Targeting the humoral immune system of patients with rheumatoid arthritis

Teng, Y.K.O.

Chapter 5

Enhanced differentiation of B-cells towards immunoglobulin-secreting cells in rheumatoid arthritis

Teng YKO
Levarht EWN
Huizinga TWJ
Toes REM
van Laar JM

Submitted

Abstract

Objectives

To investigate the distribution of lymphocytes, notably B-cells, in peripheral blood (PB) and bone marrow (BM) of RA patients as compared to healthy controls, and to analyze its association with inflammation in RA.

Methods

From 21 RA patients, positive for rheumatoid factor (RF) and anti-cyclic citrullinated protein autoantibodies (ACPAs), mononuclear cells (MCs) from PB, BM and synovium were analyzed by flowcytometry and immunohistochemistry and compared to healthy controls. Serum immunoglobulins and RA-specific autoantibodies were analyzed in relation to CRP-level, as a measure of inflammation.

Results

Significantly lower proportions of CD19+ B-cells were found in RA-PBMCs (median 6.42% [range: 1.77-18.4]) compared to healthy controls (median 9.34% [4.79-18.3]; p=0.046) and also in BMMCs (medians 7.33% [range: 2.03-24.9] and 12.8% [range: 5.71-32.7]; p=0.016, respectively). B-cell subset analysis revealed that CD27+ post-switched B-cells were reduced in RA-PBMCs (median 2.32% [range: 0.33-6.13]) versus healthy controls (median 3.93% [range: 1.93-10.2]; p=0.008) and in RA-BMMCs (medians 1.78% [range: 0.61-5.57]) versus 3.46% [range: 1.84-6.58]; p=0.004). CD3-CD38brIgD+ plasmablasts (median 0.02% [range: 0.0-0.08]) and CD3-CD38brIgD- plasma cells (median 0.16% [range: 0.02-0.96]) were significantly increased in RA-PBMCs compared to healthy controls (medians 0.002% [range: 0.0-0.02]; p=0.027 and 0.06% [range: 0.01-0.31]; p=0.009, respectively). Moreover, high CRP-levels in RA were associated with significantly higher serum immunoglobulin (mean±SEM: 18.4±1.56 g/L), ACPA-IgG (mean±SEM: 783±133) and RF-IgM levels (mean±SEM: 118±24) than low CRP-levels (mean±SEM: 14.2±1.14 g/L [p=0.05]; 433±88 [p=0.048]; 41±7.1 [p=0.016], respectively).

Conclusion

RA is characterized by inflammation-dependent, enhanced B-cell differentiation combined with functional hyperactivity of immunoglobulin-secreting cells.

Introduction

Autoimmune diseases (AIDs), such as rheumatoid arthritis (RA), are chronic inflammatory illnesses of unknown etiology, commonly accompanied by the presence of circulating autoantibodies. The humoral immune system has long been known to be involved in the pathogenesis of RA. Already in the 60s increased serum immunoglobulin levels were reported in RA patients as compared to healthy controls[1]. Moreover, the presence of rheumatoid factor (RF) or anti-cyclic citrullinated protein antibodies (ACPA) was associated with more severe disease and worse prognosis, although they are not a prerequisite for RA[2,3]. Recently, several clinical trials demonstrated the efficacy of B-cell depletion with rituximab, an anti-CD20 monoclonal antibody, in reducing RA disease activity and radiographic progression[4]. However, the exact contribution of B-cells to RA pathogenesis is still subject to debate.

The introduction of B-cell depleting therapy triggered several studies to investigate the mechanisms through which B-cell depletion ameliorated RA-related disease activity. However, even though eradication of circulating B-cells by rituximab was complete, the return of these B-cells did not uniformly precede relapse of disease[5,6]. Moreover, a recent study demonstrated that a high B-cell load in synovium was associated with non-responsiveness to treatment with rituximab[7]. Also, rituximab only led to the depletion of CD20+ B-cells leaving a significant proportion of CD20- B-lineage cells untouched[7]. Therefore, it still remains unclear how B-cell depletion interferes with pathological processes in RA.

In the present study we performed a comprehensive analysis of lymphocyte distribution, notably of B-cells, in blood, bone marrow and synovium of RA patients versus healthy controls in order to further dissect the potential pathogenetic role of B-cells in RA. In addition, the relationship between systemic inflammation and the distribution of B-cells and serum immunoglobulin was examined. We hypothesized that inflammation would drive differentiation of autoreactive B-cells towards immunoglobulin-secreting cells, resulting in altered distribution of B-cells and increased immunoglobulin-production including autoantibodies.

