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Evaluation of PBMC Distribution and TLR9 Expression in Patients with Systemic Lupus Erythematosus

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ABSTRACT

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease which results in damage to various organs. Some animal studies have revealed that activation of Toll-like receptors (TLRs) is important in the pathogenesis of SLE.

In the present study, the percentage of different immune cell subsets in 35 SLE patients and 38 control subjects was analyzed by flow cytometry. We also assessed the expression of TLR9 in the population of peripheral blood mononuclear cells (PBMCs) including T lymphocytes (CD4⁺ and CD8⁺), B lymphocytes (CD19⁺), NK cells (CD56⁺) and monocytes (CD14⁺) in SLE patients and healthy controls.

The results showed that the percentage of CD8⁺ T lymphocytes and CD14⁺ monocytes were significantly higher ($p < 0.001$) in the SLE patients than the healthy control subjects. Moreover, the percentage of CD56⁺ NK cells were significantly lower in the SLE patients than the healthy control subjects ($p = 0.001$).

The findings indicated that the expression of TLR9 was significantly higher in CD4⁺ and CD8⁺ T lymphocytes and CD19⁺ B lymphocytes of SLE patients than in control subjects (all $p < 0.05$). The difference in TLR9 expression are involved in pathogenesis of the SLE, hence it can be used as an indicator for SLE diagnosis.

Keywords: Autoimmunity; Lymphocyte; Monocyte; Systemic lupus erythematosus; Toll-like receptor 9

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex systemic autoimmune disorder that is characterized by B cell hyperactivity, abnormally activated T cells and

other cells of immune system.¹ There is a ten-fifteen times higher frequency of SLE in women.² The exact pathogenesis of SLE remains unidentified, but several genetic, environmental and hormonal factors are involved.³ B cells appear essential to the pathogenesis of SLE because dysfunctions of these cells result in the production of a wide variety of auto-antibodies and immune complex deposition in organs.⁴ Autoantibody-producing B cells may develop by several mechanisms including defective negative selection,⁴ inappropriate function of complement or inhibitory FC receptors^{5,6} and stimulation of Toll-like receptors (TLRs).^{7,8} Recent studies have shown that some abnormality of the innate immune system may also be involved in the immunopathogenesis of SLE. TLRs family is one of the most important pattern recognition receptors of the innate immune system. Toll-like receptors are involved in the immunopathogenesis of systemic lupus erythematosus.⁹ TLRs can stimulate cell by self-molecules such as immune complexes comprising DNA or RNA.^{10,11} Among TLRs, TLR7 and TLR9 may contribute to the immunological response in SLE against self-antigens such as single-stranded RNA (ssRNA) and DNA, respectively.¹² Engagement of these receptors by specific ligands leads to increased expression of proinflammatory cytokines and may increase intensity of autoimmune disease.¹³ TLRs indirectly play an important role in T cell responses through the innate immune system.¹⁴ Nevertheless, expression of TLRs in T cells, also may contribute directly to T cell-mediated immune responses.¹⁵ Although disturbed PBMCs balance might be considered as one of the pathologic mechanisms, there are also controversy about role of TLR9 in SLE. Hence, in the present study, we analyzed percent of CD4⁺ and CD8⁺ T cells, CD19⁺ B cells, CD14⁺ monocytes, CD56⁺ Natural killer cells and the level of TLR9 expression in these cells in patients with SLE compared to healthy controls.

MATERIALS AND METHODS

Patients

Thirty five SLE patients (four males and 31 females; age 37.17±11.04 years) who met the revised classification criteria for SLE from the Shariati Hospital of Tehran university of Medical Sciences were enrolled in this study from October 2011 to March 2012. Informed consent was obtained from all the

patients. 38 sex-and-age normal matched volunteers were recruited to serve as healthy control individuals (eight males and 30 females). Disease activity was scored using the SLE Disease Activity Index (SLEDAI).¹⁶ The study was approved by ethics committee of our university and written informed consent was obtained from each person prior to the study.

PBMCs Preparation

Heparinized whole blood samples were collected from each individual. PBMCs were prepared using Lymphoflot (BioRad, Germany) by density gradient centrifugation.

