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Autoreactive B cells in rheumatoid arthritis

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Chapter III

Autoreactive B cells in rheumatoid arthritis highly express Fas and CD22

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Abstract

B cell tolerance is established through the balance between activating and inhibitory signals. In mice, downregulation of immune checkpoint receptors (ICRs) leads to B cell dysregulation and autoantibody formation. Previously, we showed that autoreactive B cells expressing anti-citrullinated proteins antibodies (ACPA) in rheumatoid arthritis (RA) patients downregulated the expression of Fc-gamma-RIIb, an essential ICR that conveys inhibitory signals from immune-complexed IgG. Using differentially labelled streptavidin tetramers bound to cyclic citrullinated peptide 2 (CCP2), we extended the assessment of ICR expression by circulating, ACPA-positive memory B cells (MBC) using flow cytometry in these patients. We found that apoptotic receptor Fas expression was upregulated on ACPA-positive MBC compared to control MBC. The upregulation of Fas and downregulation of Fc-gamma-RII resembled the phenotype of tetanus toxoid (TT)-specific MBC from healthy individuals upon recent booster vaccination. The expression levels of other ICRs tested (CD5, PECAM-1, CD200R, LAIR-1, FcRL4) were expressed variably but similar between ACPA-positive MBC and control MBC. Of note, CD22 was strongly expressed by the majority of both ACPA-positive and TT-specific MBCs. In conclusion, with respect to the expression of the ICRs tested, ACPA-positive MBC are heterogenous but show consistent signs of recent antigenic and T cell-derived activation. The strong positivity of ACPA-positive MBC for Fas and CD22 suggest that these receptors may serve as targets for interventions that could lead to autoreactive B cell tolerance and/or apoptosis.

Chapter III

Introduction

B cell activation in germinal centres is hallmarked by somatic hypermutation and clonal expansion. These characteristics are reminiscent of tumour development which entails high genetic mutation load and hyperproliferation. To regulate its activation, B cells express immune checkpoint receptors (ICRs), for instance immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing receptors and apoptosis-inducing receptors. The inhibitory receptors, such as CD5, CD22, and FcγRIIB, LAIR1 and FcRL4, function by activating phosphatases at the proximity of the B cell receptor (BCR) activation signalling microdomain. Subsequently, they dephosphorylate key phosphorylated proteins in the BCR signalling pathway, thereby modulating the signal strength and the downstream effects such as cell proliferation and survival.[1] Ligation of apoptosis receptors such as Fas receptor (CD95), on the other hand, induces the formation of a death-inducing signalling complex (DISC) which promotes an activation-cascade of caspases leading to DNA degradation, membrane blebbing and other apoptosis characteristics.

The expression of ICRs is essential throughout B cell development to prevent excessive B cell responses towards foreign antigens as well as activation of autoreactive B cells. In fact, deficiency of Fas, PECAM-1 and CD22 in mice leads to autoimmune hyperproliferative syndrome, autoantibody formation and B cell hyperreactivity, respectively.[2-4] The activation of ICRs, however, does not necessarily lead to B cell apoptosis. CD5, PECAM-1 and LAIR-1 activation has been shown to protect B cells from activation-mediated cell death.[5,6] Therefore, the balance between activating signals and inhibitory signals is essential for B cell survival, development and function.

B cell dysfunction plays a central role in human autoimmune diseases, as demonstrated by the clinical efficacy of CD20-depletion therapy, rituximab, in treating rheumatoid arthritis (RA), pemphigus vulgaris and antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis.[7-9] The majority of RA patients harbours disease-specific anticitrullinated protein antibodies (ACPA), a family of citrullinated antigen-binding autoantibodies which are able to prognosticate rapid progression of RA. Previously, we showed that ACPA-positive B cells from RA patients persistently proliferated, up-regulated co-stimulatory molecules CD80/86 and excessively produced the neutrophil chemoattractant IL-8.[10] Interestingly, these cells down-regulated Fc-gamma-RII (FcγRII) which may enable them to circumvent ACPA immune complex-mediated inhibition. Notably, this finding

is in-line with autoimmune susceptibility observed in FcγRIIB-deficient mice.[11] Moreover, it is relevant not only to explain how ACPA-positive B cells remain hyperactive despite circulating ACPA and ACPA-containing IC, but also in light of efforts that evaluate FcγRIIB as target to inhibit autoreactive B cells in RA and other autoimmune diseases. As deficiency of other ICRs can also lead to autoimmunity in mice, a thorough assessment of ICR expression on ACPA-positive B cells is essential for understanding how immunological tolerance is breached and which of these receptors can be targeted. In this manuscript, we therefore assessed the expression of several ICRs, including CD5, CD22, PECAM-1, Fas, CD200R, LAIR-1 and FcRL4 on circulating ACPA-positive memory B cells (MBCs) in patients with RA. As comparator, we used tetanus toxoid (TT)-specific MBC from the same patients. Finally, we assessed possible mechanism by which ICR expression could be altered.

