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Imbalance of peripheral B lymphocytes and NK cells in rheumatoid arthritis

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Abstract

The study was focused on several cellular immune disorders correlated with the imbalance between peripheral blood B lymphocytes and NK cells in severe rheumatoid arthritis. By flow cytometry we calculated the proportions of T, T helper, T cytotoxic/suppressor, B lymphocytes and natural killer cells in peripheral blood. The mitogen-induced proliferation of peripheral lymphocytes was measured by tritium-labeld uridine incorporation. Experimental data highlight a connection between annomal values of the B to natural killer cells ratio and disorders of the peripheral mononuclear cells concentration. We also showed that the polyclonal proliferation capacity of peripheral lymphocytes in rheumatoid arthritis is solely related to the B to natural killer cells ratio or to the natural killer cells proportion. The study reveals a potential role of the imbalance between proportions of peripheral B lymphocytes and natural killer cells in the immune pathogenesis of rheumatoid arthritis, thus pointing out an interrelation between the adaptive and innate immune systems.

Keywords: rheumatoid arthritis - B lymphocytes - NK cells - lymphocyte proliferation

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory systemic disease that causes irreversible joint destruction, with substantial social effects in terms of cost and disability [1,2].

Although the pathogenesis of RA remains incompletely understood, recruitment of immune cells into the synovial membrane, a shift in the phenotype and function of synovial fibroblasts, hyperplasia of synovial lining cells are accepted as pathological coordinated events in RA [3].

Lymphocytes that accumulate in the synovial sublining tissue are often organized in functionally competent germinal center-like microstructures that sustain the chronic inflammatory process and autoimmune reactions in RA [4]. Although compelling evidence indicates that CD4+ T cells play a pivotal role in the pathogenesis of RA in different

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check points of the disease [5], therapeutic B cell depletion proved to be an effective and safe treatment [6], suggesting a critical role of B lymphocytes in RA. Beside their role of arthrogenic immunoglobulins secretors [7], B cells regulate the activation of the tissue-invading CD4+ T cells by presenting the relevant antigen, by controling T cell homing and survival or the cytokine network [8,9].

In RA most of the immune cells interact in complex networks that lead to tissue-injurious inflammatory reactions. Surprising new evidence has arised regarding the involvement of natural killer (NK) cells in RA. NK cells were detected in the synovium [10] and elevated concentrations were recorded in peripheral blood [11]. Good correlations were found between pain, serum cortisol and the NK-mediated cytotoxicity, indicating particular disturbancies of the neuroendocrine-immune axis in RA [11]. NK might contribute to the pathogenesis of RA by perforin- or granzyme-mediated cytotoxicity [10] and cytokine production [13]. It is worth noticing that an unusual subset of CD4+CD28- T cells expressing NK receptors, exhibiting autoreactivity, clonal expansion in absence of classical costimulatory signals and cytolytic functions was identified, bridging specific and non-specific immune responses [14].

RA is a systemic disease with important life-threatening extra-articular symptoms [2] with a background of disease-associated peripheral immune disorders that contribute to RA spreading. Activated T, B lymphocytes and memory cells were detected in periphery, generated either as a consequence of cell traffic between blood and synovium or persistent antigen challenge [15,16]. Accordingly, analysis of the accesible peripheral pool of leukocytes can offer valuable information regarding the immunological background of RA, both from systemic and local point of view.

Considering that a complex interrelation between the adaptive and innate immune systems was lately highlighted [17], the aim of our study was to identify a possible cellular immune disorder related to peripheral B lymphocytes and NK cells in severe RA. Our chief finding is that disorders of peripheral mononuclear blood cells concentration are related to an imbalance between proportions of peripheral B lymphocytes and NK cells, imbalance that also controls the polyclonal proliferation capacity of peripheral blood lymphocytes in RA.

Materials and methods

Chemicals

Histopaque, cytochrome *c* (from horse heart), phytohemagglutinin M (PHA), pokeweed mitogen (PWM), RPMI 1640 medium, fetal bovine serum, antibiotic-antimycotic solution, POPOP, PPO, toluene and ethanol were purchased from Sigma Chemical CO, St. Louis MO, USA. Tritium labeled uridine [^{3}H-Urd] was obtained from the Institute of Physics and Nuclear Engineering "Horia Hulubei", Magurele, Romania. Simultest^{TM} IMK-Lymphocyte Kit was purchased from Becton Dickinson, Erembodegem-Aalst, Belgium.