Flow Cytometry

PBMCs were isolated from whole blood and were divided into 1×10⁶ cells per 5 tubes. Each tube separately incubated with anti-CD4, -CD8, -CD19, -CD14, -CD56 fluorescein isothiocyanate (FITC) - conjugated monoclonal antibodies (BD PharmingenTM, San Jose, CA, USA) and then anti-TLR9 PE-conjugated antibody (Rat IgG2a, κ; BD PharmingenTM, San Jose, CA, USA) added to all of the tubes as described below. For CD markers, the flow cytometric cell surface staining method was used. We carried out cell surface staining by adding 10 µl of anti-human anti-CD marker antibodies conjugated with FITC and 100µl staining buffer. Then PBMCs were washed once with phosphate-buffered saline. For TLR9, the intracellular staining method was used and PBMCs fixed and permeabilized using BD Cytofix/cytopermTM Fixation/Permeabilization kit (San Jose, CA, USA) according to protocol. Then the cells were incubated with PE-conjugated monoclonal antibody against intracellular TLR9. Briefly, PBMCs were first stained with anti-CD marker antibodies, and then fixed and permeabilized with BD Cytofix/cytoperm TM Fixation/Permeabilization kit for 20 minutes on ice. Then the cells were incubated with TLR9 antibody. PBMCs phenotypes were determined using FACS analysis system (Becton-Dickinson). The percentages of CD4⁺, CD8⁺, CD19⁺, CD14⁺, CD56⁺ expressing TLR9 were assessed. Flow cytometry data were analyzed using FlowJo® software (TreeStar Software). In all the experiments, isotype controls were included using an appropriate monoclonal antibodies.

PBMCs Distribution and TLR9 Expression in SLE Patients

Statistical Analysis

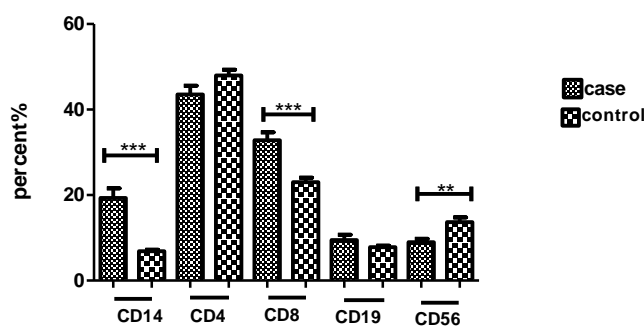
The results are expressed as means±SEM. Student's t test was performed for statistical analysis. $p < 0.05$ was considered significant. All calculations were performed on SPSS for Windows, version 20.

RESULTS

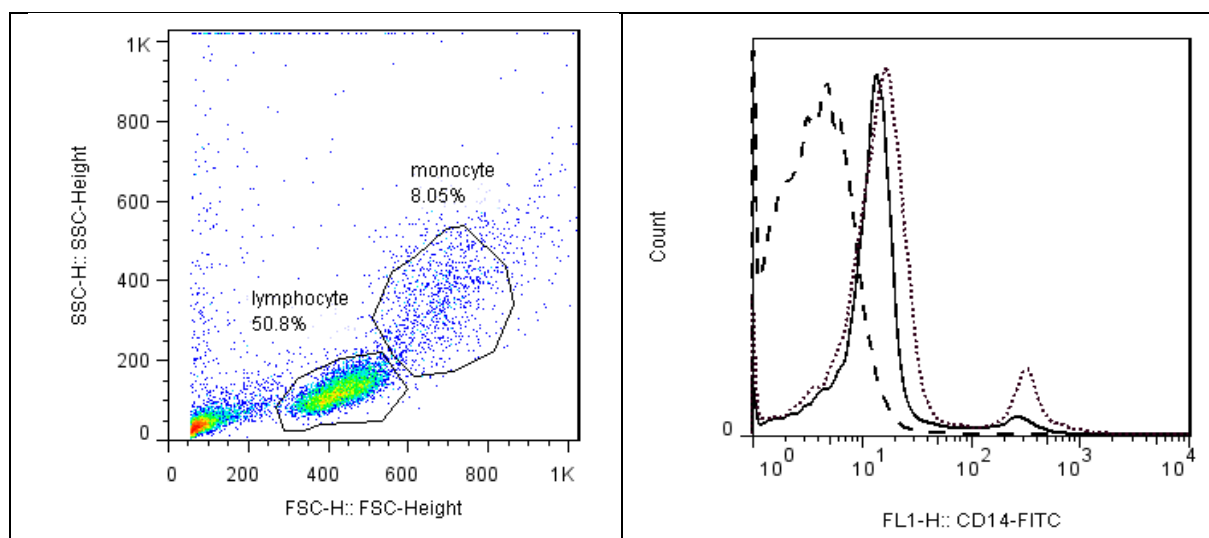
Percentage of Peripheral Blood Mononuclear Cells (PBMCs) in SLE Patients and Control Subjects