Patients & Methods

Patients

The study involved paired samples of blood and bone marrow from 21 patients with severe RA who were positive for IgM rheumatoid factor (RF-IgM) and IgG autoantibodies against cyclic citrullinated peptides (ACPA-IgG). These patients were selected from a cohort of 28 refractory RA patients who participated in a single-center, open-label, phase I/II trial to investigate the safety, feasibility and efficacy of maintenance treatment with rituximab, as described previously[7]. Seven patients were excluded for the following reasons: incomplete sampling in 3 patients and 4 patients were seronegative for RF-IgM or ACPA-IgG. The control group for analysis of peripheral blood ('PB controls') comprised 14 sex-matched healthy volunteers. The control group for analysis of bone marrow ('BM controls') consisted of 15 healthy persons, who were screened for donor eligibility in the context of allogeneic bone marrow transplantation for patients with hematologic malignancies. With the exception of age and sex, demographical and medical data of the control groups were not available for the researchers. The study protocol was approved by the Ethics Committee of the Leiden University Medical Center and all patients gave written informed consent.

Assessment of inflammation

Disease activity was assessed by the four variable disease activity score of 44 joints (DAS_{44}) according to EULAR guidelines, erythrocyte sedimentation rate and C-reactive protein. Functional disability was assessed with the Health Assessment Questionnaire (HAQ).

Flowcytometric analysis

Heparinized blood samples and bone marrow aspirates (anti-coagulated by ethylene diamine tetra-acetic acid [EDTA]) were obtained as described previously[7]. Peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) were isolated by density gradient centrifugation over Ficoll-Hypaque (LUMC, Leiden, The Netherlands). PBMCs and BMMCs were immediately stained for flowcytometric analysis. Stainings were performed by incubating fresh mononuclear cells with mouse anti-human monoclonal antibodies (mAbs) in phosphate-buffered saline (PBS)/1% bovine serum albumine (BSA) at

4°C for 10 minutes. The following mAbs were used in different combinations: anti-CD4 fluorescein isothiocyanate (FITC) mAb (clone RPA-T4); anti-CD14-FITC (MSE2); anti-CD19-FITC (H1B19); anti-CD8-PE (RPA-T4); anti-CD16-PE (B73-1); anti-CD19-PE (H1B19); anti-CD27-PE (L128); anti-CD56-PE (MY31); anti-IgD-PE (IAG-2); anti-CD19-PerCp-Cy5.5 (SJ25C1); anti-CD38-PerCp-Cy5.5 (HIT-2); anti-CD3-APC (UCHT1) (all from Benton Dickinson, San Jose, CA). After incubation cells were fixed in 4% paraformaldehyde (LUMC, Leiden, The Netherlands) and analyzed within 24-48 hrs. Stained cells were analyzed with a FACScalibur (Becton Dickinson) flowcytometer and the associated software program FlowJo (Tree Star Inc., Ashland, USA) was used to calculate frequencies within the lymphocyte population.

Measurements of serum antibody levels

Serial serum samples of each patient were analyzed for serum levels of total immunoglobulins, autoantibodies and exogenous antigen-specific antibodies. Total serum levels of IgG, IgM and IgA were measured by immunoturbidimetry on the COBAS Integra 400/700/800 (Roche Diagnostics, Indianapolis, Indiana, USA) according to the manufacturer's guidelines.

Serum levels of rheumatoid factor (RF) of the IgM isotype (RF-IgM) were measured using a standardized ELISA, as previously described[8].

Serum levels of anti-cyclic citrullinated peptide (ACPA) of the IgG isotype (ACPA-IgG), IgM-isotype (ACPA-IgM) and IgA-isotype (ACPA-IgA) were measured using a commercial ELISA (Immunoscan RA, mark 2; Euro-Diagnostica, Arnhem, The Netherlands), according to the manufacturer's instructions and as previously described[9].