Here, we describe that ICR downregulation is not a general feature of ACPA-positive MBC, as CD22, CD31, CD200R and LAIR-1 abundance on ACPA-positive MBC was similar to that on control MBC. Moreover, only a fraction of ACPA-positive MBC expressed the markers CD5 and FcRL4, similar to control B cells. Remarkably, Fas was strongly expressed by the autoreactive MBC. The upregulation of Fas and the downregulation of FcγRIIB, are compatible with recent antigenic stimulation and T cell-derived factors, as TT-specific MBC showed a similar phenotype upon booster vaccination. Together, these phenotypic findings may be relevant for future therapeutic approaches aimed at targeting autoreactive B cells in RA.

Materials and Methods

Patients and healthy donors

Eighteen ACPA-positive RA patients receiving conventional synthetic disease modifying anti rheumatic drugs (csDMARDs) and, in some cases, glycocorticoids at the outpatient clinic of the Department of Rheumatology at the Leiden University Medical Centre (LUMC) were recruited to participate in the study. The patients met the 2010 ACR/EULAR criteria for RA at the time of diagnosis and were naïve for any biologic DMARDs. All patients gave written informed consent and donated five heparin-tubes of venous blood, totalling approximately 45 ml. The ethical review board of LUMC gave permission for the conduct of the study (protocol number P17.151). Three healthy donors, one female and two males, received

tetanus toxoid (TT) booster vaccination and donated five heparin-tubes of venous blood before vaccination and at week 1, 2, 5, 10 and 22 post booster vaccination.

Cell isolation and flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood using Ficoll-Paque gradient centrifugation and stored at 4°C overnight in Iscove's Modified Dulbecco's Medium enriched with 8% heat-inactivated foetal calf serum, 100 U/ml penicillin/streptomycin and 2mM Glutamax. To determine ACPA-positive B cells, differentially labelled streptavidin tetramers bound to biotinylated second generation cyclic citrullinated peptide (CCP2) and its arginine control variant (CArgP2) were used as previously described.[12] In addition, direct conjugation of two separate fluorophores to TT proteins were used to determine TT-specific B cells.[10] To stain for the ICRs on both ACPA-positive and TT-specific B cells, PBMC from individual patients were split into three fractions. Two fractions were stained with CCP2-Brilliant Violet 605 (BV605), CCP2-APC and CArgP2-PE tetramers, anti-CD3 Pacific Blue (PB, clone UCHT1), CD14 PB (clone M5E2), CD19 APC-Cy7 (clone Sj25C1), CD20 Alexa Fluor 700 (AF700, clone 2H7, Biolegend), CD27 PE-Cy7 (clone M-T271) or CD27 PerCP Cy5.5 (clone M-T27,1Biolegend) to detect all B cell subsets of ACPA-positive B cells. At the same time, one of the two PBMC fractions was stained with anti-CD5 BV510 (clone UCHT2), CD22 BV510 (clone HIB22), CD31 FITC (clone WM59), CD95 PE-CF594 (clone DX2), CD200R PerCP eFluor710 (clone OX108), anti-FcRL4 (CD307d) PE-Cy7 (clone 413D12, Biolegend), anti-LAIR1 (CD305) Brilliant Blue 515 (BB515 clone DX26, all from BD Biosciences except when notified); while the second fraction of the two PBMC was stained with appropriate isotype controls of anti-ICR antibodies conjugated with the same fluorophores. The third fraction of the PBMC was stained with TT-PE and TT-APC and the same labelled antibodies as fraction one. Due to redundancy of some fluorophores listed, two combinations of the above-mentioned labelled antibodies were employed: first a combination of CD5, CD31, CD95 and FcRL4 staining and second CD22, CD200R and LAIR1. Consequently, samples from multiple patients were required to assess the expression of all ICRs listed. DAPI was added at a final concentration of 200nM directly before measurement on a BD LSRFortessa flow cytometer.

