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CD40 Mediates Downregulation of CD32B on Specific Memory B Cell Populations in Rheumatoid Arthritis

Xiaoyu Zhang,* Erin Burch,* Ling Cai,† Edward So,‡ Fleesie Hubbard,* Eric L. Matteson,§ and Scott E. Strome*

Altered B cell function is important in the pathogenesis of rheumatoid arthritis (RA). In this report, we show that patients with active RA have an increased frequency of CD32B low/neg cells in the CD27\(^+\)IgD\(^-\) memory B cell subset and that these changes are associated with phenotypic and functional B cell activation. Studies using PBMCs from healthy donors revealed that downregulation of CD32B on B cells is mediated by CD40–CD40L interactions and is potentiated by IL-4 and inhibited by both IL-10 and IL-21. These findings appear physiologically relevant because CD4 T cell expression of CD40L correlated with the frequency of CD32B low/neg cells in the CD27\(^+\)IgD\(^-\) memory B cell subset in patients with RA. Our data support a model in which high levels of CD40L, present on circulating T cells in patients with RA, causes B cell activation and CD32B downregulation, resulting in secondary protection of memory B cells from CD32B-mediated cell death. *The Journal of Immunology, 2013, 190: 6015–6022.

Humans have three canonical activating FcγRs (CD16, CD32A, and CD64) and one inhibitory receptor (CD32B). With the exception of CD64, these receptors are of low to moderate affinity, meaning that Fc multimers of the appropriate isotype are required for effective signaling (1). Interestingly, B cells express only the inhibitory CD32B FcγR, which is thought to serve as a physiologic brake for Ab production (2). Specifically, Ag complexed to both the BCR and Ab permits crosslinking of CD32B on the B cell surface, inhibiting B cell function via an ITIM-mediated signaling cascade (3, 4). In the absence of Ag-specific Ab, Fc-bearing immune aggregates can induce CD32B-mediated B cell apoptosis through two ITIM-independent signaling pathways (5, 6).

Recent data suggest that the aberrant expression and function of CD32B on B cells is important in the pathogenesis of various autoimmune diseases (7, 8). Specifically, CD32B gene deficiency increases both the susceptibility to autoimmunity and disease severity in mice (9–11). In humans, decreased CD32B expression on B cells has been observed in patients with Ab-dependent autoimmune diseases, such as systemic lupus erythematosus (SLE), RA and chronic inflammatory demyelinating polyneuropathy (CIDP) (12–16). The precise population of B cells that are affected by this reduced expression is restricted to specific B cell subsets (17). Peripheral B cells are subdivided into naive (CD27\(^-\)IgD\(^+\)), preswitched memory (CD27\(^+\)IgD\(^+\)), conventional memory (CD27\(^+\)IgD\(^-\)) and double-negative memory (CD27\(^+\)IgD\(^-\)) subsets based on expression of the surface markers IgD and CD27 (18). Alterations in the frequency and activation status of specific subsets of circulating B cells have been observed in various autoimmune diseases and are associated with disease duration, disease activity, and production of disease-specific autoantibodies (19–22). Despite the knowledge that B cell expression of CD32B is downregulated in patients with select autoimmune diseases, it is uncertain whether this reduced expression is restricted to specific B cell subsets. Furthermore, the mechanisms that regulate B cell–associated CD32B expression in RA are unknown.

In this report, we show that the CD27\(^+\)IgD\(^-\) memory B cell population expresses significantly lower levels of CD32B in patients with active RA. Furthermore, we found that downregulation of CD32B on CD27\(^+\)IgD\(^-\) B cells ex vivo is associated with upregulation of CD40L on CD4 T cells in RA patients. The physiologic relevance of CD40 in regulating CD32B expression is supported by data showing that CD40 ligation induces sustained reduction of CD32B expression on B cells. These findings suggest an important role of the CD40L–CD40 pathway in protecting B cells from FcγR checkpoint inhibition, and they define a potential treatment approach to restore B cell homeostasis in RA.

Materials and Methods

Patients and controls

Whole blood samples from 34 patients with rheumatoid arthritis (22 women, 12 men; mean age, 53 y; range, 26–71 y) were obtained from the Mayo Clinic College of Medicine. All patients with RA met the 1987 American College of Rheumatology classification criteria for RA (23). Disease activity was assessed using the disease activity score index (24). Patients who received rituximab (Rituxan) treatment within 2 y were excluded. The protocol was approved by the institutional review boards at the Mayo Clinic College of Medicine and the University of Maryland.

