Impaired early B cell tolerance in patients with rheumatoid arthritis

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Autoantibody production is a characteristic of most autoimmune diseases including rheumatoid arthritis (RA). The role of these autoantibodies in the pathogenesis of RA remains elusive, but they appear in the serum many years before the onset of clinical disease suggesting an early break in B cell tolerance. The stage of B cell development at which B cell tolerance is broken in RA remains unknown. We previously established in healthy donors that most polyreactive developing B cells are silenced in the bone marrow, and additional autoreactive B cells are removed in the periphery. B cell tolerance in untreated active RA patients was analyzed by testing the specificity of recombinant antibodies cloned from single B cells. We find that autoreactive B cells fail to be removed in all six RA patients and represent 35–52% of the mature naive B cell compartment compared with 20% in healthy donors. In some patients, RA B cells express an increased proportion of polyreactive antibodies that can recognize immunoglobulins and cyclic citrullinated peptides, suggesting early defects in central B cell tolerance. Thus, RA patients exhibit defective B cell tolerance checkpoints that may favor the development of autoimmunity.

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Abbreviations used: BCR, B cell receptor; CCP, cyclic citrullinated peptide; IFA, immunofluorescence assay; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TdT, terminal deoxynucleotidyl transferase; XLA, X-linked agammaglobulinemia.

Rheumatoid arthritis (RA) is a common chronic inflammatory disease that affects ~1% of the population (1). An important characteristic that distinguishes RA from other inflammatory and degenerative joint diseases is the production of autoantibodies directed against self-antigens including antibody Fc regions (rheumatoid factors), type II collagen, and cyclic citrullinated peptides (CCP; 2–5). These antibodies appear in the serum of RA patients many years before the onset of clinical disease suggesting an early break in B cell tolerance (6). However, the underlying mechanisms that account for autoantibody production in RA have not been defined. Mouse models reveal an important role for T and B cells in the development of inflammatory arthritis (7, 8). In humans, an important role for B cells in RA was recently demonstrated by successful treatment of RA patients with anti-CD20 monoclonal antibodies that eliminate B cells (9, 10). Although little is known about the characteristics of RA B cells, some express unusual B cell receptors (BCRs) with 11–amino acid–long CDR3 Igκ chains that accumulate in the joints of RA patients (11, 12). It is unclear if those unusual BCRs result from altered B cell development and reflect impaired B cell tolerance.

We previously analyzed how B cell tolerance was established in humans by following the evolution of autoantibody-producing B cells during B cell development (13). Using an RT-PCR method that allowed us to clone and express in vitro recombinant antibodies amplified from single B cells, we found that 55–75% of early B cell precursors expressed self-reactive antibodies and that autoantibody-producing B cells in healthy donors were removed from the population at two discrete checkpoints (13). The first checkpoint occurs in the bone marrow between the early immature and immature B cell stage. The second counterselection step of autoantibody-expressing B cells takes place in the periphery, at the transition from new emigrant to mature naive B cells (13). We applied the same method to characterize how B cell tolerance was established in RA patients. We found that central and peripheral B cell tolerance checkpoints were defective in RA patients and allowed the accumulation of peripheral mature naive autoreactive B cells that may contribute to RA pathogenesis.

The online version of this article contains supplemental material.

RESULTS

To determine whether B cell tolerance is established properly in RA patients, we enrolled nine active RA patients who met the Revised Criteria of the American College of Rheumatology and examined the reactivity of antibodies from six of them (Table I). Eight patients were either naive to steroids, disease-modifying antirheumatic drugs, and biologics, or off these medications for at least 4 mo (see Materials and methods and Table I). Only patient RA07 had been taking hydroxychloroquine for 2 mo before the day when blood was drawn. In total, we cloned and expressed in vitro 176 and 177 antibodies from single new emigrant ($CD19^+CD10^+IgM^+CD27^-$) and mature naive ($CD19^+CD10^-IgM^+CD27^-$) RA B cells, respectively, and compared them to control antibodies from four previously reported and three additional healthy donors (Table S1 available at http://www.jem.org/cgi/content/full/jem.20042321/DC1; 13–15).