PBMCs were gated according to their forward and side scatter characteristics. The target cell population was chosen and the percentage of different subsets of peripheral mononuclear cells in SLE patients and control subjects was identified. The percentage of different immune cell subsets in SLE patients and

control subjects are shown in Figure 1. The results revealed that the percentage of CD4⁺ T lymphocytes was lower in the SLE patients than the control subjects, but this decrease was not significant. On the other hand, the percentage of CD8⁺ T lymphocytes was significantly higher in SLE patients ($p < 0.001$). The ratio of CD4⁺ T lymphocytes to CD8⁺ T lymphocytes was significantly lower in SLE patients than control group (Figure 2). The percentage of CD19⁺ B cells was higher in SLE patients, however it was not significant. The percentage of CD56⁺ NK cells was significantly lower in the SLE patients than the control group ($p = 0.001$). The percentage of CD14⁺ monocytes was significantly higher in SLE patients than control group ($p < 0.001$).



(a)



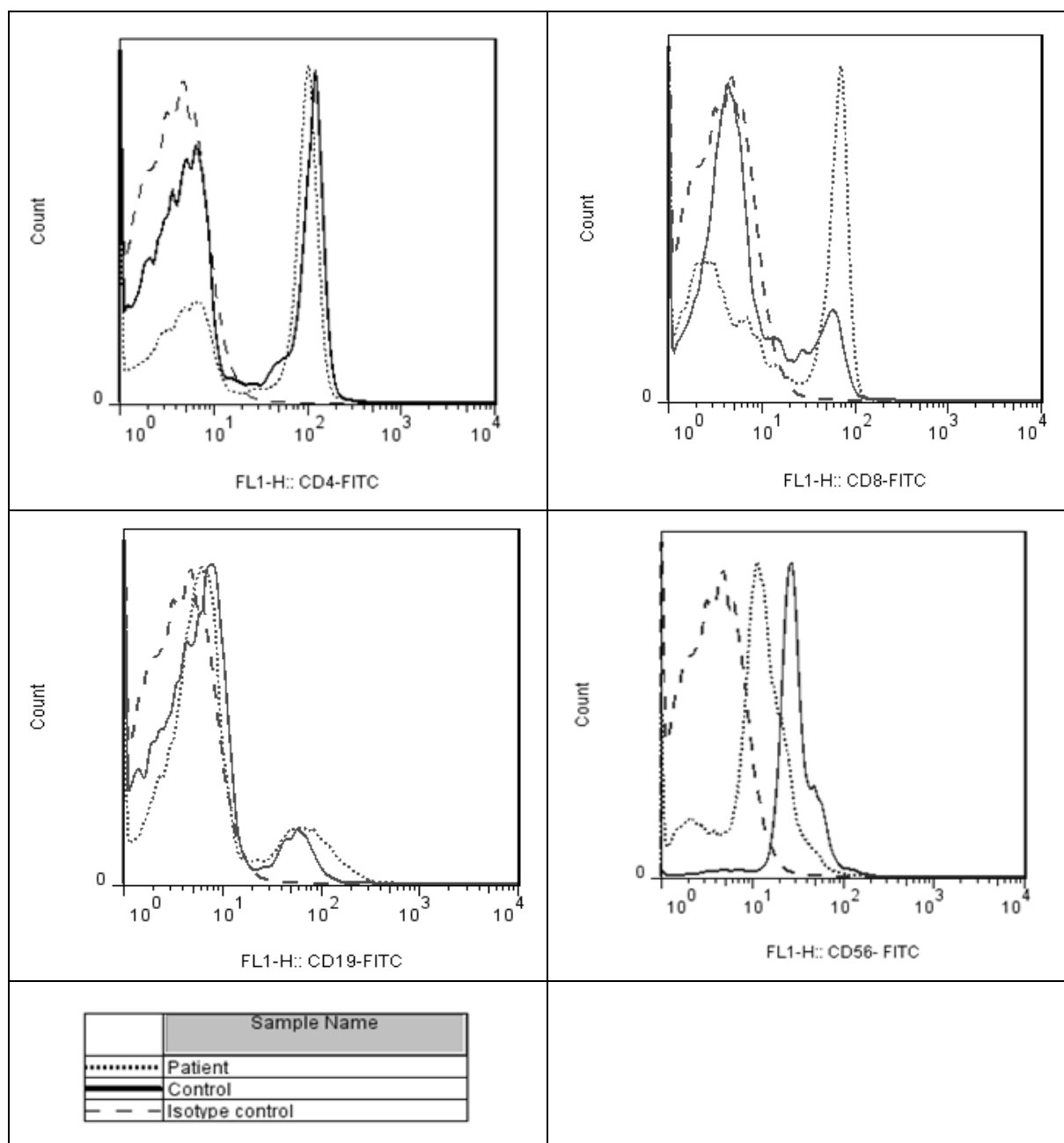


Figure1. Percentage of different immune cell subsets. (a) The percentage of peripheral blood mononuclear cells (PBMCs) in patients with SLE and healthy controls ($p=0.001$, $p<0.001$, mean \pm