For the vaccination study, PBMC were divided into three fractions in a ratio of 2:1:2 based on cell count. The third fraction was frozen for future studies while the first two were stained directly for dead cell detection using fixable violet dead

cell staining kit (Molecular probes). The first fraction was subsequently stained with TT-PE, TT-APC, anti-CD3 PB, CD14 PB, CD19 APC-Cy7, CD20 AF700, CD27 PE-Cy7, CD95 PE-CF594 and CD32 BV605 (clone FLI8.26, all from BD Biosciences). The second fraction was stained with the same set of antibodies and labelled TT but received the isotype controls of anti-CD95, and CD32 with the identical fluorophores. Next, both fractions were fixed and permeabilized (Fixation/ Permeabilization Concentrate, Diluent, and Buffer by eBioscience) to stain for Ki-67 for another study. These samples were also measured with BD LSRFortessa. All data were analysed using BD FACSDiva Software Version 9.0 (BD Biosciences). FlowJo v10.7.0 (BD) software was used for multilayer histogram data depiction of the vaccination study.

Statistical analysis

GraphPad Prism 8.1.1 (330) was used to perform statistical analyses. Friedman test, followed by Dunn's test was used to compare paired, non-parametric data of more than 2 groups. Information about the number of patients used for each marker is provided in the figure legends.

Results

Circulating ACPA-positive MBC highly expressed Fas and CD22

Previously, we developed a staining procedure for flow cytometry which enabled a direct comparison of rare, antigen-specific autoreactive versus protective B cell populations from individual patients with RA.[10] ACPA-positive MBC were detected using differentially labelled streptavidin tetramers conjugated with biotinylated CCP2 or its arginine control peptide, CArgP2. On the other hand, tetanus toxoid (TT)-specific MBC were detected using direct labelling of TT protein using two distinct fluorophores. The specificity of the staining was confirmed by immortalized monoclonal B cell lines expressing antibodies with the respective reactivities, and by blocking experiments on patient's samples.[10,12] Live B cells were identified based on CD19 positivity and negativity for CD3, CD14 (marker of T cell and monocyte, respectively) and a dead cell marker. As described previously, the majority of antigen-specific B cells expressed both CD20 and CD27, which marks the differentiation stage of memory B cells (MBC). Therefore, all subsequent comparisons of ICR expression were performed on the CD20+CD27+ MBC subset