Patients

We investigated a group of 21 outpatients with RA (RA patients), 19 females and 2 males, that fulfilled the revised criteria for the classification of RA [18], were positive for rheumatoid factor (RF) and were placed in the functional class III defined by Steinbroker. The main selection criteria of RA patients were: classical anti-rheumatic therapy with methotrexate could not control disease progression and severe side-effects were recorded, more than 7 joints were affected, high values of the C reactive protein (CRP) and of the erythrocyte sedimentation rate (ESR) were recorded (Table 1). The characteristics of investigated RA patients presented in table 1 indicate a severe, active form of disease.

Normal values of the peripheral cellular immune parameters were established in a group of 18 age-matched healthy volunteers (HV), 16 females and 2 males, presenting no clinical signs of inflammation, infection or other immune disorders (autoimmune diseases or allergies) at the moment of testing.

Informed consent was obtained from all the investigated RA patients and HV.

Immunophenotyping of peripheral lymphocytes

Fresh blood was collected by venipuncture in EDTA-coated vials. Phenotyping was performed by flow cytometry using Simultest^{TM} IMK-Lymphocyte reagents. Briefly, 100μL of blood were labeled with 10μL monoclonal antibodies for 20 min in dark. Red

Table 1 Clinical and biological parameters of RA patients (n=21).

Parameter	Mean ± SEM	Range	Normal range
Age (years)	55 ± 3	25 - 72	-
Disease duration (years)	8.6 ± 1,3	1 - 30	-
Tender joints	34 ± 4	4 - 68	-
Swollen joints	22 ± 2	3 - 43	-
Morning stiffness (min)	181.4 ± 32,2	30 - 480	-
ESR (mm/h)	60.1 ± 5,1	24 - 92	< 10 (males) < 20 (females)
CRP (mg/L)	48.0 ± 13,1	15 - 217	< 10

blood cells were removed by incubating the samples with lysing solution for 10 min in dark. Tubes were centrifuged for 5 min at 1200 rpm. Samples were treated further with washing solution and were centrifuged 5 min at 1200 rpm. Finally, cells were suspended in cell fixation solution and were ready for flow cytometry measurement. Data acquisition and processing were performed using a flow cytometer (Becton Dickinson) running with the SimulSet software. Informations regarding the percentages of peripheral T (CD3+), T helper (Th: CD3+CD4+), T cytotoxic/suppressor (Tc/s: CD3+CD8+) lymphocytes, Th to Tc/s ratio, B lymphocytes (CD19+) and NK cells (CD16+ CD56+) were obtained.

Isolation of peripheral mononuclear cells

Fresh blood was collected by venipuncture in sterile heparin-coated vials. Mononuclear cells were isolated from peripheral blood by gradient centrifugation [19] using Histopaque (specific gravity 1.077). Cells were counted in a Burger-Turck haemocytometer. Cellular viability, scored by the eosin exclusion test, exceeded 96%. Mononuclear cells were suspended in RPMI 1640 medium supplemented with 5% fetal bovine serum, antibiotic-antimycotic solution and 0.2% $NaHCO_3$ (complete culture medium) and were used for the radioisotopic proliferation assay.

Stimuli

Mononuclear cells isolated from peripheral blood, were stimulated in vitro with lectin mitogens: 10µg/mL PHA as mitogen for CD4+ and CD8+ T lymphocytes [20] and 2.5µg/mL PWM as mitogen acting on B lymphocytes and a subpopulation of T lymphocytes that provides help for B cells [20, 21].

Radioisotopic assay for lymphocyte proliferation

Lymphocyte proliferation of isolated cells, non-stimulated or *in vitro* activated using lectin mitogens, was measured by the [^{3}H-Urd] incorporation test [22]. Briefly, triplicate test samples (200µL final volume) containing mononuclear cell suspension ($2x10^6$ lymphocytes/mL) in complete culture medium, were incubated in absence or presence of lectin mitogens (PHA or PWM) in 96 well Costar plates (Corning Incorporated, Corning NY USA) for 72h at 37^0C in 5% CO_2. 18h prior to harvesting, cell cultures were labeled with 1µCi ^{3}H-Urd/sample. Cultures were harvested on Skatron filters (Skatron Instruments, Sterling USA) that were further measured for radioactivity in scintillation liquid [4 g PPO, 50 mg POPOP in 1 L of a mixture of 60% toluene and 40% ethanol (v/v)] using a β-counter. Results were expressed as pulses/min (ppm).