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School of Medicine, and all patients provided informed written consent. Thirty-one peripheral blood samples from healthy controls were obtained as whole blood or buffy coats from Biological Specialty Incorporation (Colmar, PA). Among these healthy donors, 23 (15 women, 8 men; mean age, 47 y; range, 37–71 y), were used as age- and sex-matched healthy controls for patients with RA.

Cell preparation and activation
PBMCs were isolated from whole blood or buffy coat with Ficoll density gradient centrifugation (Amersham Biosciences). B lymphocytes were purified from PBMCs by negative selection using B cell isolation kit II (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. Purity of cell separations was typically >98%. All in vitro cell cultures were performed in RPMI 1640 (Mediatech) supplemented with 10% FCS (Atlanta Biologicals), 1% penicillin/streptomycin, 1% HEPES, and 1% Glutamax (all from Life Technologies).

PBMCs or purified B cells were cultured at a concentration of 1 × 10⁶ cells/ml for 3 days (CD27+IgD− CD32B high), followed by subsequent 5 days (CD27+IgD− CD32B low). Following reagents were added at the initiation of culture: 10 µg/ml α-1gs F(ab')₂ fragments (goat α-human IgA/IgG/IgM [H+L], Jackson ImmunoResearch), 1 µg/ml purified goat α-human CD40 Ab (R&D Systems), 5 µg/ml recombinant human CD40L (R&D Systems), 2.5 µg/ml CpG (InvivoGen). The following recombinant cytokines were used to evaluate their effect on CD32B expression: 100 U/ml IL-2 (Proleukine, Chugai), 20 ng/ml IL-4 (R&D Systems), 1 ng/ml IL-10 (BD Biosciences), 50 ng/ml IL-10 (eBioscience, San Diego, CA), 100 ng/ml IL-21 (BioSource International, Camarillo, CA), 20 ng/ml BAFF (eBioscience), 500 U/ml IFN-γ (eBioscience), and 50 ng/ml TNF-α (BD Biosciences). To induce CD40L expression on T cells, PBMCs were activated with 5 µg/ml PWM (Sigma-Aldrich). Purified mouse α-human CD40L (20 µg/ml; BD Biosciences) mAb was used to block CD40-CD40L interactions.

Flow cytometric analysis
For flow cytometric analysis ex vivo, 100 µl whole blood was incubated with various combinations of conjugated mAbs to cell surface markers for 30 min at room temperature. BRCs were subsequently lysed in PBS by lysis buffer (eBioscience) and washed twice with FACS buffer (0.5% BSA in PBS). For in vitro analysis of B cell phenotypes, cells were collected from cell culture, stained with indicated mAbs in FACS buffer, and washed twice before acquisition. The flow cytometry samples were then analyzed by FACSDiva (BD Biosciences) or FlowJo software. The following reagents were used: FITC, PE, APC, and BV421-conjugated mAbs were added at the following concentrations: 0.1 µg/ml anti-CD3, 0.1 µg/ml anti-CD4, 0.1 µg/ml anti-CD8, 0.1 µg/ml anti-CD27, 0.1 µg/ml anti-CD38, 0.1 µg/ml anti-CD95, 0.1 µg/ml anti-CD154, 0.1 µg/ml anti-BR3, 0.1 µg/ml anti-TACI, and 0.1 µg/ml anti-TRANCE. peripheral blood mononuclear cells were stained with 10 µg/ml FITC-conjugated anti-CD20 mAb (BioLegend). Background staining with isotype controls was subtracted. B cell apoptosis was determined by staining with annexin V/7-aminoactinomycin D (Annexin V-PE Apoptosis Detection Kit I, BD Biosciences) according to the manufacturer’s instructions.

Measurement of serum autoantibodies
Patients’ plasma samples were collected after centrifuge separation of whole blood in an ACD-A BD Vacutainer and frozen at −80°C. The levels of rheumatoid factor (RF) IgG (Oncogene Diagnostika, Mainz, Germany) and autoantibodies (anti-CCP1, anti-CCP2, and anti-CCP3) were measured with ELISA following the manufacturer’s instructions. The cutoff level for both RF IgG and anti-CCP1, anti-CCP2, and anti-CCP3 was 5 U/mL. Greater than 60 U/mL for anti-CCP3 is considered strongly positive.