Arthroscopy, synovial tissue sampling and immunohistochemical analysis

Arthroscopy of clinically affected knees and sampling of synovial tissue specimens was done in all patients as previously described[7]. Paraffin-embedded sections were cut for staining with mouse anti-huCD20cy (clone L26), mouse anti-huCD79a (clone JCD117), mouse anti-human Ki-67 (clone MIB-1), mouse anti-huCD68 (clone KP1), rabbit anti-mouse Ig isotype control (code X0936) (all from Dako Netherlands BV, Heverlee, Belgium), rabbit anti-huCD3 (clone SP7, Neomarkers, Fremont, USA), mouse anti-huCD138 (clone B-B4, Serotec, Oxford, UK), mouse IgG_1-biotin isotype control (clone J1D9, Ancell/Kordia, Leiden, The

Netherlands) and purified mouse IgG_1 isotype control (BD Pharmingen, NJ, USA). Stained sections were coded and randomly analyzed as previously described[7].

Statistical analysis

Non-parametric tests were used to compare RA patients with healthy controls. Mann-Whitney U tests for independent samples were used to compare baseline characteristics, percentages of lymphocytes and their subsets and to compare RA patients dichotomized for CRP-level. P-values were considered significant when $p \leq 0.05$.

Results

Study Patients

Twenty-one patients with refractory RA were included in the study. The patients' median age was 53 (range: 33-75) years, 16 patients were female (76%) and the median disease duration was 13.2 (range: 1.3-53.2) years. Patients had a median DAS_{44} score of 3.70 (range: 2.17-5.02), a median erythrocyte sedimentation rate of 43 (range: 4-114) mm/1^{st} hr, and a median CRP level of 24.5 (range: 2-97) mg/L. The median Health Assessment Questionnaire (HAQ) score was 1.63 (range: 0.13-2.88) and joint damage, assessed on x-rays of hands and feet, yielded a median score of 49 (range: 20-194). With respect to the control groups, PB controls were younger with a median age of 42 years (range: 33-61; p=0.01), 9 were (64%) female (p=0.45). The BM controls had a median age of 41 years (range: 28-61; p=0.004) and 5 (33%) were female (p=0.01).

Distribution of lymphocytes in blood, bone marrow and synovium of RA compared to healthy controls

In peripheral blood, we observed that RA patients and healthy controls had comparable proportions of CD3+CD4+ T-helper cells, CD3+CD8+ cytotoxic T-cells, CD3+CD16/56+ natural killer (NK-) T-cells, CD3-CD16/56+ NK-cells and CD14+ monocytes (Table 1). RA patients had significantly lower proportions of CD19+ B-cells (median 6.42% [range: 1.77-18.4]) as compared to healthy controls (median 9.34% [4.79-18.3]; p=0.046). Also, significantly more circulating CD3-CD38br immunoglobulin-secreting cells were observed in RA (median 0.21% [range: 0.03-1.01]) as compared to healthy controls (median

Table 1 Distribution of lymphocytes in *peripheral blood* of RA patients compared to healthy controls

	RA Median [range]	**Healthy Median [range]**	**p-value**
Peripheral Blood			
T-helper cells CD3+CD4+	24.2 [2.82-57.3]	33.8 [6.69-65.5]	0.552
Cytotoxic T-cells CD3+CD8+	20.1 [6.54-52.9]	30.3 [16.1-65.4]	0.100
Natural killer T-cells CD3+CD16/56+	3.80 [0.51-22.5]	6.54 [2.40-26.2]	0.278
Natural killer cells CD3+CD16/56+	9.56 [3.59-31.3]	11.5 [5.88-17.7]	0.612
Monocytes* CD14+	11.5 [3.97-26.5]	14.2 [10.3-30.1]	0.177
B-cells CD3-CD19+	6.42 [1.77-18.4]	9.34 [4.79-18.3]	**0.046**
Plasma cells CD3-CD38br	0.21 [0.03-1.01]	0.06 [0.01-0.33]	**0.004**
Bone Marrow			
T-helper cells CD3+CD4+	14.7 [3.62-54.7]	13.7 [8.25-27.3]	0.640
Cytotoxic T-cells CD3+CD8+	13.7 [2.27-50.0]	11.6 [9.86-26.2]	0.959
Natural killer T-cells CD3+CD16/56+	2.37 [0.35-15.9]	1.78 [0.22-4.94]	0.119
Natural killer cells CD3+CD16/56+	9.08 [2.75-16.1]	6.84 [2.19-17.2]	0.136
Monocytes/Macrophages* CD14+	10.5 [3.65-35.4]	4.64 [2.38-9.65]	**0.001**
B-cells CD3-CD19+	7.33 [2.03-24.9]	12.8 [5.71-32.7]	**0.016**
Plasma cells CD3-CD38br	0.37 [0.06-1.07]	0.37 [0.10-0.69]	0.822