Table 2 Peripheral immune cellular status of RA patients. Healthy volunteers (HV) were used as control. NS –statistically not significant (p_2>0.05).

Cellular immune parameter (units)	RA patients (n=21)	HV (n=18)	Statistical difference
Peripheral lymphocytes counts (cells/mL blood)	(1.68 ± 0.25) x10^6	0.91 ± 0.07 x10^6	p_2=0.006
Peripheral monocytes counts (cells/mL blood)	(0.53 ± 0.11) x10^6	0.20 ± 0.02 x10^6	p_2=0.006
Lymphocyte to monocyte ratio	3.9 ± 0.4	5.8 ± 0.7	p_2=0.030
T lymhocytes (%)	76.2 ± 2.2	75.8 ± 1.2	NS
Th lymhocytes (%)	53.1 ± 2.0	47.4 ± 1.9	p_2=0.043
Tc/s lymhocytes (%)	22.3 ± 1.5	27.5 ± 2.4	p_2=0.038
Th to Tc/s ratio	2.6 ± 0.2	1.9 ± 0.2	p_2=0.012
B lymphocytes (%)	11.9 ± 1.2	10.4 ± 0.7	NS
NK cells (%)	10.5 ± 1.7	11.9 ± 1.5	NS
Baseline of lymphocyte proliferation (ppm)	2493 ± 421	4185 ± 1205	NS
PHA-induced lymphocyte proliferation(ppm)	16617 ±2588	18357 ± 2096	NS
PWM-induced lymphocyte proliferation (ppm)	14613 ± 2170	16051 ± 2358	NS

Statistical analysis

Results were expressed as mean value ± standard error of the mean (SEM). Statistical comparison between groups/ subgroups of RA patients and HV were performed using the t-test with unequal variances, while the t-test for paired samples was used for comparison between parameters within a group/subgroup. P-one tail or -two tail (p_1 and p_2) were used for statistical significance where appropriate. Correlations were established by using Pearson correlation test (PC).

Results

The study was focused on a group of 21 RA patients with severe, active disease, refractory to classical therapy with methotrexate. A group of 18 age- and gender-matched HV was used as control group.

Peripheral immune celullar status of RA patients

RA patients exhibited statistically significant disorders of their peripheral leukocytes counts and proportions, when compared to HV.

RA patients presented moderately high lymphocyte counts paralleled by markedly elevated monocyte counts (PC=0.94), leading to low values of the lymphocyte to monocyte ratio (Table 2).

Analysis of T lymphocytes subsets indicated that RA patients had normal T cells proportions, but elevated relative values of the Th subset and a shift of the Tc/s proportion towards lower values were noticed (Table 2). The recorded high values of the Th to Tc/s ratio were mainly due to reduced Tc/s lymphocytes percentage (PC=-0.86).

The polyclonal proliferation capacity of peripheral lymphocytes isolated from RA patients was within

normal range for both unstimulated and mitogen-activated cells (Table 2) and was not affected by the abnormal lymphocyte to monocyte or Th to Tc/s ratios.

Although percentages of peripheral blood B lymphocytes and NK cells in RA patients were within normal ranges (p_2=0.382, p_2=0.536) (Table 2), we found features of these cells that correlate to other immune parameters in RA patients, but not in HV. The imbalance between B lymphocytes and NK cells was best described by the B to NK cells ratio.

Correlation between B to NK cells ratio and peripheral mononuclear cells counts

We sorted RA patients in three subgroups characterized by low (subgroup 1), median (subgroup 2) and