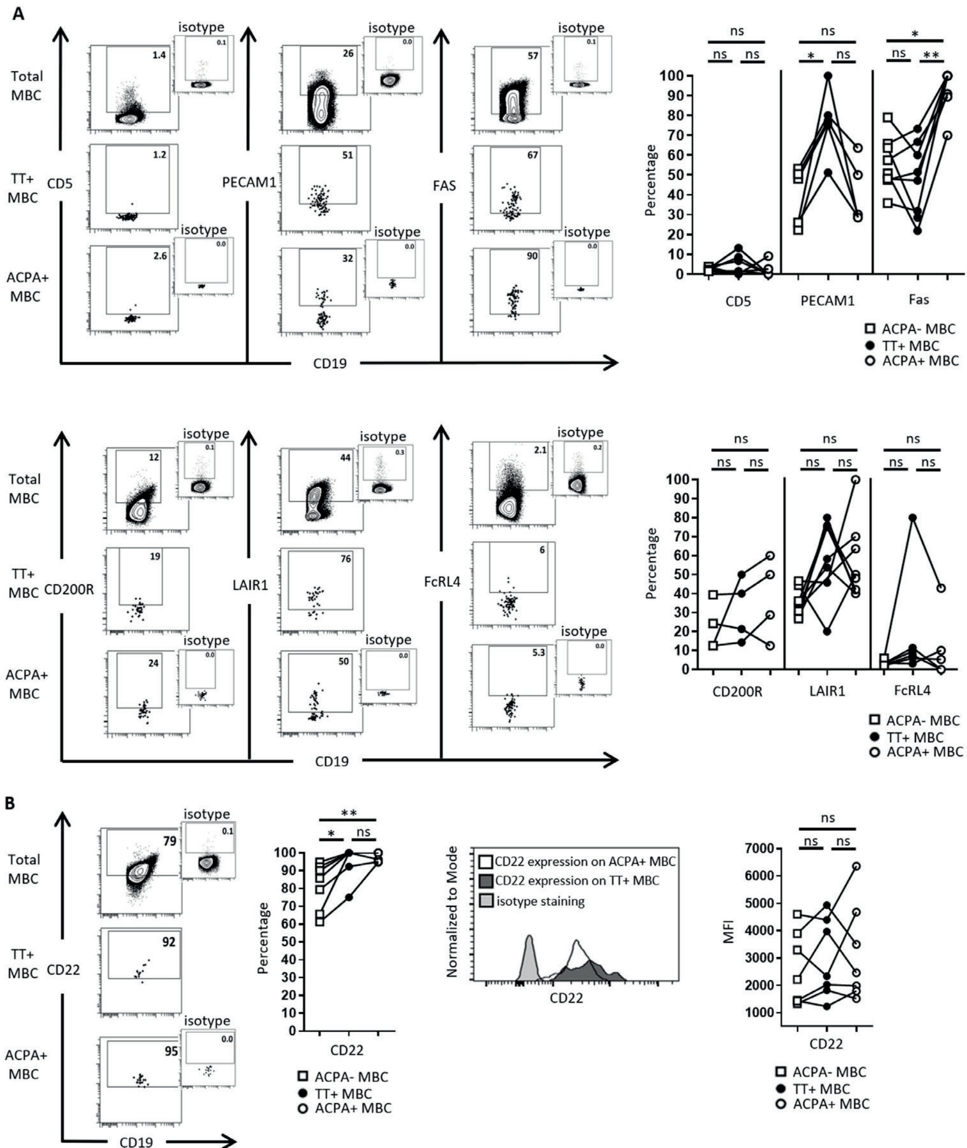

Figure 1. The expression of immune checkpoint receptors on circulating ACPA-positive, TT-specific and total MBC from RA patients. (A) The percentage of CD5, PECAM1, Fas, CD200R, LAIR1, FcRL4 positive cells of Total, TT-specific and ACPA-positive memory B cells (MBC) in PBMC from a total of 18 individuals RA donors. **(B)** The percentage (left side) and median fluorescence intensity (MFI, right side) of CD22-positive cells from respective cell populations. Connected dots depict data from individual patient samples. ns = non significant, * $p \leq 0.05$, ** $p \leq 0.01$. Friedman test with Dunn's multiple comparison test.

of ACPA-positive-, TT-specific- and total B cells only.

Analysis of peripheral blood samples from patients with established RA receiving csDMARDs showed that a median of 30% (range 29%-64%), 39% (13%-60%), 49% (40%-100%) of ACPA-positive MBC expressed PECAM-1, CD200R and LAIR, respectively (Figure 1A). These proportions were similar to control MBCs. Moreover, only a small number of ACPA-positive MBC expressed CD5 and FcRL4 (CD307d), 0% (0%-9%) and 5% (0%-43%) respectively. Also these percentages were similar to both TT-specific and total MBC (Figure 1A). In contrast, more than 95% of ACPA-positive and TT-specific MBC expressed CD22, exceeding the frequency of total MBC positive for this marker. The median expression level per cell, determined by the median fluorescence intensity of CD22, was, however, similar between all subsets. (Figure 1B). Finally, Fas was expressed on virtually all ACPA-positive MBC (median of 100% (70%-100%)) while the frequency of Fas-positive TT-specific MBC and total MBC was significantly lower (50% (22%-73%) of TT-specific MBC ($p<0.01$) and 54% (36%-79%) of total MBC ($p<0.05$)) (Figure 1B). These findings indicate that ACPA-positive MBC comprise a heterogeneous population. With regard to the ICR tested, and except for the upregulation of Fas as well as downregulation of CD32 previously described, no consistent additional disturbance of ICR expression was noted on these cells compared to controls.

ICR expression pattern of ACPA-positive MBC resembles that of recently boosted TT-specific MBC

Out of eight different ICRs we have tested so far, only FcγRIIB (CD32B) and Fas displayed dysregulated expression by ACPA-positive MBC compared to control MBC. To understand how these autoreactive MBCs downregulate FcγRIIB and

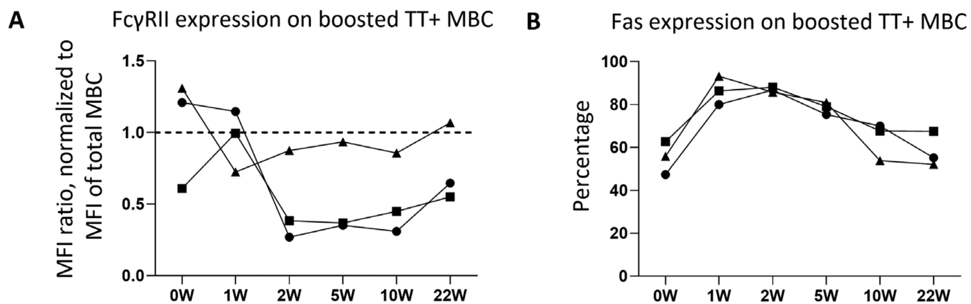

Figure 2. The expression of FcγRII and Fas on circulating TT-specific MBC from healthy individuals following tetanus booster vaccination. (A) The Median Fluorescence Intensity (MFI) of FcγRII expression and **(B)** the percentage of Fas-positivity on circulating TT-specific MBC in PBMC from three healthy individuals. Connected dots depict data from individual donor.

