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Rheumatoid arthritis is associated with signaling alterations in naturally occurring autoreactive B-lymphocytes

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Abstract

Immune tolerance established during the development of B lymphocytes can be subverted in mature cells and lead to autoimmunity. This study focuses on the recently discovered subset of CD19⁺CD27⁺IgD⁺IgM^{low/-} B cells that recognize self-antigens and have the capacity to produce autoantibodies, but under normal conditions do not generate autoimmune response due to intrinsic signaling inhibition (a condition known as clonal anergy and characterized by impaired antigen receptor signaling). Phosphorylation of intracellular signaling proteins and Ca²⁺ responses in anergic B cells were measured by multicolor flow cytometry. Our results demonstrate a distinct phosphorylation pattern for major signal transduction proteins, which distinguishes anergic B cells. Comparison of B cell signaling properties in Rheumatoid Arthritis patients and healthy controls revealed a reversal of pTyr and Ca²⁺ anergic signaling features in patients, accompanied by phosphorylation decreases of Blnk, Syk, SHP2, CD19. We identified BCR signaling pathway alterations associated with the loss of anergic B cell tolerance in Rheumatoid Arthritis.

Keywords

B cells; tolerance; clonal anergy; Rheumatoid Arthritis; BCR signal transduction; protein phosphorylation; Ca²⁺ signaling

INTRODUCTION

Alterations in B cell immune tolerance are increasingly recognized as an important factor in the development of autoimmune rheumatoid arthritis (reviewed in [1]). Developing B cells undergo multiple consecutive tolerance checkpoints (clonal deletion, receptor editing and receptor “tuning” also known as clonal *anergy*) that are responsible for the formation of immune tolerance by eliminating or silencing of self-reactive clones. Growing evidence suggests that these tolerogenic mechanisms can be compromised during autoimmune

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AUTHORSHIP

G.A.L. and H.C.A. performed experiments and analyzed data; C.C.S. recruited patients, analyzed clinical data and contributed to addressing Reviewer's comments; K.E.F. analyzed clinical data and recruited patients; L.A.D. contributed to human protocol design and recruitment of healthy control subjects; V.M.H. contributed to the interpretation of results and writing the paper; T.L. designed the study, performed experiments, analysed data and wrote the paper.

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processes (reviewed in [2]). The latter mechanism, clonal *anergy* is defined as a state of immune unresponsiveness in which lymphocytes recognize the antigen but are unable to produce immune response due to intracellular signaling mechanisms that inhibit activation signals elicited by the antigen receptor. Thus, anergy can prevent autoreactive B cells that have escaped prior tolerogenic mechanisms from responding to self-antigens. This study is focused on the role of B cell anergy in Rheumatoid Arthritis (RA). We obtained peripheral blood B cells from a cohort of RA patients to characterize signal transduction mechanisms underlying the maintenance and changes in B cell anergy, and to assess the activation of autoreactive B cells that contribute to the development of RA.

The ability of the immune system to make a distinction between similar antigens is a fundamental mechanism for avoiding cross-reactivity which gives rise to autoimmunity. A recently recognized concept of the “digital” (on/off) nature of regulation in intracellular signal transduction pathways emphasizes a delicate balance between stimulatory and inhibitory signaling cascades that allows lymphocytes to distinguish between very closely related antigens (Germain, 2005). One major biophysical parameter believed to determine the outcome of Ag-specific lymphocyte activation is the lifetime of the Ag/receptor complex [3]. For instance in T cells, a single amino acid substitution in the antigen peptide-MHC complex can decrease the average lifetime of pMHC/TCR by only several seconds while dramatically reducing the immunogenicity of the mutated pMHC to the order of 10^4 fold [4]. Considering the high speed of initial signaling events that follow antigen receptor ligation (i.e. intracellular calcium release commences within the first minute), the lifetime of Ag/receptor interaction becomes an essential factor. Relatively small variations in the timeliness of Ag/receptor associations are amplified into large differences in downstream signaling output by a signal transduction pathway with many steps, each with its own timing parameters, and requiring a continued upstream ligand–receptor interaction to occur.

Recent studies in healthy human subjects have identified a subset of potentially autoreactive CD19⁺CD27[−]IgD⁺IgM^{low/−} late transitional cells that constitute ~2.5% of peripheral blood B cells and have anergic signaling properties [5]. These cells are hyporesponsive to antigen receptor stimulation *in vitro* and have the capacity to produce autoreactive Abs (anti-dsDNA and anti-HEp-2). This autoreactivity appears innate, as variable Ig genes show no evidence of somatic mutations, indicating that it was not generated during adaptive immune response. Two key signaling properties distinguish anergic cells from other types of B lymphocytes: reduced amplitude of phospho-protein and Ca²⁺ responses to antigen receptor ligation and elevated baseline protein phosphorylation in intact cells. These signaling features, described in both mice and humans [5, 6], reflect chronic stimulation of anergic B cells with cross-reactive autoantigens paralleled by continuous inhibition of intracellular BCR signaling through downregulatory mechanisms, and represent an important mechanism that prevents autoimmunity under normal conditions.