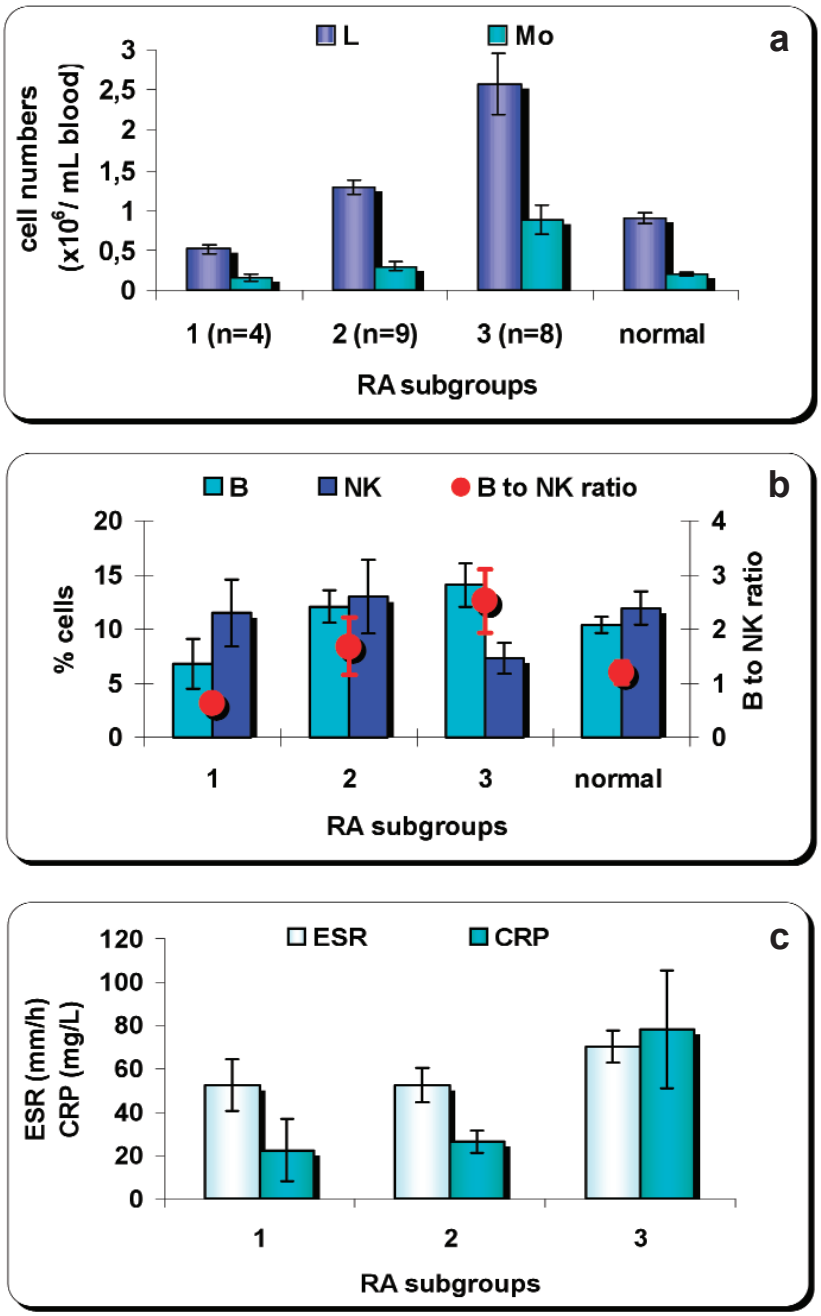


Fig. 1 The relation between peripheral blood lymphocytes counts and the B to NK cells ratio. a) Subgroups of RA patients sorted according to peripheral lymphocytes numbers; b) Percentages of B lymphocytes, NK cells and the B to NK cells ratio; c) Biological parameters.

high (subgroup 3) levels of peripheral blood lymphocytes (Fig. 1a). Experimental data show that peripheral blood monocyte numbers parallels lymphocyte counts (Fig. 1a).

Disorders of peripheral blood lymphocyte and monocyte numbers are accompanied by an imbalance of B lymphocytes and NK cells proportions, that was best highlighted by representing the B to NK cells ratio (Fig. 1b).

Low lymphocyte counts (subgroup 1, p_2=0.0003) were correlated with a decreased B to NK cells ratio (p_2=0.036) mainly determined by low proportions of B lymphocytes (p_1=0.047), while high lymphocyte counts (subgroup 3, p_2=0.002) were associated with abnormal high values of the B to NK cells ratio (p_1=0.029) (Fig. 1b). RA patients with lymphocytosis presented elevated levels of CRP (p_1=0.047) and, to a lesser extent, of ESR (p_1=0.063) (Fig. 1c), indicating an acute phase response and progressive joint disease [23].

No other variations of the investigated cellular parameters were correlated to the described disorders of peripheral lymphocyte and monocyte counts (data not shown), suggesting that the imbalance of peripheral blood B lymphocytes and NK cells plays an important role in RA.

Correlation between B to NK cells ratio and proliferation capacity of peripheral blood lymphocytes

Our experimantal results emphasize a new relation between B to NK cells ratio and the polyclonal mitogen-induced proliferation of peripheral blood lymphocytes in RA patients.