SE). (b) Representative dot plot analysis of flow cytometry showing the gating for peripheral blood mononuclear cells (PBMCs) and histogram Overlay data in patients and healthy controls were analyzed using FlowJo® software.

Expression of TLR9 on PBMCs

As shown in Figure 3, the expression of TLR9 was significantly higher in CD4⁺ and CD8⁺ T lymphocytes and CD19⁺ B lymphocytes of SLE patients than in control subjects (all $p < 0.05$). The percentage of TLR9

was slightly higher in CD56⁺ NK cells of SLE patients than in control subjects, however it was not significant. TLR9 expression in CD14⁺ monocytes was not different between SLE patients and control subjects.

PBMCs Distribution and TLR9 Expression in SLE Patients

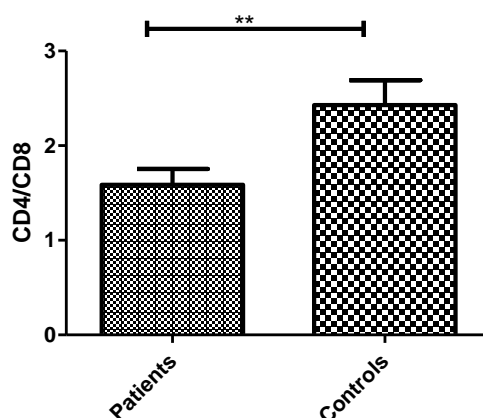


Figure2. Ratio of CD4⁺ to CD8⁺ T lymphocytes. The ratio of CD4⁺ to CD8⁺ T lymphocytes was compared between SLE patients and control subjects. ($p=0.009$, mean \pm SEM).

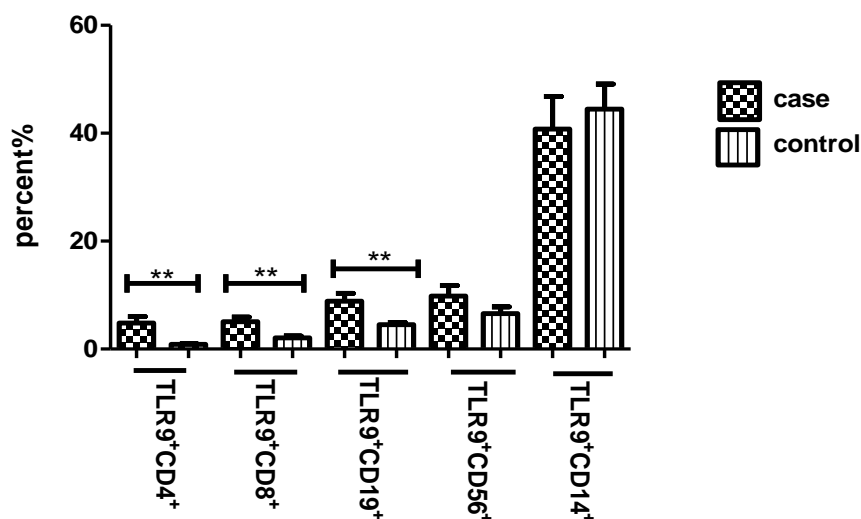


Figure3. Expression of TLR9 in different immune cell subsets. The percentage of TLR9 in CD4⁺ T lymphocytes ($p=0.003$, mean \pm SEM), CD8⁺ T lymphocytes ($p=0.001$, mean \pm SEM), CD19⁺ B lymphocytes ($p=0.003$, mean \pm SEM), CD56⁺ NK cells (mean \pm SEM), CD14⁺ monocytes (mean \pm SEM) were compared between SLE patients and control subjects.