*In contrast to all other lymphocytes subsets, CD14+ cells were analyzed as a percentage of all living cells

0.06 [range: 0.01-0.33]; p=0.004). In bone marrow significantly lower frequencies of CD19+ B-cells were found in RA patients (median 7.33% [range: 2.03-24.9]) than in healthy controls (median 12.8% [range: 5.71-32.7]; p=0.016) were found. Again, both groups did not differ with respect to CD3+ T-cell subsets and NK cell subsets, yet did for CD14+ monocytes (median 10.5% [range: 3.65-35.4]) in RA compared to healthy controls (median 4.64% [range: 2.38-9.65]; p=0.001) (Table 1). In synovium, RA patients showed typical infiltration of CD3+ T-cells (median 3 [range 0-4]), CD20+ (median 2 [range 0-4]) and CD79a+ B-cells (median 2 [range 0-4]), CD138+ plasma cells (median 1 [range 0-4]) and CD14+ macrophages (median 3 [range 1-4]).

Skewed distribution of B-lymphocyte subsets towards terminally differentiating, immunoglobulin-secreting cells

Focusing on B-cell subsets, RA patients had significantly lower proportions of CD19+CD27+ post-switched B-cells (median 2.32% [range 0.33-6.13]) and CD19+IgD+ naïve B-cells (median 3.58% [range 0.60-15.4]) in blood as compared to healthy controls (median 3.93% [range 1.93-10.2]; p=0.008 and median 8.11% [range 3.90-15.7]; p=0.009, respectively) (Table 2). Also, with respect to ISCs, CD3-CD38brIgD+ immature plasmablasts (median 0.02% [range 0.0-0.08]) and CD3-CD38brIgD- mature plasma cells (median 0.16% [range 0.02-0.96]) were significantly increased in RA patients compared to healthy controls (median 0.002% [range 0.0-0.02]; p=0.027 and median 0.06% [range 0.01-0.31]; p=0.009, respectively). In bone marrow, the CD3-CD19+CD27+ post-switched B-cells were significantly lower in RA patients (median 1.78% [range 0.61-5.57]) compared to healthy controls (median 3.46% [range 1.84-6.58]; p=0.004) (Table 2). Taken together, these data demonstrated that B-cell distribution in RA patients was skewed towards the stadium of ISCs with reduced proportions of non-secreting, matured B-cells.

A high degree of inflammation in RA is associated with increased immunoglobulin secretion

To further investigate whether the observed skewing in B-cell distribution towards ISCs was related to inflammation, we dichotomized the group of RA patients according to their CRP levels. Using a median CRP level of 24.5 g/L as cut-off, we compared serum immunoglobulin levels and autoantibody levels between the high CRP-group (patients with CRP > 24.5 g/L) and the low CRP-

Table 2 Distribution of B-lymphocyte subsets in peripheral blood and bone marrow of RA patients compared to healthy controls.

Subset	RA Median [range]	Healthy Median [range]	p-value
Pre-switched B-cells CD3- CD19+ IgD+			
Peripheral blood	3.58 [0.60-15.4]	8.11 [3.90-15.7]	**0.009**
Bone marrow	5.09 [0.61-21.2]	7.20 [2.23-12.2]	0.160
Post-switched B-cells CD3-CD19+CD27+			
Peripheral blood	2.32 [0.33-6.13]	3.93 [1.93-10.2]	**0.008**
Bone marrow	1.78 [0.61-5.57]	3.46 [1.84-6.58]	**0.004**
Early plasma cells CD3-CD38br IgD+			
Peripheral blood	0.018 [0.0-0.081]	0.002 [0.0-0.021]	**0.027**
Bone marrow	0.043 [0.0-0.274]	0.077 [0.034-0.233]	0.114
Late plasma cells CD3-CD38br IgD-			
Peripheral blood	0.157 [0.024-0.959]	0.063 [0.011-0.305]	**0.009**
Bone marrow	0.294 [0.056-0.854]	0.245 [0.070-0.461]	0.349

group (patients with CRP ≤ 24.5). We observed that the high CRP-group had significantly higher serum levels of total immunoglobulins (mean±SEM: 18.4±1.56 g/L) than the low CRP-group (mean±SEM: 14.2±1.14 g/L; p=0.05) (Figure 1A). All isotypes were increased in the high CRP-group, notably IgM (mean±SEM: 2.07±0.31 versus 1.20±0.19, respectively [p=0.02]) and IgA (mean±SEM: 3.36±0.57 versus 2.08±0.28, respectively [p=0.05]). With respect to autoantibodies, we found that ACPA-IgG levels (mean±SEM: 783±133) and rheumatoid factor IgM (mean±SEM: 118±24) were significantly elevated in the high CRP-group as compared to the low CRP-group (433±88; p=0.048 and 41±7.1; p=0.016 respectively) (Figure 1B). Of note, IgG-antibodies against exogenous antigens, i.e. rubella, mumps and measles, were not significantly different between the high and low CRP-group: circulating levels of anti-rubella