We divided both RA patients and HV in subgroups characterized by low (subgroup 1), median (subgroup 2) and high (subgroup 3) values of the B to NK cells ratio (Fig. 2a,b). The dependence of lymphocyte polyclonal proliferation on the B to NK cells ratio is presented in Fig. 3 a,b.

A noticeable difference between RA patients and HV is found in the subgroups with median values of B to NK cells ratio (subgroup 2). RA patients, but not HV, exhibit functional suppresion of PWM-activated B lymphocytes (p_2=0,031) and, to a lesser extent, of PHA-activated T lymphocytes (p_1=0,054). The difference between RA and HV in subgroup 2 is statistically significant (p_2=0,010 for PWM stimulation, p_1=0.038 for PHA stimulation). The described functional suppression of peripheral lymphocytes is corre-

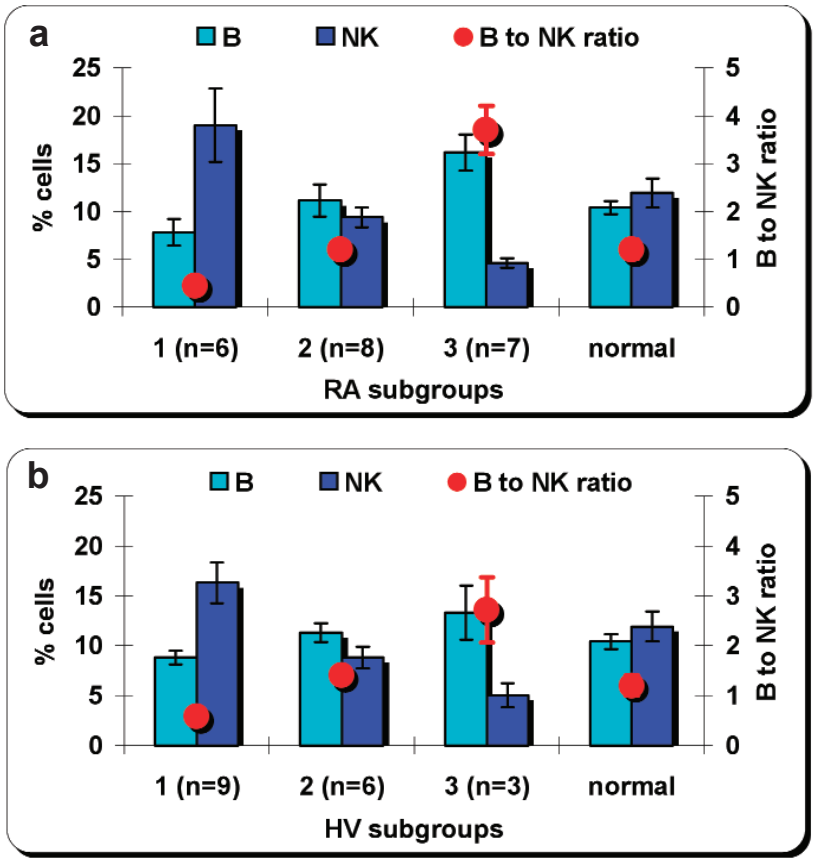
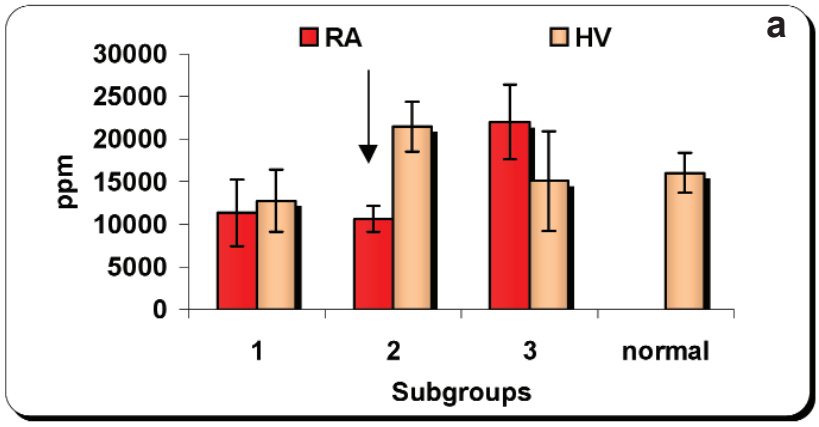


Fig. 2 Subgroups of RA patients (a) and HV (b) sorted according to the value of their B to NK cells ratio.