Anergic B cells have not been characterized in RA (or any other human autoimmune disorder to date). Pathogenic Abs and other clinical manifestations of RA are detectable only after a prolonged period of autoantibody expansion [7], and analysis of B cell activation markers expression and B cell-derived cytokines [8] have not identified disease-specific changes to date. However, it has been demonstrated that elevated phospho-activation in CD20⁺ B cells positively correlates with RA assessment scores [9].

Our recent findings in murine experimental arthritis model demonstrate the loss of B cell anergy during the onset of disease, and a distinct BCR signal inhibitory pathway that clearly distinguishes anergic cells from other B cell types [10]. This study expands our approach into human RA. The results, based on analysis of 8 key signaling proteins involved in BCR signal transduction, demonstrate that the balance of stimulatory and inhibitory signaling

cascades is clearly altered in anergic/autoreactive B cells in RA. Our study also evaluates the use of phosphoprotein activation patterns and pairwise pathway correlations as signaling-based biomarkers that associate with phenotypical/functional changes and the loss of anergy in autoreactive B cells during the onset of RA.

MATERIALS AND METHODS

Human subjects

32 healthy blood donors (mean age 35.7; male/female ratio 0.68) and 20 RA patients (mean age 55.9; male/female ratio 0.54) from UC Denver Hospital Rheumatology Clinic were recruited for this study. All RA patients have been diagnosed in recent years, are RF/anti-CCP positive and have not undergone B cell targeted treatments. Demographic and clinical data on the subjects are shown in Figure 1. Subjects were recruited according to the Colorado Multiple Institutional Review Board guidelines (Protocol # 10-0250) with informed consent agreement.

Flow cytometry analysis

PBMCs were isolated from freshly collected blood samples by Ficoll density gradient. Cells were washed with IMDM supplemented with 5% FCS and stained with fluorescently labeled Abs. For Ca^{2+} analysis by flow cytometry, cells were incubated with Indo-1AM (Carlsbad, CA) at 5 mM for 30 min at 37°C and co-stained with a cocktail of anti-human CD27 Alexa405, IgD PE, IgM Cy5 (monovalent Fab), CD19 APC-Cy7. Abs specific to surface markers were obtained from Beckton Dickinson (San Jose, CA), Jackson ImmunoResearch (West Grove, PA) and eBioscience (San Diego, CA). Polyclonal goat F(ab')₂ anti-human Ig(H+L) (Southern Biotech, Birmingham, AL), referred to in the text as anti-BCR, was used to stimulate cells *in vitro*. Monovalent Fab used to label IgM on B cell surface does not crosslink surface IgM molecules and thus does not stimulate B cells [11], unlike anti-BCR F(ab')₂. For intracellular phosphoprotein staining, purified PBMCs were stimulated with anti-BCR for 10 min (5 µg/ml), then rapidly fixed and permeabilized in 20x volume of BD Cytfix/Cytoperm buffer and stained with a cocktail of surface marker Abs (as described above) and with phospho-specific Abs followed by FITC labeled secondary Ab. Experiments were performed as described [12] on BD LSR II flow cytometer (Beckton Dickinson, San Jose, CA). B cell subsets of interest were gated upon as indicated in Figure 2A, B and protein phosphorylation intensity (mean fluorescence intensity, MFI) or intracellular Ca^{2+} influx (Indo-1AM Ex/Em ratio units) were analyzed as described [12]. Fluorescence intensity of phospho-specific Ab staining within the gated subsets was compared between stimulated and nonstimulated cells. Online Supplement Figure 1 illustrates experimental design and data analysis algorithm. Figure 2D, C show representative examples of the actual flow data (histograms) from which MFI or Ca^{2+} peak values were collected. Statistical analysis was performed with Prism software (GraphPad, San Diego, CA) and JMP software (SAS Institute, Cary, NC).

RESULTS

CD19⁺CD27⁻IgD⁺IgM^{low/-} B cells from healthy control subjects exhibit anergic signaling features

We recruited 32 healthy blood donors (mean age 35.7; male/female ratio 0.68) to study normal human anergic B cells for characteristic signaling properties (Figure 1). Freshly isolated live PBMCs were stained with a cocktail of fluorescently labeled Abs specific to human B cell surface markers (CD19, CD27, IgD, IgM). First, we verified the presence of the previously described [5] CD19⁺CD27⁻IgD⁺IgM^{low/-} anergic B cells (referred to further in the text as B_{an}) in peripheral blood of healthy subjects. The results confirmed the

occurrence of this subset (Figure 2A) and that the overwhelming majority of CD19⁺IgD⁺IgM^{low/-} B_{an} cells are CD27⁻ (Figure 2B). Minor CD27⁺ subpopulations also present in the gated CD19⁺IgD⁺ subsets consist of IgM⁺ memory cells and IgM⁻ Cδ class switched B cells [13] whose signaling characteristics are beyond the scope of this study. We also assessed surface IgG expression on anergic and non-anergic B cells and found no significant differences. This is consistent with literature data [5].