Quantitative RT-PCR
Total RNA was isolated using the SV Total RNA Isolation System (Promega), and cDNA was synthesized using the Maxima First Strand cDNA synthesis kit (Fermentas Life Sciences). Real-time quantitative PCR was performed using 7500-Fast Real-Time PCR Systems (Applied Biosystems, Foster City, CA) with specific primers, probes, and software (Applied Biosystems). The level of CD32b mRNA was quantified based on a titrated standard curve co-run in the same experiment and calibrated with the expression level of acidic ribosomal protein 36B4. All samples were done in triplicate.

Statistical analysis
Differences between defined patient and control groups were compared using the two-tailed nonparametric Mann–Whitney U test. Correlation was examined using Spearman rank correlation analysis; p < 0.05 is considered statistically significant. Data were analyzed using GraphPad Prism software (GraphPad Software).

Results
Peripheral B cell subsets are altered in patients with RA
We first determined the frequency of distinct B cell subsets, defined by the expression of CD27 and IgD, in the peripheral blood of healthy controls and patients with RA (Fig. 1). Patients with active RA had a significantly higher frequency of CD27+IgD− memory B cells and lower frequency of CD27+IgD− naive B cells than patients with inactive disease. In addition, patients with active RA had a significantly higher frequency of CD27+IgD− memory B cells than did healthy controls, but not compared with patients with inactive RA. These data demonstrate that patients with active RA have increases in their memory B cell subsets and select decreases in naive B cells that cannot be adequately characterized by CD27 staining alone.

Expression of CD32B is reduced on CD27+IgD− memory B cells in patients with active RA and is associated with high levels of autoantibody production
CD32B expression on circulating B cells is downregulated in several autoimmune diseases. For example, CD27 positive memory B cells have decreased CD32B expression in patients with RA (14). Despite this knowledge, it is unclear whether this downregulation is restricted to specific memory B cell subsets or select populations within these subsets. To address these questions, we first compared the expression pattern of CD32B on specific B cell subsets from healthy donors. In contrast to the uniform expression of CD32B on IgD+ B cells (both CD27+ and CD27− memory), IgD− B cells (including CD27+IgD− and CD27−IgD− memory subsets) demonstrated a heterogeneous pattern of CD32B expression in select donors (Fig. 2A). Specifically, there was a distinct subpopulation of CD32B low/neg B cells in both subsets of IgD− B cells. The prevalence of these populations is age dependent, because donors younger than 30 y displayed a significantly lower frequency of CD32B low/neg B cells in CD27+IgD− B cells (Fig. 2B).

Subsequent analysis (Fig. 3) revealed that this CD32B low/neg CD27+IgD− population was significantly increased in RA patients with active disease compared with age matched healthy controls. Furthermore, patients with high serum levels of RF IgG or α-cyclical citrullinated peptide (CCP) autoantibodies had significantly higher frequencies of CD32B low/neg CD27+IgD− cells compared with both patients with low serum autoantibodies and healthy controls. In contrast, the frequency of CD32B low/neg B cells in CD27−IgD− B cell subsets did not differ between patients and healthy donors. There was also no correlation between the frequency of CD32B low/neg CD27+IgD− B cells with disease activity or serum levels of autoantibodies in RA patients.

Frequency of CD32B low/neg CD27+IgD− memory B cells correlates with B cell activation in RA
Ex vivo analysis of B cell activation revealed (Supplemental Fig. 1) that the CD27+IgD− memory B cell subset expressed the highest
levels of activation markers in both RA patients and healthy donors. Therefore, we wanted to understand whether there was a correlation between CD32B downregulation and CD27+IgD+ memory B cell activation in vivo. As shown in Fig. 4A, the frequency of CD32B low/neg B cells was significantly correlated with the expression levels of CD80, CD86 and CD95 on the CD27+ IgD- B subset in RA patients. However, there was no correlation between CD32B and CD80 or CD86 on CD27+ IgD- B cells from healthy donors.

To place these findings in context, we evaluated the expression of CD32B on germinal center (GC) and naive B cells from hypertrophic human tonsil as a surrogate for the rheumatoid synovium (25). IgD+CD38hi naive B cells were CD32BhiCD86loCD80loCD95lo, and IgD-CD38+ GC B cells were CD32BloCD86hiCD80hiCD95hi (Fig. 4B). Although these findings must be interpreted with caution, they suggest that CD27+IgD- memory B cells from patients with RA are in a chronic state of activation, analogous to that observed in GC B cells from human tonsil.