upregulate Fas, we vaccinated three healthy donors with TT booster vaccine and followed the expression of these markers on TT-specific MBC before and after boost vaccination. We found that TT-specific MBC lowered their FcγRIIB expression two weeks post-boost vaccination. This reduced expression level slowly reverted with time but did not achieve its pre-boost level until 22 weeks after vaccination (Figure 2A). Moreover, more TT-specific MBC expressed Fas one week after vaccination. As for CD32, this enhanced expression gradually decreased over time, approximating pre-boost levels at 10 weeks after vaccination (Figure 2B). FcγRIIB and Fas expression patterns on TT-specific MBC at the 2nd week after vaccination were similar to those observed on ACPA-positive MBCs. These findings suggest that circulating ACPA-positive MBCs may have received recent B cell receptor (BCR) activation and received T-cell derived factors, notably CD40 ligation.

Discussion

B cell survival and development require a balance between stimulatory and inhibitory signals from the local B cell environment that are transmitted intracellularly through the expression of activation and immune checkpoint receptors (ICRs). ICR downregulation leads to defects in B cell selection and predisposes mice to autoimmunity.[3,11,13] The functional roles of ICRs on B cells in human autoimmune disease are, however, less clear. Previously, we showed that autoreactive ACPA-positive B cells in RA patients downregulated their expression of FcγRII, a key ICR, which may help these cells to escape from inhibitory signals.[10] Here, we extended our assessment of ICR expression on ACPA-positive B cells. We hypothesized that ACPA-positive B cells may display additional abnormalities in ICR expression to support their survival and to ultimately induce autoimmunity toward citrullinated self-antigens. In addition, dominant ICR expression on these cells could be attractive targets for inducing immunological tolerance in RA.

We found that a median of 100% of ACPA-positive MBC expressed Fas. This percentage of Fas-positive cells is significantly higher than that of total and TT-specific MBC in the steady state, in the same patients. Given that Fas-induced apoptosis is instrumental in limiting the development of autoreactive MBC in the germinal centres, our finding may seem surprising.[14-16] It was reported, however, that Fas-induced apoptosis can indeed be circumvented by simultaneous activating signals. Concurrent BCR signalling protects B cells from Fas-induced apoptosis through upregulation of caspase-8 inhibitor, c-FLIP.[17,18] Furthermore, Fas

activation contributes to B cell activation, proliferation and differentiation when BCR and CD40 pathways are simultaneously activated.[19,20] Given that ~40% of ACPA-positive MBC express the proliferation marker Ki-67[10], it is possible that increased Fas expression on these cells may be accompanied by increased BCR and CD40 signalling which both promote survival and proliferation. Possibly, this putative elevated BCR signalling may originate from BCR binding to citrullinated self-antigens in germinal centres, or relate to increased tonic BCR activation due to Fab glycan-induced modulation of BCR signalling.[21] If correct, inhibiting BCR signalling pathways may predispose ACPA-positive MBC to Fas-induced apoptosis.

We also found that the expression levels of CD22, PECAM1, LAIR1 and CD200R on circulating ACPA-positive MBC were similar to total MBC from the same patients. A median of 98% of ACPA-positive MBC expressed CD22, at levels comparable to those found on total and TT-specific MBC. Moreover, a median of 30%, 49% and 39% of ACPA-positive MBC expressed PECAM1, LAIR1 and CD200R, respectively, demonstrating the variability among these cells. Overall, however, these data indicate that the overall expression levels of ICR are not altered on ACPA-positive B cells.