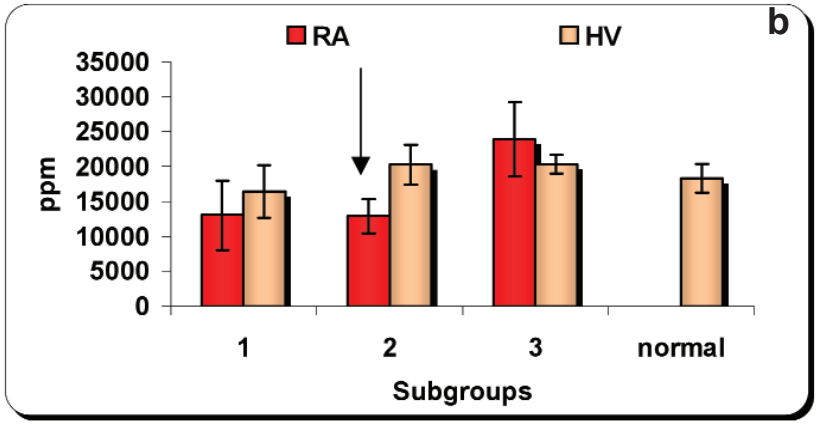


Fig. 3 The relation between B to NK cells ratio and polyclonal proliferation of peripheral blood lymphocytes in RA and HV subgroups defined in Fig. 2. a) PWM-induced proliferation; b) PHA-induced proliferation.

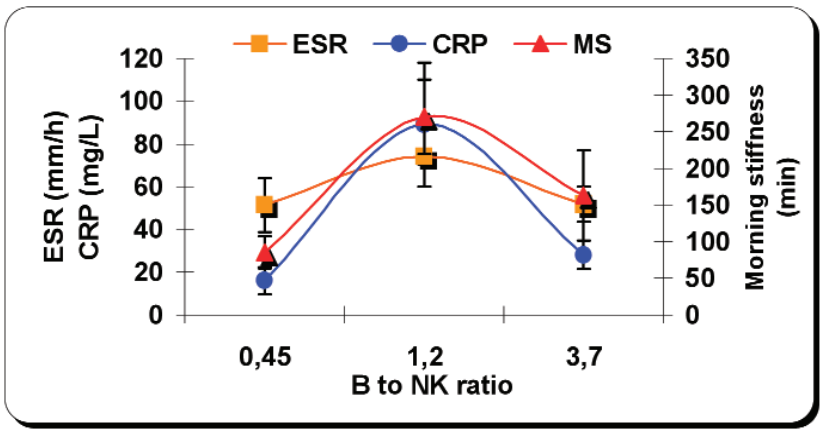
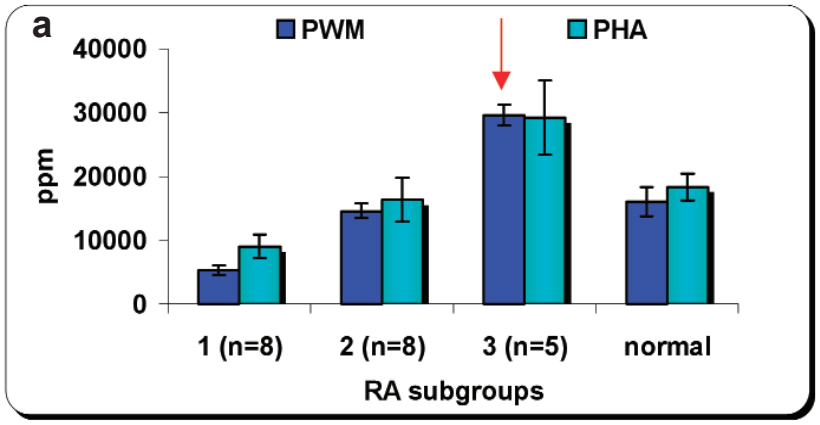


Fig. 4 Clinical and biological parameters of RA patients sorted according to the value of the B to NK cells ratio (subgroups defined in Fig. 2a).

lated with high values of CRP (p_2=0,038), ESR (p_2=0.037) and of the morning stiffness (p_2=0.008) (Fig. 4), indicating severe inflammation and joint injury [23].