CD40 signaling is important for the downregulation of CD32B on activated B cells

The fact that the increased frequency of the CD32B low/neg CD27+IgD- memory B cell subset correlates with cell activation in RA suggests that cellular activation can cause CD32B downregulation on B cells. We therefore asked what activation signals are required for this effect. Purified B cells from healthy donors were activated through BCR crosslinking, CD40 ligation, or TLR stimulation. Stimulation of purified B cells with α-CD40 resulted in significant downregulation of CD32B expression in a dose-dependent fashion (Supplemental Fig. 2). This effect was enhanced when B cells were stimulated concurrently with α-Ig or CpG. In contrast, CpG stimulation did not induce the downregulation of CD32B, whereas α-Ig led to a slight reduction (Fig. 5A). Next, we used recombinant CD40L, which lacks an Fc domain, to activate B cells in the presence of BCR crosslinking. Like α-CD40, CD40L stimulation mediated downregulation of CD32B on α-Ig activated B cells (Fig. 5B). Furthermore, the addition of α-CD40L blocking mAb to PWM activated PBMCs, partially reversed these effects, indicating that the reduction of CD32B expression can occur as a result of naturally occurring CD40L-CD40 interactions (Fig. 5C). In keeping with these observations, α-CD40 stimulation decreased CD32B mRNA expression (Fig. 5D).

To understand whether the observed loss of CD32B could be attributed to selective activation induced apoptosis of the CD32B high population, we cultured B cells in the presence of α-CD40 and studied the survival of CD32B low/neg and CD32B high subsets. Our data demonstrate that, following exposure to α-Ig α-CD40, both CD32B low/neg and CD32B high B cells have similar rates of apoptosis (data not shown). Importantly, this response appears to be durable, as prolonged exposure of B cells to α-CD40, results in sustained decreases in the levels of CD32B expression (Fig. 5E). Collectively, these data demonstrate that CD40 ligation induces persistent downregulation of CD32B expression on B cells and that this response is not simply a result of deleting the CD32B high B cell subset.

**FIGURE 1.** B cell subsets in RA patients and healthy controls separated according to surface expression of CD27 and IgD. (A) Representative dot plot of IgD versus CD27 expression on peripheral blood CD19+ B cells from a healthy donor. (B) The frequency of each subset of peripheral blood B cells was compared among patients with active RA (n = 12) or inactive disease (n = 16) and healthy donors (n = 21). Results are shown as box plots displaying the median, 25th and 75th percentiles as the box, and the 5th and 95th percentiles as whiskers. *p < 0.05, **p < 0.005.

**FIGURE 2.** CD32B low/neg population identified in peripheral CD27+ IgD- and CD27+ IgD+ B cell subsets. (A) Histograms from a healthy donor showing CD32B expression in four subsets of peripheral blood B cells. The values represent the frequencies of CD32B low/neg in each B cell subset. (B) Box plots representing the 5th, 25th, 50th (median), 75th, and 95th percentiles of the frequency of CD32B low/neg cells in peripheral CD27+IgD- and CD27+ IgD- B cell subsets in age groups <30 (n = 7) and >30 (n = 21) of healthy donors. *p < 0.05.
CD40-mediated suppression of CD32B is potentiated by IL-4 and reversed by IL-21 and IL-10

Recent evidence suggests that binding of the soluble Fc component of i.v. Ig to DC-SIGN leads to upregulation of CD32B on regulatory macrophages in an IL-33– and IL-4–dependent fashion (26). Based on these data, we sought to determine whether specific cytokines could work in concert with α-CD40 to modulate B cell expression of CD32B. In our initial studies, we screened a panel of cytokines known to modulate B cell activation or function (Fig. 6A). When used in combination with α-Ig, α-CD40, or α-Ig/α-CD40, IL-4 further reduced the expression of CD32B, whereas IL-21 induced CD32B upregulation. Interestingly, IL-6 and IL-10 also upregulated CD32B expression, but only in the presence of both α-Ig and α-CD40 stimulation. Importantly, whereas CD40

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**CD27 IgD**

FIGURE 3. Frequencies of CD32B low/neg B cells are increased in the CD27* IgD* memory B cell subset from RA patients with high levels of serum autoantibodies. CD32B expression on CD27* IgD* and CD27* IgD* B cell subsets was compared between RA active (n = 15) versus inactive disease (n = 14), RF IgG-positive (n = 16) versus -negative (n = 8), and α-CCP IgG/IgA strong positive (n = 21) versus low/negative (n = 8), respectively. Data from aged-matched healthy controls (n = 16) were included in each comparison. Results are shown as box plots displaying the median, 25th, and 75th percentiles as the box, and the 5th and 95th percentiles as whiskers. *p < 0.05.

