear (16, 17).

In some specific cases SLE patients have liver disfunction such as autoimmune hepatitis and primary billiary liver cirrhosis. Treatement of SLE patients depends on individual clinical manifestations and there is no absolute guide in clinical management of lupus nephritis or about SLE in general. The regulator T cells are important in the autoreactivity control and in pathogenesis of many autoimmune inflamatory diseases, as well. The SLE patients can have pasive or active form of disease which is caused by immune complexes accumulation in different tissues and organs such as kidneys, skin, blood vessels and CNS. The hyperactivity of B lymphocytes caused by large amount of autoantibodies, increase the level of cell lymphoid apoptosis and IL-10 is also specific SLE characteristic. In pathoetiology of SLE CD4+T cells are also important (18, 19, 20, 21).

Molecular genetic studies have shown the changes in expression of more than one thousand genes in the cases of systemic lupus erythematosus and rheumatoid arthritis (21) and also changed values of CD4 (22, 23, 24) and CD8 and CD28 molecules on T-lymphocytes, as well (25).

In modern SLE clinical management of disease activity, the level of CD3+, CD4+, CD8+, CD16+CD56+NK cells, is monitored by flow cytometry, for all of these parameters. In some cases the presence of DNT cells (double negative CD4-CD8- T cells) in blood specimens is unusual occurrence. But mainly, for SLE patients, one of the characteristics are, increased level of CD4+ and CD 8+ T-cells which is directly poportional to level of anti-ds-DNA, IgG and erythrocytes sedimentation, but oposite to C3 and C4 components level in serum specimens. Because of all that, these parameters are important in disease activity monitoring. We performed quantitative measurements of these biomarkers by nephelometry, ELISA-Hycor and Flow cytometry is SLE patients serum specimens. Manly, we obtained expected results but in some cases the results were specific for some SLE clinical phenotypes.

We determined the low level of C3 and C4 complement componets. The increasement of immunoglobulin values were regularry in corelation with phenotyping parameters. In most cases we deteced increased levels of CD3 (T-Ly), and decreased levels of CD3+/HLA-DR and total HLA-DR expressed on lymphocyte surfaces. The level of C3 and C4 is monitored several weeks because SLE patients with persistant C3 and C4 low level must be subjected to complement detailed analyses.

5. CONCLUSIONS

Our investigations showed positive correlation between C3, C4 complement components and immunophenotyping parameters and between CD3 (T-Ly) and CD3+/HLA-DR and total HLA-DR. Regadless to SLE specific manifestations the values of CD4:CD8 i CD4 (Th) were always stable and in referal limits. These parameters are not proved as good indicators of SLE disease activity or therapy effectiveness.

In SLE acute phase, decreased level of CD19 (B Ly) and CD16+56+ (NK) is determined and increased level of CD3 (T Ly) i CD8 (CTL) but high level of activated T lymphocytes. In SLE+RA (combination of SLE and rheumatoid arthritis features) clinical phenotypes, low level of NK cells and increased level of T lyphocytes, was determined.

Flow cytometry indicated that therapy was more effective at SLE patients for T lympocytes normalisation, but in any case despite of therapy, the extremelly low level of B cells (CD19) remains stable. The therapy showed more influence to T-cells in comparison to B cells. It would be useful, in further investigations, to take a look to the research of IL-10, IL-15, MMP-3, CD44 i CD25 parameters and determine their role for some specific SLE clinical phenotypes, taking into consideration individual genetic variation.