RA new emigrant B cells express remarkable antibody repertoires

The B cells from our nine patients revealed at least three distinct patterns of antibody sequences. Consistent with previous reports on RA, the proportion of Igκs with unusual ≥11–amino acid–long CDR3s in new emigrant B cells was significantly increased from 0 to 4.1% in controls to 5.7 to 17.9% in RA patients ($P = 0.0008$, Fig. 1 and Tables S2–S16 available at http://www.jem.org/cgi/content/full/jem.20042321/DC1; 11, 12). The Igκ antibody repertoires of RA01, RA02, and RA08 new emigrant B cells were also remarkable in that they displayed significantly increased upstream Jκ1 usage (RA01: 53.1%, $P = 0.025$; RA02: 51.4%, $P = 0.02$; and RA08: 47.9%, $P = 0.027$; Fig. 2 A). In contrast, Jκ1 usage frequency in new emigrant B cells from seven control healthy donors and from the other RA patients ranged from 21 to 36% and 21 to 41%, respectively (Fig. 2 A; 13, 14). Jκ1 Ig segment is eliminated when upstream Vκ genes delete preexisting VκJκ1 genes by recombining with downstream Jκ genes (16). We found that upstream Vκ gene usage in new emigrant B cells was decreased in RA01 (9.5%), RA02 (8.5%, $P = 0.044$), and RA08 (14.3%) when compared with controls (21–36%; Fig. 2, B and C). Upstream Vκ gene usage was also decreased in RA01 and RA02 mature naive B cells (RA01: 5.2%, $P = 0.0048$; RA02: 16.1% $P = 0.3$ vs. 27.7% in control mature naive B cells; Tables S8 and S9 available at http://www.jem.org/cgi/content/full/jem.20042321/DC1 and

Table I. Patient characteristics

	RA01	RA02	RA03	RA04	RA05	RA06	RA07	RA08	RA09
Background	black	Asian	Hispanic	white	white	black	black	Hispanic	Hispanic
Age	60	62	62	36	30	64	44	28	52
Gender	F	M	F	F	F	F	F	M	F
Age RA onset	47	60	59	35	28	62	42	26	51
Years of disease	13	2	3	1	2	2	2	2	1
Joints involved	Virtually all	Knees, elbows wrists, PIPs	IP, MCP, wrists, elbows, neck, TMJ	Hands, knees	Left wrist, ankle	Shoulder, right hand, diffuse arthralgias	Knees, hands	Diffuse symmetrical (PIPs, MCPs, wrists, knees, ankles)	Hands, wrists, knees, ankles
Prior medication	Intermittent steroids, hydroxychloroquine, methotrexate	None	Steroids, methotrexate	None	None	None	Hydroxychloroquine (for 2 mo)	Steroids, hydroxychloroquine, methotrexate	None
Months off DMARDS/steroids	5	NA	4	NA	NA	NA	0	10	NA
RF	<20	848	1,110	55	48	474	267	258	36
Anti-CCP	32	88	240	63	<20	>250	218	80	150
X-ray erosions	+	+	−	−	+ (by MRI)	−	−	−	+ (by ultrasound)
Comorbidities	Hypertension	Anal fistula, cardiomegaly	TB exposure	None	Childhood seizures	Asthma	Prior cocaine use until 12/03, TB exposure	Polysubstance abuse on methadone	Hypothyroidism, gastric reflux
Family history (autoimmune)	−	Brother with "arthritis"	−	Cousin with SLE	Father and mother with thyroid disease	Parents with "arthritis"	−	−	Sister with "arthritis"

Patients were considered rheumatoid factor or anti-CCP positive when their antibody titers were >20. DMARDs, disease-modifying anti-rheumatic drugs; CCP, cyclic citrullinated peptides; PIPs, proximal interphalangeal joints; IPs, interphalangeal joints; MCPs, metacarpophalangeal joint; TMJ, temporomandibular joint; TB, tuberculosis bacillus.

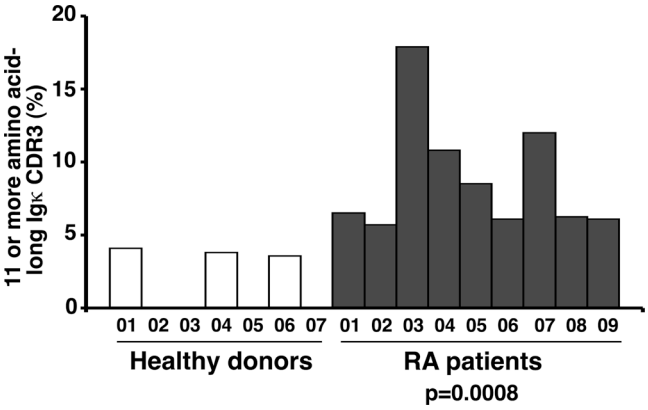

Figure 1. RA B cells express antibodies with unusually long CDR3s. Increased frequency of Igκ genes with unusually long ≥11–amino acid CDR3s in new emigrant B cells from RA patients. Differences in frequency of Igκ genes with ≥11–amino acid CDR3s between healthy donors and RA patients were found to be statistically significant (P = 0.008).