IgG (mean±SEM: 197±46.4 IU/mL versus 140±43.7 IU/mL; p=0.38), anti-measles IgG (mean±SEM: 3.61±0.45 AU/mL versus 3.68±0.49 AU/mL; p=0.74) or anti-mumps IgG (mean±SEM: 2.66±0.27 AU/mL versus 2.28±0.38 AU/mL; p=0.11) (data not shown). Collectively, these data functionally supported the notion that B-cell distribution in RA was skewed towards the stadium of ISCs, which was related to the level of inflammation.

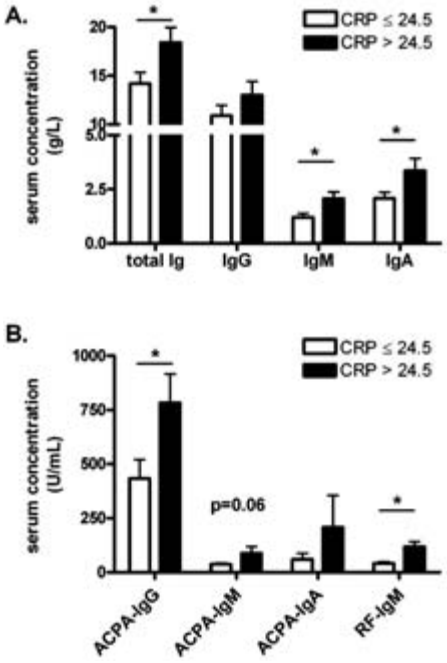

Figure 1 A: Serum immunoglobulins and B: autoantibodies of RA patients dichotomized for the level of inflammation, measured by C-reactive protein (group median of 24.5 g/L was used as cut-off)

Discussion

The present study demonstrated that of all lymphocyte subsets, mainly the proportion of B-lymphocytes in peripheral blood and bone marrow differed between RA patients and healthy controls, i.e. by being significantly lower. B-lymphocyte subset analysis revealed that a reduction of post-switched B-cells was predominantly responsible for the observed differences. In contrast, a higher circulating fraction of phenotypic ISCs was present in RA, the functional relevance of which was underpinned by the association between inflammation and high serum levels of total immunoglobulins and RA-specific autoantibodies. Collectively, these data indicated that RA patients showed enhanced differentiation of B-cells towards plasma cells.

The present study is the first to report on B-cell distribution in serum, blood, bone marrow and synovium of refractory RA patients. In line with previous studies in SLE patients[10,11] and patients with Sjögren's syndrome[12], enhanced B-cell differentiation towards the plasma cell stage was now also found in refractory RA patients. A possible explanation for these observations was proposed by Bernasconi *et al.*, who postulated that in healthy persons polyclonal activation of memory B-cells was responsible for a continuing process of B-cell differentiation resulting in longstanding detectable antibody levels of different specificities[13]. Indeed, the reported in vitro experiments demonstrated a new and plausible concept for building long-lived humoral memory. However, if the presented concept held true in vivo, B-cells from patients with AID were chronically exposed to a pro-inflammatory environment implicating that memory B-cells underwent continuous activation and differentiation towards plasma cells. Our data showed that the specific disturbances in B-cell distribution found in blood and bone marrow of RA patients were in line with the latter concept. Of note, to investigate whether B-cells were polyclonally activated, we examined serum levels of RA-specific autoantibodies as well as antibodies against exogenous antigens. Despite the increased serum levels of total immunoglobulins, we only found autoantibody levels to be increased. However, the data on antibodies against exogenous antigens were limited by the low number of specificities (n=3) tested and the biological relevance of the small differences found is unclear. Thus, the observation of increased serum levels of immunoglobulins and autoantibodies supports the observations of enhanced differentiation of B-

cells towards ISCs in RA patients. The question remains whether B-cell differentiation was cause or consequence of pro-inflammatory signals in RA, indirectly implicating that effective anti-inflammatory treatment might reverse the skewed B-cell distribution.