In conjunction with the surface B cell markers, total tyrosine phosphorylation (pTyr) was measured in fixed/permeabilized cells co-stained intracellularly with fluorescently labeled anti-pTyr mAb (PY20). Cells were (or were not) stimulated with polyclonal anti-human Ig (anti-BCR) as indicated. An example of primary data recorded by the flow cytometer is in Figure 2D (duplicate phosflow assay). Mean fluorescence intensity (MFI) values were extracted from histograms and results from multiple donors were averaged. Data acquisition algorithm is shown in online Supplement Figure 1. Anti-BCR F(ab')₂ isotype control was established early on in the study and demonstrated that an “irrelevant” goat anti-human CD3 F(ab')₂ Ab did not stimulate Ca²⁺ and pTyr responses in B cells regardless of the dose (unpublished observations). Top left panel in Figure 3A (highlighted in grey) demonstrates that intact B_{an} cells exhibit significantly elevated baseline pTyr levels ($p=0.023$) and reduced amplitude of response to anti-BCR stimulus (89 vs. 34.2% increase over baseline) as compared to the general population of CD19⁺IgD⁺IgM⁺ B cells (referred to further in the text as IgM⁺).

We also examined baseline intracellular Ca²⁺ levels and amplitudes of BCR-mediated Ca²⁺ responses in these two subsets. B cells were labeled with the same surface staining Ab cocktail as above, and with cell-permeable Ca²⁺ probe Indo-1AM. Ca²⁺ responses of B_{an} cells to anti-BCR were reduced when compared to the “bulk” of IgM⁺ B cells (Figure 2C and averaged data in Figure 4A (control)). No significant differences in baseline Ca²⁺ levels were observed (averaged data not shown).

Comparison of anergic signaling properties between B_{an} cells from healthy controls and patients with active RA

We recruited a group of 20 RA patients (mean age 55.9; male/female ratio 0.54) from UC Denver Hospital Rheumatology Clinic who have been diagnosed in recent years, are RF and anti-CCP positive and have not undergone B cell targeted treatments (Figure 1). Other clinical parameters examined in this study (see Discussion) were Erythrocyte Sedimentation Rate (ESR) and C-reactive protein (CRP in mg/dL) that are lab measures of inflammation and are commonly used as surrogates of RA disease activity, and CDAI (Clinical Disease Activity Index) that is a clinical composite index based entirely on clinical criteria. It is calculated as: CDAI = TJC28+SJC28+Patient GA+Physician GA; TJC28 is tender joint count of 28 standard joints (fingers, wrists, elbows, shoulders, knees); SJC is swollen joint count of the same joints; GA is Global Health assessment measured from 0 to 10; CDAI>22 – High disease activity, 10.1–22 – Moderate disease activity, 2.8–10 – Low disease activity, <2.8 – Remission. No significant phenotypic or quantitative differences with the control group were found in IgM⁺ and B_{an} subsets obtained from RA patients (data not shown). Phosphoprotein responses (averaged MFI values) of IgM⁺ and B_{an} cells from RA patients are shown in Figure 3B.

We identified the following hierarchy of parameters for comparing phospho-protein responses between the subject groups:

1. first, based on mean MFI values derived from multiple individual samples (duplicate phosflow assay per sample, Figure 2D), the average amplitude of B cell responses to anti-BCR was calculated as % increase over a corresponding non-

stimulated control (i.e. anti-BCR stimulation had increased total pTyr levels by 89% in IgM⁺ B cells and by 34.2% in B_{an} cells from healthy subjects (Figure 3A, top left panel)). This was designated as parameter $X = ((MFI_{a-BCR} \times 100\%) / MFI_{nonstimul.}) - 100\%$

2. then, in order to account for baseline differences, a relative difference between response amplitudes of IgM⁺ and B_{an} cells was calculated (i.e. each bar in Figure 5A shows that B_{an} response to anti-BCR is $Y\%$ higher/lower than that of IgM⁺ within the same donor group). This was designated as parameter $Y = X_{B_{an}} - X_{IgM^+}$
3. finally, Y_{RA} was directly compared to $Y_{control}$ (Figure 5B).

As baseline protein phosphorylation activity is increased in normal anergic B cells, one might expect the loss of anergy in autoimmunity to be accompanied by a reduction in baseline pTyr levels. However, comparison of B_{an} baseline protein phosphorylation activity between RA and control groups revealed that it was not reduced in RA (in fact, tended to be ~30% higher, although the difference did not reach statistical significance; Figure 4B, top left panel). This indicates that, unlike the control group, in RA B_{an} cells have even higher stimulation threshold due to increased baseline signaling activity caused by greater autoantigen load and further elevated intrinsic signaling inhibition.