DISCUSSION

In this study, we examined the percentage of peripheral blood mononuclear cells in SLE patients and compared with control group. We found that the percentage of CD8⁺ T lymphocytes was significantly higher and CD4/CD8 ratio was decreased in SLE patients. No significant difference was seen in the percentage of B cells. Blanco et al. revealed that increase in number and function of CD8⁺ T

lymphocytes may lead to release of higher amounts of auto-antigens.¹⁷ On the other hand increased immune complexes may result in cross presentation by dendritic cells which lead to differentiation of CD8⁺ T lymphocytes.^{17,18} This findings support self-sustaining of autoimmunity in SLE.¹⁷ SLE T cells contribute to a skewing of the T cell response towards heightened effector and deficient regulatory functions.³ Studies showed that in SLE disease, T cells are abnormal in the phenotype, receptor and signaling, gene transcription,

and regulatory lymphocytes.¹⁵

Our results showed that the percentage of CD56⁺ NK cells was significantly lower in the SLE patients than the control group. In accordance to our finding, Park et al.¹⁹ reported that percentages and absolute numbers of NK cells, cytotoxic activities were significantly lower in the peripheral blood of SLE patients than in healthy control subjects. Previous studies also reported that NK cell numbers and activities are reduced in patients with systemic autoimmune diseases.^{20,21} B. Hervier et al.²² in accordance with our study showed that the absolute NK cell count was decreased in patients with active SLE. This impairment might be due to a defect in T cells function which is important in NK cell generation and activation. Functional assay for CD8⁺ and NK cells may provide better understanding of SLE pathogenesis.

We showed that the percentage of CD14⁺ monocytes was significantly higher in SLE patients. Recent studies emphasize the importance of innate immunity in SLE. Monocytes and macrophages are major components of the innate immune system. In comply with our findings abnormalities in phenotype and function of monocyte are reported in SLE.²³ A rise in the activation of monocytes resulting in immune dysregulation has been observed.²⁴

There is several evidence reporting that TLRs as another part of innate immunity, participate in the SLE autoimmune responses.²⁵ The precise effect of TLR9 in SLE is not clear and there are controversial findings.¹² In the present study the TLR9 expression on different subsets of PBMCs also were studied in SLE patients. Our findings revealed that the expression of TLR9 was significantly higher in CD4⁺ and CD8⁺ T lymphocytes of SLE patients than in control subjects (Figure 3). Increased expression level of TLR9 in CD4⁺ and CD8⁺ T lymphocytes of SLE is in line with the previous studies.^{25,26} It has been established that TLRs expressed on T cells function as a costimulatory receptor in activated T cells.²⁷ Previous studies suggested evaluation of TLRs expression on T-cell sub-populations in various autoimmune diseases.²⁸ Our results showed significant increased expression of TLR9 in CD19⁺ B cells from SLE patients. Other studies demonstrated that TLR9 expressing proportion of plasma cells and memory B cells were higher in active SLE patients and TLR9 expression levels of B cells were increased in SLE patients.^{29,30} Migita et al. and Nakano et al. showed that human peripheral blood

B cells express TLR9 and its expression is increased in patients with SLE. This elevated expression of TLR9 in B cells may be related to the abnormal B cell hyperactivity in patients with SLE.^{30,31} Our results similarly confirmed this augmentation. There is the perpetuation of autoimmune responses to nucleic acid self-antigens in SLE which is related to TLR9 and TLR7.¹² Immune complexes formed during the pathogenesis of SLE bind to FcγRIIIa on plasmacytoid dendritic cells (pDCs) and they be internalized to interact with endosomal TLRs. Then pDCs release interferon alpha (IFN-α) which influences pDCs for being more efficient antigen presenting cell (APC) for autoreactive T cells. Immune complexes may trigger B cells by engagement of BCR/TLR which results in B cell activation and production of autoantibodies. These autoantibodies perpetuate formation of immune complexes.¹²