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**CD27 IgD**

FIGURE 4. Frequency of CD32B low/neg CD27* IgD* B cells correlates with B cell activation in RA. (A) Expression of CD32B, CD86, CD80, and CD95 on the peripheral blood CD27* IgD* B cell subset were analyzed by FACS. The correlations between CD32B expression and CD86, CD80, and CD95 expression were compared between patients (n = 22 for CD86 and CD95, n = 17 for CD80) and healthy donors (n = 21) using Spearman rank correlation analysis. (B) Tonsillar mononuclear cells were analyzed for the expression of CD32B, CD80, CD86, and CD95 on naive (IgD+CD38*) and GC (IgD-CD38*) B cells. Data are representative of three experiments from different donors.
stimulation downregulated CD32B on both CD27+IgD− memory and CD27−IgD+ naive B cells, when used in isolation, IL-4 only affected naive B cells. However, the combination of IL-4 and CD40 worked synergistically to downregulate CD32B on both subsets of B cells in vitro (Fig. 6B).

Correlation between CD32B downregulation on CD27+IgD− B cells and CD40L T cells in patients with RA

Having demonstrated that CD40 signaling mediated CD32B downregulation on B cells from healthy donors in vitro, we next sought to understand the role of CD40/CD40L interactions in regulating
CD27⁺IgD⁻ memory B cell specific CD32B downregulation in vivo in patients with RA. We first compared CD40L expression on resting and activated CD4 T cells between patients with RA and healthy controls, and we confirmed previously reported significant increases in CD40L expression in RA patients (Fig. 7A, 7B) (27). Furthermore, we found a significant correlation between CD4 T cell expression of CD40L and the size of the CD32B low/neg subpopulation in CD27⁺IgD⁻ B cells in RA (Fig. 7C). These data provide indirect evidence that the high levels of CD40L found in patients with RA might contribute to the reduced levels of CD32B on select B cell subsets.

Discussion

In this study, we show that patients with active RA have a significantly higher frequency of CD27⁺IgD⁻ memory B cells and a lower frequency of CD27⁺IgD⁺ naive B cells compared with patients with inactive disease. Furthermore, we identify a subpopulation of CD32B low/neg cells in the CD27⁺IgD⁺ peripheral memory B cell subset, which is increased with age in both healthy individuals and in patients with RA. These data are consistent with previous reports, which showed that patients with active RA have reduced levels of CD32B expression on CD27⁺ memory B cells (14). Our findings demonstrate that this downregulation of CD32B on memory B cells is not present in all memory subsets; rather, it is primarily restricted to the CD27⁺IgD⁻ B cell population. Specifically, unlike CD27⁺IgD⁻ naive B cells, the expression pattern of CD32B on CD27⁺IgD⁻ B cells is heterogeneous, with a primary population of CD32B high and a subpopulation with CD32B low/neg expression. The increased frequency of this CD32B low/neg population in the CD27⁺IgD⁻ memory B subset is associated with enhanced B cell activation and autoantibody production. Whereas these changes in the absolute percentage of B cell expression of CD32B are relatively small, murine studies demonstrate that even small differences in B cell expression of CD32B have profound biologic significance (17), and they suggest that our findings are physiologically relevant.

Importantly, whereas phenotypic decreases in CD32B might be confounded by RF- or α-CCP-containing immune complexes interference with α-CD32B staining, these factors cannot completely account for our observations. Specifically, in vitro preincubation of B cells in serum with different levels of autoantibodies does not result in differential binding of detecting α-CD32B Ab on the B cell surface. (Supplemental Fig. 3) Furthermore, our data demonstrate that, in patients with high levels of RF and/or α-CCP, the levels of CD32B low/neg cells were selectively increased in the CD27⁺IgD⁻ subset, but not in the CD27⁺IgD⁺ population. In addition, both CD27⁺IgD⁺ memory and CD27⁻IgD⁻ naive B subsets from patients with RA express CD32B at similar levels as healthy controls (data not shown). If steric hindrance were artificially inhibiting α-CD32B staining, we would expect that these three B cell subsets would exhibit changes in CD32B expression analogous to those observed in the CD27⁺IgD⁻ B subset in patients with high levels of autoantibodies.