The absolute amplitude of B_{an} pTyr response to anti-BCR ($X_{B_{an}}$) was also not reduced in the RA group and, akin to the baseline, the response was slightly higher: $X_{B_{an}(RA)} = 44.5\%$ and $X_{B_{an}(control)} = 34.2\%$ (online Supplement Figure 3 highlighted in grey). However, when normalized to the bulk of IgM⁺ B cells within the same group of donors (parameter $Y_{B_{an}}$), the response of B_{an} subset in the RA group appeared decreased by 35.6%: $Y_{B_{an}(RA)} = 19.3\%$ vs. $Y_{B_{an}(control)} = 54.9\%$ (Figure 5A, highlighted in grey) due to the higher baseline. These results indicate that despite the already elevated activation threshold in RA B_{an} cells, stimulation with anti-BCR drives the signaling even further and overcomes anergy, as evidenced by higher than control group MFI phosphorylation values in RA B_{an} cells (compare B_{an} bars in top left panels of Figure 3A and Figure 3B).

Similar characteristics were also found when comparing Ca²⁺ signaling response amplitudes in RA vs. control B_{an} cells. While in healthy controls B_{an} responses to anti-BCR were significantly lower than those of the bulk of IgM⁺ cells ($p=0.0073$), in RA this difference was insignificant (Figure 4A). No significant differences in baseline B_{an} Ca²⁺ levels between RA and control groups were found (data not shown).

Overall, we observed that in RA, in contrast to the control group, B_{an} total pTyr and Ca²⁺ responses to anti-BCR resemble those of normal non-anergic IgM⁺ B cells. This suggests the reversal of anergy in RA.

Comparison of B_{an} phosphoprotein signaling patterns between RA and control groups

In order to characterize signaling pathways that underlie total pTyr and Ca²⁺ signaling features of B_{an} cells described above, we examined phosphorylation of key signaling molecules involved in BCR-mediated pathways. We focused on several key signal transduction proteins (Blnk, Syk, SHP2, CD19, Erk1/2, Jnk, PLCγ2) involved in main outcomes of B lymphocyte activation: Ca²⁺ mobilization, lipid raft aggregation, BCR internalization, integrin-dependent cytoskeletal rearrangements and transcription factors activation (highlighted in Figure 6).

Comparison of baseline phosphorylation levels of individual signaling molecules between nonstimulated RA and control B_{an} cells revealed pronounced increases in Blnk, SHP2 and Jnk in the RA group (Figure 4B). Statistically significant results (p values of unpaired two-

tailed t-test <0.05) are marked with α . Percentile divergences between the MFI values of Figure 4B are shown in online Supplement Figure 2. Noteworthy, baseline levels of Syk and CD19 also tended to be higher in the RA group, although statistical significance was insufficient for this data set.

Next, we compared $X_{B_{an}(RA)}$ and $X_{B_{an}(control)}$ parameters for each signaling protein. Blnk, SHP2 and PLC γ 2 exhibited 2-fold or more differences in the absolute amplitude of B_{an} response to anti-BCR in the RA group (online Supplement Figure 3). Normalization of these numbers to the responses of IgM⁺ B cells within the same group of donors (parameter $Y_{B_{an}}$) revealed marked differences in relative phospho-response amplitudes of Blnk, Syk, SHP2, CD191, Erk1/2 and Jnk between RA and control groups: $Y_{B_{an}(RA)}$ and $Y_{B_{an}(control)}$ are shown in Figure 5A and constitute a distinct pattern which is graphically presented as a color map in Figure 5B. Schematic summaries of B_{an} signaling patterns in RA and control groups in the context of BCR signal transduction pathways are shown in Figure 6.

Using linear regression analysis for the combined group (i.e. RA patients and normal controls) adjusted for study subjects age and gender we observed that there was a modest trend for a lower tyrosine phosphorylation in response to BCR cross-linking with age ($R^2=0.12$, $p=0.0518$). However, at the same time, in RA group there was a significantly greater response to BCR stimulation in IgM⁺ B cell population as compared to normal control subjects (adjusted Mean $\pm$ SE % increase in phospho tyrosine was $121.4\pm30.6\%$ in the RA group, as compared to $22.5\pm31.0\%$ increase in this cell population in the normal control group, $p=0.05$) suggesting the break of anergy in RA patients.

Correlations between phosphoprotein responses to anti-BCR in B_{an} cells from healthy controls and RA patients

To access pairwise and higher relationships among phospho-proteins involved in BCR-triggered B_{an} signaling pathways, we analyzed correlations between original MFI values for individual signaling molecules within RA and control groups. Spearman's multivariate analysis algorithm (JMP/SAS software) revealed correlations shown in Figure 7A. Positive numbers indicate that increases in the phosphorylation of signaling proteins are pairwise linked and therefore the two proteins are involved in the same signaling pathway(s), i.e. Blnk and Syk in the top left table. Statistically significant values ($|p|<0.05$) are highlighted in grey. Bottom panels in Figure 7A display scatterplot correlation matrices for each group (each dot represents individual sample). Alterations in pairwise relationships observed in the RA group are highlighted in red (Figure 7A, top right panel). Graphical representations of pairwise relationships among phospho-proteins are shown in Figure 7B.