REFERENCES

1. Doyle HA, Yan J, Liang B, Mamula MJ. Lupus autoantigens: their origins, forms, and presentation. Immunol. Res. 2001; 24; 131-147.
2. Tan EM. Autoantibodies to nuclear antigens: Their immunobiology and medicine. Adv Imm. 1982; 33: 167-239.
3. Wakeland EK, Liu K, Graham RR, Behrens TW. Delineating the genetic basis of systemic lupus erythematosus. Immunity. 2001, 15: 397-408.
4. Gaffney PM, Moser KL, Graham RR, Behrens TW. Recent advances in the genetics of systemic lupus erythematosus. Rheum Dis Clin North Am 2002, 28: 111-126.
5. Okumura N, Nomura M, Tada T. Effects of sample storage on serum C3 assay by immunonephelometry. Clin Lab Sci. 1990; 3; 54-57.
6. Cohen M. Systemic Lupus Erythematosus: diagnosis and classification. Intern Med J. 2004; 34(12): 701-702.
7. Deng YJ, Huang ZX, Zhou CJ, Wang JW, You Y, Song ZQ, Xiang MM, Zhong BY, Hao F. Gene profiling involved in immature CD4+ T lymphocyte responsible for systemic lupus erythematosus. Molecular Immunology. 2006; 43: 1497-1507.
8. Filaci G, Bacilieri S, Fravega M, Monetti M, Contini P, Ghio M, et al. Impairment of CD8+T suppressor cell function in patients with active systemic lupus erythematosus. J Immunol, 2001; 166: 6452-6457.
9. Kavanaugh A, Tomar R, Reveille J, Solomon DH, Homburger HA. Guidelines for clinical use of the antinuclear antibody test and tests for specific autoantibodies to nuclear antigens. American College of Pathologists. Arch. Pathol.Lab. Med. 2000; 124: 71-81.
10. Procedure for C3/C3c and C4/C4c quantitative determination. Dade Behring, Germany, 2005.
11. Hycor Biomedical Autostat TM-II anti-cardiolipin enzyme linked immunosorbent assay (ELISA). Hycor Bic Medic al Inc., USA, 2006.
12. Qian Yu Wei, Chungwen Eun-Hyung Lee F, et al. Elucidation of seventeen human peripheral blood B-cell subsets and quantification of the tetanus response using a density-based method for the automated identification of cell populations in multidimensional flow cytometry data". Cytometry Part B: Clinical Cytometry, 2010; 78B-S69.
13. Loken MR. Immunofluorescence Techniques in Flow Cytometry and Sorting (2nd ed.). Wiley.1990: 341-353.
14. Ornatsky O, Bandura D, Baranov V, Nitz M, Winnik MA, Tanner S. Highly multiparametric analysis by mass cytometry. Journal of Immunological Methods. 2010; 361 (1-2): 1-20.
15. Liu C, Ahearn J. The search for Lupus Biomarkers. Best PractRes Clin Rheumatol. 2009; 23(4): 507-523.
16. Rivest C, Lew RA, Welsing PM, Sangha O, Wright EA. et al. Association between clinical factors, socioeconomic status, and organ damage in recent onset systemic lupus erythematosus. J Rheumatol. 2000; 27: 680-684.
17. Karamehić J, Subašić Đ, Gavrankapetanović F, Zečević L, Eminović I, Memić S, Šerić N, Drače Z. The incidence of antinuclear antibodies (ANA) detected by indirect immunofluorescence Assay (IFA method). Med Arh. 2007; 61 (1), 16-19.
18. Subašić Đ, Karamehić J, Ljuca F, Gavrankapetanović F, Delić-Šarac M, Eminović I, Kovačević D. Correlation of autoantibodies presence detected by IFA-anti ds DNA, IFA-AMA and immunoblotting with corresponding data in clinical management of autoimmune diseases. Bosnian Journal of Basic Medical Sciences. 2008; 8(1): 86-92.
19. Karamehic J, Subasic D, Kasumovic M, Hodzic H, Prljaca-Zecevici L, Tufekcic M, Aganovic-Musinovic I. Clinical management of patients with systemic lupus erythematosus (SLE) with different C1q-CIC and C3 concentrations. Med Arh. 2010; 64(2): 75-79.
20. Subašić Đ, Karamehić J, Gavrankapetanović F, Tvrtković E, Mekić M, Prljača-Zečević L, Muminagić S, Hasković E, Eminović I, Ridić O. Correlation of C3d effector molecules concentrations, C1q-CIC level and disease activity in patients with rheumatoid arthritis. Health Med, 2010; 4(4) supp-1; 1113-1119.
21. Mandel M, Gurevich M, Pauzner R, Kaminski N, Achiron A. Autoimmunity gene expression portrait: specific signature that intersects differentiates between multiple sclerosis and systemic lupus erythematosus. Cli Exp Immunol. 2004; 138: 164-170.
22. Krishnan S, Warke VG, Nambiar MP, Tsokos GC, Farber DL. The FcR gamma subunit and Syk kinase replace the CD3 zeta-chain and ZAP-70 kinase in the TCR signaling complex of human effector CD4 T cells. J Immunol. 2003; 170: 4189-4194.
23. Nambiar MP, Mitchell JP, Ceruti RP, Malloy MA, Tsokos GC. Prevalence of T cell receptor zeta chain deficiency in systemic lupus erythematosus. Lupus. 2003; 12: 46-51.
24. Duriagin S, Ofori H, Jagodzinski PP. Flow cytometric analyzis of CD4+ T cell receptor zeta chain deficiency in patients with systemic lupus erythematosus. Adv In Med Sci. 2006; 51; 181-183.
25. Liu Z, Tugulea S, Cortesini R, Suciu-Foca N. Specific suppression of T helper alloreactivity by allo-MHC class I-restricted CD8, CD28 T cells. Int Immunol. 1998; 10: 775-83.