In addition to RA, the dysregulation of CD32B on B cells has been reported in several other autoimmune diseases (13–15). However, the mechanisms responsible for the B cell–specific downregulation of CD32B are uncertain. Based on the fact that (1) in vitro cell activation has been linked to the downregulation of CD32B on B cells (28) and (2) B cells in autoimmune diseases are in an abnormal state of activation (14, 29), we compared the role of different activation signals in regulating B cell–associated CD32B expression. In keeping with previous reports (28), CD40 stimulation alone causes significant downregulation of CD32B expression on B cells from healthy donors. Furthermore, although TLR stimulation alone has no effect and BCR crosslinking only induces an intermediate level of CD32B loss, the combination of CD40 costimulation with these signals produces synergistic downregulation of B cell–associated CD32B.

Compared with naive B cells, CD27⁺IgD⁻ memory B cells from both healthy donors and RA patients had an activated phenotype (Supplemental Fig. 1), as evidenced by enhanced expression of CD86, CD80, and CD95. Interestingly, there was no correlation between CD27⁺IgD⁻ memory B cell expression of CD32B and cell surface expression of CD80 and CD86 in healthy donors. In contrast, the frequency of CD32B low/neg in CD27⁺IgD⁻ memory B cells from patients with RA was strongly correlated with phenotypic and functional activation parameters. Considering that different B cell activation signals lead to different levels and different subset specific regulation of CD32B expression, we postulate that the mechanisms that induce B cell activation differ between patients with RA and healthy controls.

**FIGURE 7.** Inverse correlation between expression of CD32B on B cells and CD40L on T cells in patients with RA. (A and B) CD40L expression is enhanced on T cells from patients with RA. Peripheral blood CD4 T cells were analyzed for baseline (A; n = 14 for healthy, n = 26 for RA) and PWM activation induced (B; n = 7 for healthy, n = 12 for RA) CD40L expression by flow cytometry. The line indicates the median. *p < 0.05, **p < 0.005. (C) The correlation between ex vivo expression of CD40L on CD4⁺ T cells with CD32B on CD27⁺IgD⁻ B cells in patients with RA (n = 30) was analyzed using Spearman rank correlation analysis.
CD40-CD40L engagement plays a pivotal role in the pathogenesis of autoimmune diseases (30). CD40L is expressed mainly on activated CD4+ T cells. In concordance with previous reports (27), we observed that expression of CD40L is increased on both resting and activated CD4+ T cells in patients with RA. This increase might reflect an augmented and prolonged Ag driven activation of lymphocytes in patients. Alternatively, a recent study revealed that increased CD40L mRNA expression is associated with DNA demethylation in female patients with RA (31). Importantly, a significant correlation was noted between CD4+ T cell expression of CD40L and the frequency of CD32B low/neg B cells in the CD27+IgD− memory B cell subset in our study. The biologic relevance of this finding is supported by the data showing that RA CD27+IgD− memory B cell have a similar phenotype as in vitro α-CD40–activated B cells from healthy donors and GC B cells from hypertrophic human tonsils. Despite the fact that disease duration and varied treatment algorithms can differentially influence T cell expression of CD40L and B cell expression of CD32B, our data suggest that intensive CD40-CD40L interactions have an important role in downregulating CD32B expression on specific B cell subsets in patients with RA.

Following CD40 ligation, the absolute levels of CD32B expression on B cells are tightly regulated by the cytokine microenvironment, with IL-4 inhibiting and IL-6, IL-10, and IL-21 potentiating expression. IL-4 is reported to reduce the expression of CD32B on activated murine B cells (32). Our data indicate that IL-4 alone affects CD32B expression on CD27+IgD− naive B cells in humans. When used in combination with α-CD40, the function of IL-4 is potentiated, facilitating CD32B loss on both naive and memory B cell subsets. Interestingly, memory T cells from the synovia of patients with RA have limited ability to produce IL-4 (33), and IL-4 can be used to treat murine models of RA. Furthermore, IL-21R−deficient mice do not develop disease in a K/BxN murine model of RA (34). Therefore, our studies suggest that following CD40 ligation, these therapies could paradoxically enhance the ability of B cells to survive by limiting the effects of Ab-Ag–mediated signaling through CD32B.