Lastly, atypical B cell populations have been reported to be increased in patients with RA. First, the "age-associated B cell" or ABC population was found primarily in aged women with RA. This cell population overexpresses CD19, CD80, CD86, HLA and Fas which is similar to ACPA-positive MBC. While the ABC population was reported to also overexpress CD5, however,[22] ACPA-positive MBCs did not express this marker. Second, FCRL4-expressing B cell population was found in the joints of RA patients.[23] A small fraction of these cells showed BCR reactivity to citrullinated peptides.[24] FcRL4 is also expressed by B cells which fail to clear chronic viral infections. These phenotypically "exhausted" B cells are less proliferative and have reduced differentiation potential.[25] Our data showed that only 5% of circulating ACPA-positive MBC expressed FcRL4, similar to control MBCs. As we currently only tested FcRL4 expression on ACPA-positive B cells from peripheral blood, it will be interesting to test this marker on these cells from synovial fluid.

We acknowledge limitations of our study. First, we did not test all ICRs that are known to be expressed by B cells. This is due to limitations of our flow cytometry technique which only allows a limited set of marker combinations for reliable measurement of fluorophores. Single cell sequencing may circumvent this issue but may not detect lowly expressed transcripts. Alternatively, mass cytometry can be

used to detect the expression of virtually all ICRs known to be expressed by B cells. We also did not test the expression of these receptors at the site of inflammation, and whether the signalling pathways of these receptors are still intact on ACPA-positive MBC. These interesting aspects, however, warrant further study. Finally, the small numbers of sample may not bring to light the subtle, yet biologically relevant differences of ICR expression between ACPA-positive MBC and control MBCs.

In conclusion, we showed that ACPA-positive MBC are heterogeneous, with differential expression of several ICRs at levels comparable to control MBC (Table 1). Interestingly, our previous and current findings regarding ICR showed that Fas was upregulated and FcγRII was downregulated by ACPA-positive MBC.[10] These characteristics phenocopied recently boosted TT-specific MBC observed in healthy individuals, pointing to possible molecular mechanisms that shape the phenotype of ACPA-positive B cells, namely continuous B cell receptor activation and T-cell derived factors. In addition, the expression CD22 by more than 95% of ACPA-positive MBC may serve as a promising target to induce tolerance for targeted therapy or vaccines against ACPA-positive RA. Finally, the biological significance of Fas over-expression on ACPA-positive MBC remains to be studied.

Table 1. The expression of immune checkpoint receptors (ICRs) on circulating ACPA-positive MBC from RA patients receiving conventional DMARDs

[a] median and range; [b] compared to circulating tetanus toxoid-specific MBC and total MBC from the same RA patients.

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	ICR	Ligand	Function	Expression on circulating ACPA+ MBCs	
				Percentage[a]	Levels[b]
1	LEU1 (CD5)	CD5[26] CD72[27]	Increases B cell activation threshold and prevents BCR-mediated cell death.[5] CD5+ B cells are enriched in elderly women who suffer from RA, referred to as “age-associated B cells”.[28]	0% (0-9%)	unaltered
2	SIGLEC-2 (CD22)	Sialic acid[29]	Knocked-out mouse showed B cell hyperactivation and enhanced chemotaxis.[4]	98% (91-100%)	unaltered
3	PECAM-1 (CD31)	CD31, αvβ3 integrin[29] CD38[31]	Adhesion molecule. Knocked-out mouse displayed B cell hyperresponsiveness and autoantibodies formation.[3]	30% (29-64%)	unaltered
4	FCyRIIB (CD32B)	IgG Fc tail	Inhibits antigen-specific B cells through antigen-IgG immune complex-mediated FcγRIIB and BCR co-ligation. Knocked-out mouse showed hypergammaglobulinemia and susceptibility to anaphylactic reaction and autoimmunity.[32]	30% (29-64%)	decreased[10]
5	Fas (CD95)	Fas ligand	Primarily an apoptosis receptor. Knocked-out mouse showed autoantibody formation and lethal lymphoproliferation.[13]	100% (70-100%)	increased
6	CD200R	CD200 (OX-2)	Modulates cellular activation. CD200R activation alleviates collagen-induced arthritis in mice.[33]	39% (13-60%)	unaltered
7	LAIR-1 CD305)	Collagen domain, including C1q[34]	Activation of LAIR1 on B cells inhibits IgG, IgE, IL-8 and TNFα production.[35] Knocked-out mice showed dysregulation of humoral immune response.[36]	49% (40-100%)	unaltered
8	FcRL4 (CD307d)	IgA Fc tail[37]	FcRL4+ B cells in RA synovial fluid express TNF α and RANKL mRNAs.[23] Expressed on a B cell population in the peripheral blood of patients with chronic viral infections, referred to as “exhausted” B cells.[38,39]	5% (0-43%)	unaltered

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