DISCUSSION

This report presents comparative analysis of signaling properties of CD19⁺CD27⁻IgD⁺IgM^{low/-} autoreactive anergic B cells [5] isolated from patients with active RA and healthy control subjects. Comparison between RA patients and healthy control subjects did not reveal statistically significant differences in percentages of CD19⁺IgD⁺IgM⁺CD27⁻ cells. This is consistent with results reported by in healthy individuals [5]. For each group of subjects, we identified a distinct phosphorylation pattern of key signaling proteins involved in BCR signal transduction, which distinguishes anergic cells from other B lymphocytes. Based on these data we evaluate the use of characteristic phosphoprotein activation patterns and signaling pathway correlations as signaling-based biomarkers for autoreactive B cells that produce pathogenic autoreactive Abs in RA.

The main goal of this study is to establish differences between signaling properties of anergic B cells in RA patients and healthy controls, with the primary focus on immediate

intracellular signal transduction events following BCR engagement (protein phosphorylation and Ca^{2+} influx). Polyclonal activators such as anti-BCR provide a robust stimulus and a well-established *in vitro* experimental setting [14] to study these signaling events. Responses of anergic B cells to specific autoreactive immunogens warrant evaluation of long-term B cell activation effects that involve transcriptional changes and are beyond the scope of this report, and will be the focus of future studies in our laboratory.

Under normal conditions (in healthy controls), intact B_{an} cells exhibit significantly elevated baseline pTyr levels (Figure 3A, top left panel – compare clear bars ($p=0.023$)), which is consistent with the “high idle” signaling activity due to receptor tuning mechanisms that continuously inhibit signals elicited by the autoreactive antigen receptor [15] and distinguish this subset from other IgM^+ B cells. Consistent with the constant BCR occupancy and resultant continuous activation signals, baseline phosphorylation levels of Erk1/2, Jnk and PLC γ 2 (key BCR signaling intermediaries) were also significantly elevated in nonstimulated $B_{an(\text{control})}$ cells (Figure 3A, $p<0.05$ highlighted in grey). However, baseline phosphorylation of inhibitory phosphatase SHP was not changed, which suggests the involvement of other inhibitory pathways. In conjunction with the elevated baseline, $B_{an(\text{control})}$ cells have a reduced amplitude of pTyr response to anti-BCR stimulus as compared to the general population of IgM^+ B cells (Figure 3A, top left panel, 89 vs. 34.2% increase over baseline). Ca^{2+} responses were also significantly reduced in this subset (Figure 4A, control).

These pTyr and Ca^{2+} signaling features of B_{an} cells are consistent with at least three previously described functional properties of clonal B cell anergy (reviewed in [6]): (i) increased baseline signaling activity, (ii) reduced amplitude of BCR-triggered responses, (iii) lower BCR expression levels (particularly IgM) and support the hypothesis that in the anergic state of immune unresponsiveness B_{an} lymphocytes can recognize antigens, but are unable to produce immune responses due to intracellular signaling mechanisms that inhibit activation through antigen receptor. This mechanism is believed to play a major role in preventing autoreactive B cells that have escaped other tolerogenic mechanisms from responding to self-antigens.

Normal B_{an} cells have a unique protein phosphorylation pattern when stimulated with anti-BCR, which distinguishes this subset from the general IgM^+ B cell population (Figure 3A, Figure 6(left)). Moderately increased phosphorylation of CD19 (Figure 5B, $Y_{B_{an}(\text{control})}$ CD19 < 20% (yellow)) is consistent with the higher BCR occupancy and thus, a higher initial formation rate of BCR/CD19/Ig α / β membrane signaling complexes. However, this does not result in the enhanced activation of downstream BCR-associated signaling cascades. To the contrary, phosphorylation levels of major BCR signaling intermediaries were decreased: PLC γ 2 (–65.9%), Blnk (–276.3%), Erk1/2 (–46.7%) and Jnk (–57.6%) (Figure 5B, color-coded $Y_{B_{an}(\text{control})}$ values). The fact that $B_{an(\text{control})}$ cells exhibited these reductions in the phosphorylation of key signaling proteins involved in BCR-triggered activation pathways explains their diminished responsiveness and can too be attributed to receptor tuning signaling mechanisms that inhibit activation signals elicited by the antigen receptor.