Our study is limited by the lack of information regarding CD32B expression on specific B cell subsets in diseased synovium. To gain insight into whether the observed downregulation of CD32B in specific B cell subsets is relevant to synovitis in RA, we analyzed the phenotype of GC B cells from tonsils with chronic inflammation. Our rationale for analyzing tonsillar tissue was that synovial tissue from patients with active RA forms ectopic lymphoid microstructures that are similar to those found in secondary lymphoid organs such as human tonsils (25). Our data demonstrate that IgD−CD38+ GC B cells express low levels of CD32B and high levels of activation markers. The relevance of these findings is supported by a recent study (35) demonstrating that CD27+IgD− B cells preferentially localize in the synovial compartment. Considering these facts and the recognized limitations of using surrogate tissue for analysis, we postulate that the decreased expression of inhibitory Fc receptor on CD27+IgD− B cells participates in the pathogenesis of synovitis in RA.

Human and mouse CD32B have similar inhibitory functions on B cells; however, they are dissimilar in their relative abilities to bind IgGs and in their patterns of cellular expression (36). Our human B cell data, when taken in context with published findings in mice, suggest that the regulation of CD32B expression is also different between human and mouse B cells. For example, mouse B cells are reported to upregulate CD32B in response to LPS or α-BCR stimulation, whereas CD40 stimulation alone does not influence CD32B expression (32, 37). In contrast, expression of CD32B on human B cells is downregulated in the presence of either CD40 stimulation or BCR crosslinking, but is not influenced by CpG stimulation. In vitro, IL-4 reduces CD32B expression on activated B cell from both human and mouse. Furthermore, although CD32B is upregulated on GC B cells in normal mice (38), our study confirmed that it is downregulated on human GC B cells (39). Considering the essential role of CD40-CD40L interactions in the GC response, we postulate that the differential regulation of CD32B expression induced by CD40 stimulation of human and mouse B cells, might contribute to the observed disparity in CD32B expression on GC B cells between humans and mice in vivo.

Genetic variants of CD32B gene are reported to affect the expression and function of this receptor and be associated with select autoimmune conditions in both mice and humans (40–42). We cannot completely exclude the possibility that specific CD32B polymorphisms, known to affect CD32B expression, partially account for our observations in RA; however, the extremely low prevalence of these polymorphisms limits their potential influence on our findings (41, 42).

In summary, we identified a subpopulation of CD32 low/neg B cells in the CD27+IgD− memory B cell subset in patients with RA, which is associated with disease activity and autoantibody production. This CD32B downregulation is mediated by CD40-CD40L interactions and is secondarily regulated by the cytokine milieu. Understanding of these regulatory pathways provides potential sites, alone and in combination, for therapeutic intervention in autoimmune disease.

Disclosures
S.E.S. is a cofounder and major stockholder in Gliknik Inc., a biotechnology company. He also receives royalties from the Mayo Clinic College of Medicine for licensure of intellectual property pertaining to the use of CD137 and B7-H1 to third parties. The other authors have no financial conflicts of interest.

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Supplemental figure 1. Phenotype of peripheral B cell subsets in healthy and RA.

The expression of CD86, CD80, and CD95 on peripheral blood B cell subsets were compared between RA patients and healthy donors. P values were determined by the Mann-Whitney test.

* p<0.05, **p<0.01.
Supplemental Figure 2. Anti-CD40 mediates down regulation of CD32B on peripheral B cells in a dose-dependent fashion.

Purified peripheral blood B cells from healthy donor were stimulated with different concentrations of α-CD40 Ab for 2 days. Expression of CD32B on the CD27+IgD- and CD27-IgD+ B cell subsets was analyzed by FACS staining. Data are representative of two individual experiments using different donors.
Supplemental figure 3. The binding of anti-CD32B Ab on the B cell surface is not affected by the level of serum RF IgG.

Raji cell (A) and purified peripheral blood B cell from one healthy donor (B) were pre-incubated in 50% serum from RA patients and healthy donors. Binding of α-CD32B Ab on cell surface was then assessed by FACS analysis. The expression of CD32B on Raji cell line is shown as MFI, whereas percent of CD32B low/neg in the CD27⁺IgD⁻ memory B cell subset is shown for peripheral blood B cells. Compared with serum from healthy donors (n=5) and RF negative patients (n=8), pre-incubation of Raji cells and purified B cells with RF positive serum (n=19) does not decrease the binding of α-CD32B antibody on cell surface.