RA patients and HV in subgroup 3 (Fig. 2a,b) showed high values of their B to NK cells ratio, mainly due to an extremely low NK percentage (p_2=0,020). In RA patients, but not in HV, the increase of B to NK cells ratio from median (subgroup 2) to elevated values (subgroup 3) is accompanied by an augmentation of the mitogen-induced proliferation of B lymphocytes (p_2=0,045) and, to a lesser extent, of T lymphocytes (p_1=0,048) (Fig. 3a,b).

We better emphasized the relation between high values of B to NK ratios and increased lymphocyte proliferation by sorting RA patients and HV according to the magnitude of Urd incorporation. Three subgroups were defined, characterized by low (subgroup 1), median (subgroup 2) and high (subgroup 3) proliferation intensity of PWM-activated B lymphocytes (Fig. 5a,b).



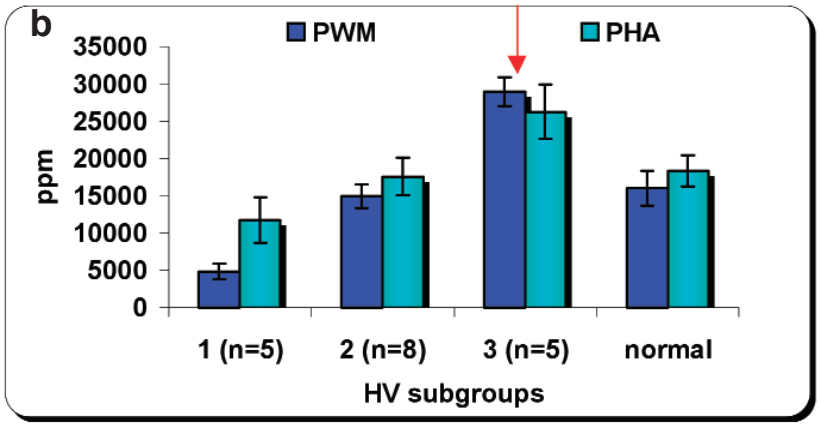
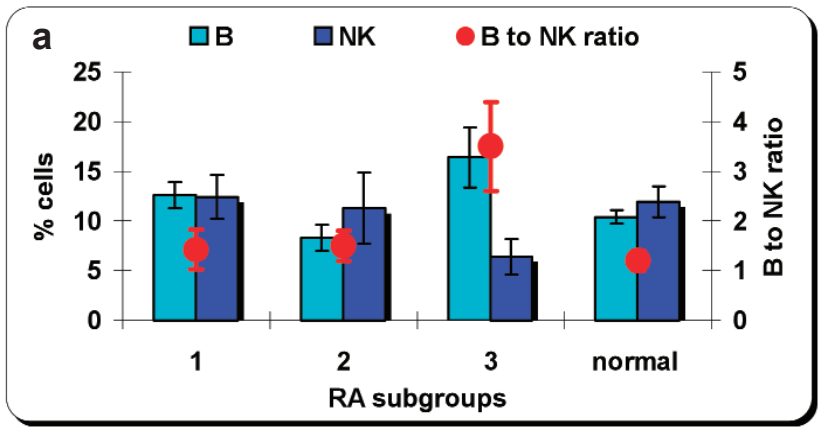


Fig. 5 Subgroups of RA patients (a) and HV (b) sorted according to PWM-induced proliferation of peripheral blood lymphocytes.



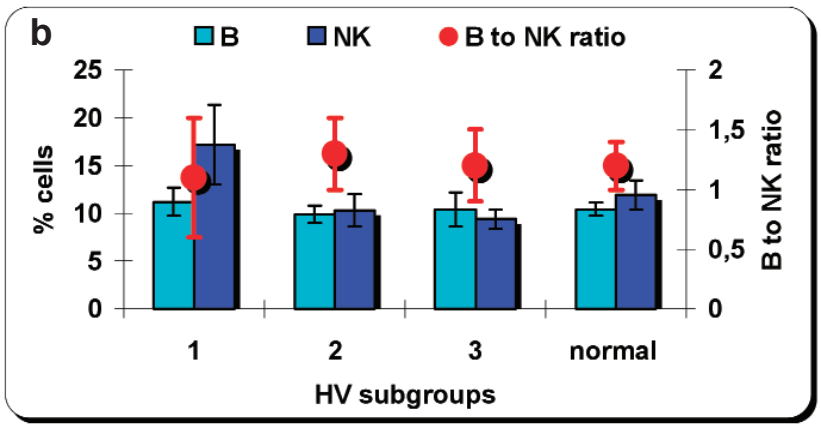


Fig. 6 The relation between proliferation capacity of peripheral blood lymphocytes and B cells proportions, NK cells proportions and B to NK cells ratios for the subgroups of RA patients (a) and HV (b) defined in Fig. 5.