Comparison of B_{an} phosphoprotein signaling patterns between RA and control groups revealed pronounced increases in baseline activity of Blnk, SHP2 and Jnk in the RA group (MFI values in Figure 4B and percentile differences in online Supplement Figure 2). Statistically significant increases in baseline phosphorylation levels of these proteins in the RA group indicate simultaneous activation of both signal amplificatory (Blnk, Jnk) and inhibitory (SHP) pathways in intact $B_{an(\text{RA})}$ cells. These reflect RA-induced changes in the anergic status of this B cell subset in that the increased autoantigen load promotes more BCR signaling activity, which, unlike under normal conditions (healthy controls), triggers

SHP-mediated signal inhibitory pathway. Apparently, this inhibitory pathway, aimed to offset B_{an} activation by auto-antigens, is ineffective in RA.

Comparison of relative anti-BCR phospho-response amplitudes of individual signaling molecules in B_{an} cells from RA and controls (parameters $Y_{B_{an}(RA)}$ and $Y_{B_{an}(control)}$) revealed distinctly different patterns in each group (Figure 5A, B; signaling pathways in Figure 6). When compared to controls, $B_{an}(RA)$ cells exhibited pronounced relative decreases in phosphorylation of Blnk, Syk, SHP2, CD19 and increases in Erk1/2 and Jnk.

Qualitative phospho-protein signaling differences between RA and control B_{an} cells are summarized in Figure 5C. The increased activation of Erk and Jnk upon BCR engagement in RA B_{an} cells is likely related to higher rates of transcription factors initiation and synthesis of pathogenic Abs as both Erk and Jnk are further downstream of BCR signaling pathways and both translocate into the nucleus. On the other hand, the diminished phosphorylation of more direct/upstream intermediaries of initial BCR signaling events such as Blnk, Syk and CD19 is indicative of an inhibitory pathway, though the latter is not SHP2-mediated (this phosphatase was reduced as well).

Correlation analysis of phosphoprotein responses to anti-BCR in B_{an} cells within control and RA groups revealed pairwise relationships among phospho-proteins involved in BCR signaling pathways (Figure 7A). If the matrix of intermolecular signaling correlations in the control group is to be considered the feature of “normal” signaling pathways in anergic B cells, then alterations observed in the RA group are associated with changes in the B_{an} immune tolerance status and development of the disease (Figure 7A, red highlights in top right panel). Illustrations of pairwise relationships among phospho-proteins are shown in Figure 7B.

Using linear regression model analysis adjusted for patients age and gender we did not find any significant associations between ESR or serum CRP levels and Ca^{2+} responses, baseline tyrosine phosphorylation and tyrosine phosphorylation in response to BCR crosslinking in anergic B cells of these patients. A significant positive correlation between CDAI and baseline as well as BCR-triggered total tyrosine phosphorylation was observed in the anergic IgM^- B cells of RA patients ($R^2=0.60$, $p=0.0147$, $R^2=0.67$, $p=0.007$ for baseline pTyr levels and BCR-triggered pTyr, respectively) with 2.9 unit increase in baseline pTyr and 4.4 unit increase in BCR-triggered pTyr MFI for each unit of CDAI (online Supplement Figure 4). Similarly a significant positive correlation between the CDAI and the degree of Syk and Erk phosphorylation in anergic IgM^- B cells in response to BCR stimulation was observed ($R^2=0.71$, $p=0.0023$ and $R^2=0.67$, $p=0.0113$ for pSyk and pErk, respectively) (online Supplement Figure 5). Of interest, we observed a trend for positive correlation between CDAI and peak Ca^{2+} responses in the anergic B cells, with a 6.2 unit increase in MFI for each unit increase in CDAI disease score ($R^2=0.67$, $p=0.0579$). Given relatively small sample size in this study we would like to further explore these relationships in the future study. Murine models that utilize specific signaling protein deficiencies as well as direct substrate-base protein kinase assays are also among future approaches to validate and expand our findings.

We cannot rule out a possibility that immunosuppressant and other therapeutic drugs currently used in RA, such as Methotrexate and Humira (anti-TNF shown to disrupt germinal center reactions through effects on follicular dendritic cells [16]) can have general effects on B cell signal transduction, including that in anergic B cells. Detailed information on medications received by RA patients at the time of the study is in Figure 1. Cells were studied *in vitro* after multiple washes with fresh culture media, which eliminates continuous exposure to the drugs. Furthermore, our data was subjected to multi-level normalizations,

i.e. *in vitro* cell responses to anti-BCR were calculated as % increase over corresponding non-stimulated controls separately for IgM⁺ and B_{an} cell subsets for each individual donor/sample (parameter $X_{B_{an}}$), followed by the calculation of relative differences between response amplitudes of IgM⁺ and B_{an} cells (parameter $Y_{B_{an}}$) within RA and control groups independently. Thus, any systemic/unanticipated drug effects potentially influencing signal transduction in RA B cells are reflected in these normalizations. Finally, due to the broad spectrum of lymphocyte subsets that may be affected by these drugs, isolated effects on anergic B cells are unlikely. However, we plan to address this caveat in our future studies by enrolling RA patients who have not been under treatment or had withdrawn from treatment.