Our results show slight but no significant increase of intracellular TLR9 protein expression in CD56⁺ NK cells from SLE patients. NK cells present regulatory function in normal situation but deleterious role in pathogenesis of autoimmune diseases.³² Increased TLR expression on NK cells was observed in some autoimmune diseases.^{33,34} Chronic activation of these molecules induce the expansion of atypical NK cells in mouse models of SLE.³⁵ In the current study, the percentage of TLR9 was to some extent increased in CD56⁺ NK cells of SLE patients, though it was not significant. Further studies will be requisite to determine the particular role of NK cells in SLE.

To put in a nut shell, we showed that PBMCs balance is disturbed in SLE patients and difference in TLR9 expression can be attributed as an indication of functional deviation.

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REFERENCES

1. Mok C, Lau C. Pathogenesis of systemic lupus erythematosus. *J Clin Pathol* 2003; 56(7):481-90.
2. Means TK, Luster AD. Toll-like receptor activation in the pathogenesis of systemic lupus erythematosus. *Ann N Y Acad Sci* 2005; 1062:242-51.
3. Crispín JC, Liossis S-NC, Kis-Toth K, Lieberman LA,

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- Kyttaris VC, Juang Y-T, et al. Pathogenesis of human systemic lupus erythematosus: recent advances. *Trends Mol Med* 2010; 16(2):47-57.
- Mackay F, Schneider P, Rennert P, Browning J. BAFF AND APRIL: a tutorial on B cell survival. *Annu Rev Immunol* 2003; 21(1):231-64.
 - Carroll MC. A protective role for innate immunity in systemic lupus erythematosus. *Nat Rev Immunol* 2004; 4(10):825-31.
 - McGaha TL, Sorrentino B, Ravetch JV. Restoration of tolerance in lupus by targeted inhibitory receptor expression. *Science* 2005; 307(5709):590-3.
 - Marshak-Rothstein A. Toll-like receptors in systemic autoimmune disease. *Nat Rev Immunol* 2006; 6(11):823-35.
 - Christensen SR, Shlomchik MJ, editors. Regulation of lupus-related autoantibody production and clinical disease by Toll-like receptors. *Semin Immunol* 2007; 19(1):11-23.
 - Guggino G, Giardina AR, Ciccia F, Triolo G, Dieli F, Sireci G. Are toll-like receptors and decoy receptors involved in the immunopathogenesis of systemic lupus erythematosus and lupus-like syndromes? *Clin Dev Immunol* 2011; 2012:135932.
 - Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol* 2003; 21(1):335-76.
 - Baccala R, Hoebe K, Kono DH, Beutler B, Theofilopoulos AN. TLR-dependent and TLR-independent pathways of type I interferon induction in systemic autoimmunity. *Nat Med* 2007; 13(5):543-51.
 - Kim WU, Sreih A, Bucala R. Toll-like receptors in systemic lupus erythematosus; prospects for therapeutic intervention. *Autoimmun Rev* 2009 ; 8(3):204-8.
 - Rahman AH, Eisenberg RA. The role of toll-like receptors in systemic lupus erythematosus. *Springer Semin Immunopathol* 2006; 28(2):131-43.
 - Liossis S, Ding XZ, Dennis GJ, Tsokos GC. Altered pattern of TCR/CD3-mediated protein-tyrosyl phosphorylation in T cells from patients with systemic lupus erythematosus. Deficient expression of the T cell receptor zeta chain. *J Clin Invest* 1998; 101(7):1448-57.
 - Mak A, Kow NY. The pathology of T cells in systemic lupus erythematosus. *J Immunol Res* 2014; 2014.
 - Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH. Derivation of the SLEDAI. A disease activity index for lupus patients. *Arthritis Rheum* 1992; 35(6):630-40.
 - Blanco P, Pitard V, Viallard JF, Taupin JL, Pellegrin JL, Moreau JF. Increase in activated CD8+ T lymphocytes expressing perforin and granzyme B correlates with disease activity in patients with systemic lupus erythematosus. *Arthritis Rheum* 2005; 52(1):201-11.
 - Dhodapkar KM, Krasovsky J, Williamson B, Dhodapkar MV. Antitumor monoclonal antibodies enhance cross-presentation of cellular antigens and the generation of myeloma-specific killer T cells by dendritic cells. *J Exp Med* 2002; 195(1):125-33.
 - Park YW, Kee SJ, Cho YN, Lee EH, Lee HY, Kim EM, et al. Impaired differentiation and cytotoxicity of natural killer cells in systemic lupus erythematosus. *Arthritis Rheum* 2009; 60(6):1753-63.
 - Green M, Kennell A, Larche M, Seifert M, Isenberg D, Salaman M. Natural killer cell activity in families of patients with systemic lupus erythematosus: demonstration of a killing defect in patients. *Clin Exp Immunol* 2005; 141(1):165-73.
 - Gonzalez-Amaro R, Alcocer-Varela J, Alarcon-Segovia D. Natural killer cell activity in the systemic connective tissue diseases. *J Rheumatol* 1988; 15(8):1223-8.
 - Hervier B, Beziat V, Haroche J, Mathian A, Lebon P, Ghillani-Dalbin P, et al. Phenotype and function of natural killer cells in systemic lupus erythematosus: Excess interferon- γ production in patients with active disease. *Arthritis Rheum* 2011; 63(6):1698-706.
 - Li Y, Lee PY, Reeves WH. Monocyte and macrophage abnormalities in systemic lupus erythematosus. *Arch Immunol Ther Exp (Warsz)* 2010; 58(5):355-64.
 - Byrne JC, Ní Gabhann J, Lazzari E, Mahony R, Smith S, Stacey K, et al. Genetics of SLE: functional relevance for monocytes/macrophages in disease. *Clin Dev Immunol* 2012; 2012:582252.
 - Klonowska-Szymczyk A, Wolska A, Robak T, Cebula-Obrzut B, Smolewski P, Robak E. Expression of toll-like receptors 3, 7, and 9 in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. *Mediators Inflamm* 2014; 2014.
 - Wong C, Wong P, Tam L, Li E, Chen D, Lam C. Activation profile of Toll-like receptors of peripheral blood lymphocytes in patients with systemic lupus erythematosus. *Clin Exp Immunol* 2010; 159(1):11-22.
 - Komai-Koma M, Jones L, Ogg GS, Xu D, Liew FY. TLR2 is expressed on activated T cells as a costimulatory receptor. *Proc Natl Acad Sci U S A* 2004; 101(9):3029-34.
 - Chuang T-H, Ulevitch RJ. Triad3A, an E3 ubiquitin-protein ligase regulating Toll-like receptors. *Nat Immunol* 2004; 5(5):495-502.
 - Papadimitraki ED, Choulaki C, Koutala E, Bertias G,