First, we noticed that in RA patients the proliferation capacity of lymphocytes is not dependent on the applied mitogen and the targeted lymphocyte subset, as long as highly responsive lymphocytes to PWM are also responsive to PHA (p_2= 0.939, PC=0.92) (Fig. 5a, subgroup 3). The relation is less obvious in the case of HV (p_2= 0.625, PC=0.63) (Fig. 5b, subgroup 3).

RA patients with intense lymphocyte proliferative activity (subgroup 3) showed an abnormal high B to NK cells ratio (p_1=0.035) mainly determined by low NK proportions (p_2=0.038) (Fig. 6a,b). The described intense proliferation of PWM-activated lymphocytes in RA patients could not be atributed to the proportion of B lymphocytes, only moderately higher than normal mean values (p_1=0.066) (Fig. 5a).

We point out that the above mentioned disorders of lymphocyte proliferation are solely correlated with the B to NK cells ratio. No relation could be established with variations in T, Th, Tc/s proportions or in the lymphocyte to monocyte ratio (data not shown).

Discussion

Our study was focused on a group of RA patients with severe, refractory disease, presenting various peripheral immune disorders. We evidentiated high values of the Th to Tc/s ratio that were mainly determined by abnormally low proportions of Tc/s lymphocytes and, to a lesser extent, by elevated proportions of Th lymphocytes. These results are in agreement with other reported observation [24] and theoretically point out active immune reactions, but the functional experiments we performed do not indicate an activated state of peripheral blood lymphocytes as reflected by their polyclonal proliferation ability.

The chief finding of this study is that the imbalance between proportions of peripheral B lymphocytes and NK cells has a potential role in the immune pathogenesis of RA.

We highlighted the relation that connects abnormal values of the B to NK cells ratio and altered peripheral blood mononuclear cells number, suggesting the existence of a compensatory mechanism connecting B lymphocytes and NK cells in RA. Lymphopenia was associated with low values of the B to NK cells ratio, mainly determined by a reduced B lymphocyte proportion that might have pathological consequences. It is known that the loss of peripheral B lymphocytes could be determined by their recruitment into the inflammatory synovium or antigen encounter and further repertoire contraction [25]. Lymphocytosis, which is associated with abnormal high values of B to NK cells ratio, mirrors systemic inflammation and progressive joint disease [23].

We also showed that the proliferation capacity of peripheral blood lymphocytes in RA is related to B to NK cells ratio or to NK cells proportion and not, as expected, to T, Th or Tc/s lymphocytes percentages. We emphasize that peripheral blood NK cells proportion might control the mitogen-induced proliferation capacity of peripheral blood lymphocytes, but the mechanism underlying this experimental observation is obscure. D'Orazio and Stein Streiler [26] show that activated NK cells (HLA-DR+CD56+) can function as superantigen presenting cells that induce nonspecific stimulation of T lymphocytes, but this mechanism does not fit our results showing that intense proliferation of both B and T lymphocytes was associated with low NK proportions.

We have also identified a subgroup of RA patients, characterized by normal values of B to NK cells ratio, that showed a particular pattern of functional suppression of peripheral lymphocytes in association with marked signs of systemic inflammation and joint damage. Activated lymphocytes were probably preferentially recruited into the synovial cavity where they amplified the inflammatory process, while the remainder peripheral population displayed reduced polyclonal proliferation.

Compensatory mechanisms seem to be active at the level of B lymphocytes and NK cells proportions and polyclonal lymphocyte proliferative potential. Previous reports revealed particular NK-B cells circuits in RA mediated by RF that modulate the expression of FcγRIIIa (CD16) on NK cells, thus enhancing NK cytokines production [27], while in turn NK cells control the production of RF by B lymphocytes [28].

Further investigations on absolute counts of NK cells, their activation state and the functional competence of NK-T cells might clarify the connection between the innate and adapative immune systems we revealed in this study.

Taking into account that B and NK cells can influence autoimmunity through connected pathways, combined therapy might be efficient, aiming to simultaneously modulate innate immune pathways and the adaptive memory response.

Acknowledgements

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