CONCLUSIONS

In summary, our results confirmed two previously described anergic signaling features of potentially autoreactive CD19⁺CD27[−]IgD⁺IgM^{low/−} B cells - reduced total pTyr and Ca²⁺ responses to BCR ligation in healthy humans.

Normal B_{an} cells exhibit significantly elevated baseline pTyr levels due to increased BCR signaling activity and continuous inhibition thereof; consistent with the increased antigen receptor occupancy, baseline phosphorylation levels of key BCR signaling intermediaries Erk1/2, Jnk and PLCγ2 are significantly elevated in this subset. Normal B_{an} cells exhibit reduced phosphorylation of PLCγ2, Blnk, Erk1/2, Jnk in response to a-BCR stimulus; reductions in the phosphorylation of these key signaling proteins involved in BCR activation pathways are the result of anergic receptor “tuning” inhibitory mechanisms and are consistent with the reduced total pTyr and Ca²⁺ signaling responses in this subset.

B_{an} cells from RA patients show pronounced increases in baseline activity of Blnk, SHP2 and Jnk that reflect simultaneous and continuous activation of both signal amplificatory and inhibitory pathways in B_{an}(RA) cells; increased autoantigen load triggers an additional SHP2-mediated signal inhibitory pathway in RA. B_{an}(RA) cells exhibited pronounced relative decreases in anti-BCR phospho-response amplitudes Blnk, Syk, SHP2, CD19 (direct/upstream intermediaries of initial BCR signaling events subjected to inhibitory influences within lipid raft signaling complexes), and increases in Erk1/2 and Jnk (downstream proteins that translocate into the nucleus and are involved in transcription factors initiation and synthesis of pathogenic Abs). Differences in pairwise relationships among phospho-proteins in RA and control B_{an} cells reflect the loss of anergy and are associated with autoimmune activation.

Overall, signaling features reported in this study reflect chronic stimulation of anergic B cells with cross-reactive autoantigens paralleled by continuous inhibition of intracellular BCR signaling through downregulatory mechanisms, and represent an important mechanism that prevents autoimmunity under normal conditions. This unique balance of stimulatory and inhibitory BCR signaling cascades is clearly altered in RA and presents the opportunity to use phosphoprotein activation patterns as signaling-based biomarkers for autoreactive B cells in RA. Based on the improved understanding of the altered intracellular signal cascades in RA patients, new treatments may be developed that specifically target Blnk, Jnk, SHP2, CD19, Syk and other BCR signaling intermediaries involved in the loss of B cell anergy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Characteristic phosphoprotein signaling patterns in autoreactive anergic B cells
- Loss of anergic B cell tolerance in Rheumatoid Arthritis
- Alterations in BCR inhibitory signaling pathway in B cells producing autoAbs

RA patients										Healthy Control Subjects	
subject #	Gender	anti-CCP	RF	1st positive test	ESR	CRP	CDAI	current medication	subject #	Gender	
1	F	yes	yes	Jan-11	17	<0.5	9	plaquenil	1	F	
2	F	yes	yes	Aug-11	n/a	n/a	n/a	methotrexate (20mg/week), orenica, prednisone (5 mg/day)	2	M	
3	F	yes	yes	Aug-10	18	0.8	2	methotrexate (stopped 3mo before study)	3	M	
4	F	yes	yes	Feb-05	n/a	<0.5	n/a	methotrexate (10mg/week), humira	4	M	
5	F	yes	yes	Jun-08	6	0.7	3	plaquenil, arava, prednisone (5mg/day)	5	F	
6	M	yes	yes	Feb-07	11	0.8	5	methotrexate (17.5 mg/week)	6	F	
7	M	yes	no	Dec-08	16	<0.5	n/a	methotrexate (25mg/week)	7	M	
8	M	yes	yes	Jul-08	n/a	1.4	n/a	methotrexate (10mg/week), enbrel	8	F	
9	M	yes	yes	Jul-08	7	<0.5	20	methotrexate (20mg/week), sulfasalazine, prednisone (5 mg/day)	9	M	
10	F	yes	yes	Aug-07	18	0.6	0	methotrexate (10mg/week)	10	M	
11	M	no	yes	Apr-11	n/a	n/a	19.5	plaquenil, methotrexate (10mg/week stopped 2wks before study)	11	M	
12	F	yes	yes	Aug-10	11	0.8	0	methotrexate (15mg/week), enbrel	12	M	
13	F	yes	yes	Mar-11	20	1.0	n/a	orencia, prednisone (5-10 mg/day)	13	F	
14	M	yes	yes	Aug-04	2	<0.5	n/a	sulfasalazine 1500 bid and plaquenil 200 bid	14	M	
15	F	yes	yes	Oct-05	34	<0.5	3	enbrel, prednisone (5-10 mg/day)	15	F	
16	F	yes	yes	Feb-05	7	<0.5	4	methotrexate (10mg/week), remicade	16	M	
17	F	yes	yes	Jan-11	7	<0.5	14	orencia, prednisone (5mg/day), methotrexate (stopped 1mo before)	17	F	
18	M	yes	yes	Mar-11	9	<0.5	47	methotrexate (10mg/week), prednisone 40mg/day	18	F	
19	F	yes	yes	Dec-05	17	<0.5	n/a	naproxen, prednisone (5mg/day), sulfasalazine	19	F	
20	F	no	yes	Jan-06	6	<0.5	8	methotrexate (20mg/week), plaquenil	20	F	
M/F 0.54										21	F
										22	F
										23	M
										24	F
										25	F
										26	F
										27	F
										28	M
										29	M
										30	F
										31	F
										32	F
										M/F 0.68	

median age (range)