A limitation of our study was the lower age of healthy controls compared to RA patients. Previous studies showed that age affected the distribution of B-cell subsets by reducing the proportion of memory B-cells and increasing that of naive B-cells[14,15]. However, in the present study we did not find a relation between age and proportion of B-cells or its subsets. Moreover, the age difference between both groups was too small to expect large discrepancies in the proportion of B-cell subsets. Also, one would have expected an increased proportion of naïve B-cells in the older RA group, which was not the case. Another possible bias was the redistribution of B-lineage cells towards the inflamed synovium of RA patients that could account for the reduced proportions of B-cells. However, if redistribution of lymphocytes was of influence on our flowcytometric data in blood and bone marrow, reductions in T-cells and macrophages would have been expected as these are the dominant lymphocyte subset in inflamed synovium of RA patients.

In conclusion, the present study showed enhanced B-cell differentiation in RA, providing new insights in the homeostasis of B-lineage cells in RA patients who are chronically exposed to a pro-inflammatory environment. As a consequence, adequate control of inflammation in RA patients is not only of importance to control disease activity but may also contribute to the restoration of homeostasis of the B-cell compartment in RA. The latter may be one of the mechanisms which are positively influenced by rituximab treatment.

References

1. Kunkel HG *et al*. Studies on the isolation and characterization of the "rheumatoid factor". *J Clin Invest* **38**: 424-34 (1959)
2. Ronnelid J *et al.* Longitudinal analysis of citrullinated protein/peptide antibodies (anti-CP) during 5 year follow up in early rheumatoid arthritis: anti-CP status predicts worse disease activity and greater radiological progression. *Ann Rheum Dis* **64**: 1744-9 (2005)
3. van der Helm-van Mil AH *et al*. Antibodies to citrullinated proteins and differences in clinical progression of rheumatoid arthritis. *Arthritis Res Ther* **7**: R949-58 (2005)
4. Cohen SB *et al.* Rituximab for rheumatoid arthritis refractory to anti-tumor necrosis factor therapy: Results of a multicenter, randomized, double-blind, placebo-controlled, phase III trial evaluating primary efficacy and safety at twenty-four weeks. *Arthritis Rheum* **54**: 2793-806 (2006)
5. Cambridge G *et al.* Circulating levels of B lymphocyte stimulator in patients with rheumatoid arthritis following rituximab treatment: relationships with B-cell depletion, circulating antibodies, and clinical relapse. *Arthritis Rheum* **54**: 723-32 (2006)
6. Roll P *et al*. Regeneration of B-cell subsets after transient B-cell depletion using anti-CD20 antibodies in rheumatoid arthritis. *Arthritis Rheum* **54**: 2377-86 (2006)
7. Teng YK *et al.* Immunohistochemical analysis as a means to predict responsiveness to rituximab treatment. *Arthritis Rheum* **56**: 3909-18 (2007)
8. van Esch WJ *et al.* Differential requirements for induction of total immunoglobulin and physiological rheumatoid factor production by human peripheral blood B-cells. *Clin Exp Immunol* **123**: 496-504 (2001)
9. Verpoort KN *et al.* Isotype distribution of anti-cyclic citrullinated peptide antibodies in undifferentiated arthritis and rheumatoid arthritis reflects an ongoing immune response. *Arthritis Rheum* **54**: 3799-808 (2006)
10. Dorner T *et al*. Correlation of circulating CD27high plasma cells and disease activity in systemic lupus erythematosus. *Lupus* **13**: 283-9 (2004)
11. Lipsky PE. Systemic lupus erythematosus: an autoimmune disease of B-cell hyperactivity. *Nat Immunol* **2**: 764-766 (2001)
12. Moutsopoulos HM *et al*. Immunopathogenesis of Sjogren's syndrome: "facts and fancy". *Autoimmunity* **5**: 17-24 (1989)
13. Bernasconi NL *et al*. Maintenance of serological memory by polyclonal activation of human memory B-cells. *Science* **298**: 2199-202 (2002)

14. Kruetzmann S *et al.* Human immunoglobulin M memory B-cells controlling Streptococcus pneumoniae infections are generated in the spleen. *J Exp Med* **197**: 939-45 (2003)
15. Shi Y *et al.* Regulation of aged humoral immune defense against pneumococcal bacteria by IgM memory B-cell. *J Immunol* **175**: 3262-7 (2005)