- Tsatsanis C, Gergianaki I, et al. Expansion of toll-like receptor 9-expressing B cells in active systemic lupus erythematosus: Implications for the induction and maintenance of the autoimmune process. *Arthritis Rheum* 2006; 54(11):3601-11.
30. Migita K, Miyashita T, Maeda Y, Nakamura M, Yatsushashi H, Kimura H, et al. Toll-like receptor expression in lupus peripheral blood mononuclear cells. *J Rheumatol* 2007; 34(3):493-500.
31. Nakano S, Morimoto S, Suzuki J, Nozawa K, Amano H, Tokano Y, et al. Role of pathogenic auto-antibody production by Toll-like receptor 9 of B cells in active systemic lupus erythematosus. *Rheumatology* 2008; 47(2):145-9.
32. Tian Z, Gershwin ME, Zhang C. Regulatory NK cells in autoimmune disease. *J Autoimmun* 2012; 39(3):206-15.
33. Richez C, Blanco P, Rifkin I, Moreau J-F, Schaefferbeke T. Role for toll-like receptors in autoimmune disease: the example of systemic lupus erythematosus. *Joint Bone Spine* 2011; 78(2):124-30.
34. Tadema H, Abdulahad WH, Stegeman CA, Kallenberg CG, Heeringa P. Increased expression of Toll-like receptors by monocytes and natural killer cells in ANCA-associated vasculitis. *PloS one* 2011; 6(9):e24315.
35. Voynova EN, Skinner J, Bolland S. Expansion of an Atypical NK Cell Subset in Mouse Models of Systemic Lupus Erythematosus. *J Immunol* 2015; 194(4):1503-13.