Group	Median Age (years)	Range (years)
RA patients	~35	25 - 65
Healthy Control Subjects	~55	35 - 80

Figure 1.

Groups of 20 RA patients (mean age 55.9; male/female ratio 0.54) and 32 healthy control subjects (mean age 35.7; male/female ratio 0.68) recruited for the study. All RA patients are RF and anti-CCP positive and have not undergone B cell targeted treatments.

Figure 2.

(A) Gating strategy to identify a subset of CD19⁺CD27⁻IgD⁺IgM⁻ B cells (B_{an}) in healthy controls. (B) The majority of B_{an} lymphocytes gated in (A) are CD27⁻. Minor CD27⁺ subpopulations also present in the gated CD19⁺IgD⁺ subsets consist of IgM⁺ memory cells and IgM⁻ Cδ class switched B cells. (C) Ca²⁺ responses to anti-BCR stimulation in IgM⁺ and CD19⁺IgD⁺IgM^{low/-} B cells. (D) total pTyr responses to anti-BCR stimulation in CD19⁺ cells (fluorescence intensity shift after the stimulation; duplicate experiments).

Figure 3.

(A) Phosphorylation levels of intracellular signaling proteins (baseline and after stimulation with anti-BCR Ab) in IgM⁺ and B_{an} subsets of B cells isolated from healthy controls; mean fluorescence intensity (MFI); p values of paired two-tailed t-test. (B) Phosphorylation levels in B cells from patients with active RA.

Figure 4.

(A) Intracellular Ca²⁺ response amplitude (% increase over baseline after stimulation with anti-BCR) in IgM⁺ and B_{an} B cells isolated from healthy controls or RA patients. (B) Comparison of baseline protein phosphorylation levels in B_{an} cells from healthy controls and RA patients; mean fluorescence intensity (MFI); p values of unpaired two-tailed t-test; statistically significant differences marked with α .

Figure 5.
(A) Comparison of B_{an} phospho-response amplitudes (% increase over baseline after anti-BCR stimulation normalized to IgM^+ response within each group) demonstrates relative signaling differences between B_{an} cells from healthy controls and RA patients; each bar shows that B_{an} response is $Y\%$ higher/lower than IgM^+ in the same donor group; parameters $Y_{B_{an}(RA)}$ and $Y_{B_{an}(control)}$ are shown. (B) Color map representation of the numerical data in Figure 5A (parameters $Y_{B_{an}(RA)}$ and $Y_{B_{an}(control)}$). (C) Qualitative phospho-protein signaling differences between RA and control B_{an} cells; $Y_{B_{an}(RA)}$ increase (↑) or decrease (↓) relative to $Y_{B_{an}(control)}$.

A**healthy controls** B_{an} phosphorylation response to anti-BCR (ρ)

	Blnk	Syk	SHP2	CD19	JNK	PLC γ 2	ERK
Blnk	0.8654						
Syk	0.1589	0.0973					
SHP2	0.4409	0.7061	0.1335				
CD19	0.5031	0.7713	-0.0085	0.9713			
JNK	0.6618	0.6849	0.1686	0.7971	0.8046		
PLC γ 2	0.4730	0.7823	-0.1118	0.9485	0.9790	0.7199	
ERK							

RA patients

	Blnk	Syk	SHP2	CD19	JNK	PLC γ 2	ERK
Blnk	0.4569						
Syk	-0.3601	0.2783					
SHP2	0.3902	0.7963	0.5181				
CD19	0.0631	0.1652	-0.0181	0.2922			
JNK	-0.0765	0.4202	0.9036	0.7694	0.1833		
PLC γ 2	0.3847	0.9181	0.4206	0.8773	0.2284	0.6011	
ERK							

B**healthy controls B_{an} cells** **RA patients B_{an} cells** **Figure 7.**

(A) Correlations between phosphoprotein responses to anti-BCR in B_{an} cells from healthy controls and RA patients (nonparametric Spearman's multivariate analysis). Statistically significant pairwise correlations ($|\rho| < 0.05$) highlighted in grey. Bottom panels display scatterplot correlation matrices for each group (each dot represents individual sample). Alterations in pairwise relationships observed in the RA group are highlighted in red. (B) Graphical representation of pairwise relationships among phospho-protein responses to anti-BCR in B_{an} cells from healthy controls and RA patients.