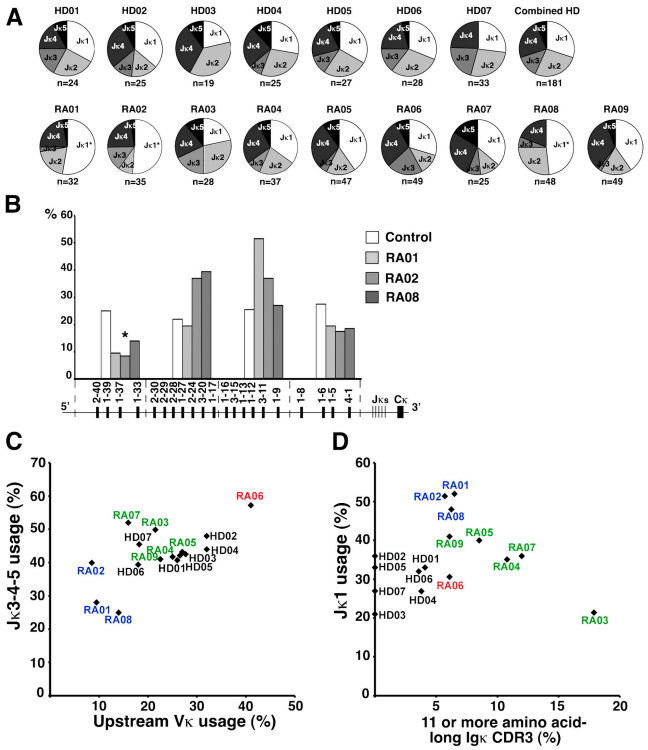

Figure 2. RA B cells can express an antibody repertoire consistent with either decreased or increased secondary V(D)J recombination. (A) Increased Jκ1 usage in new emigrant B cells from RA01, RA02, and RA08 patients. Pie charts show the proportion of different Jκ genes. The number of sequences analyzed in each fraction is indicated below the pie charts. Control healthy donor new emigrant B cells from HD01, HD02, and HD03 were previously reported (13, 14). *, Indicates statistically significant difference (RA01: 53.1%, P = 0.025; RA02: 51.4%, P = 0.02; and RA08: 47.9%, P = 0.027). (B) Decreased upstream (5′) Vκ usage in RA01, RA02, and RA08 new emigrant B cells. The proximal Vκ locus involved in 95% of Vκ–Jκ rearrangements is shown clustered into four groups of Vκ genes. Genes from the distal locus were combined to the upstream Vκ group. The percent of each Vκ group is indicated on the Y axis. *, Indicates statistically significant difference (RA02: P = 0.044; combined RA01, RA02, RA08: P = 0.0042). (C) The new emigrant B cell Igκ repertoire in RA06 is consistent with increased secondary V(D)J recombination in developing B cells. Patients' and healthy donor controls' upstream Vκ usage frequency was plotted against that of downstream Jκ3-4-5 usage. Increased upstream Vκ usage in RA06 was found to be statistically significant (P = 0.033). (D) Increased Jκ1 usage in some RA patients is associated with a lower frequency of ≥11–amino acid–long Igκ CDR3s in new emigrant B cells. Patients' and healthy donor controls' Jκ1 usage frequency was plotted against that of ≥11–amino acid–long Igκ CDR3s.

Tables S9 and S10 reported in reference 13). In contrast, new emigrant B cells from another RA patient, RA06, showed increased downstream Jκ3-4-5 combined with upstream Vκ gene usage (Fig. 2 C). In addition, increased Jκ1 usage was found in RA patient new emigrant B cells that displayed the lowest frequency of unusual ≥11–amino acid–long Igκ CDR3s (Fig. 2 D). We conclude that RA new emigrant B cells express at least three distinct patterns of Igκ antibody repertoires: unusually long Igκ CDR3s, an Igκ repertoire biased toward either upstream Jκ1 and downstream Vκ genes or downstream Jκ3-4-5 and upstream Vκ genes consistent with decreased or increased secondary V(D)J recombination, respectively.

Defective peripheral B cell tolerance checkpoint in RA patients

The self-reactivity of the recombinant antibodies from six RA patients was first tested against HEp-2 cells by ELISA and indirect immunofluorescence assay (IFA; 13, 17). We found that the proportion of HEp-2–reactive antibodies in new emigrant B cells from RA patients ranged from 31.8 to 55.8% (Fig. 3 A) and was not significantly different from that of control new emigrant antibodies previously reported (31–40.7%; 13, 14). In contrast, all six RA patients revealed an increase of HEp-2–reactive mature naive B cells that reached statistical significance in five of them (Fig. 3 B). In these patients, mature naive B cells expressing HEp-2–reactive antibodies represented 35.0–51.7%, whereas they only account for 20.4% of the control mature naive B cell compartment (RA01: 42.9%, P = 0.026; RA02: 40.0%, P = 0.031; RA03: 48.4%, P = 0.005; RA04: 51.7%, P = 0.002; RA05: 35.0%, P = 0.24; RA06: 44.8%, P = 0.015; Fig. 3 B). Antibodies from new emigrant and mature naive B cells from RA patients gave IFA patterns similar to their control counterparts, staining mainly the cytoplasm of HEp-2 cells (unpublished data). We conclude that RA patients fail to remove autoreactive B cells in the periphery between the new emigrant and mature naive B cell stages, revealing a defective second checkpoint in B cell tolerance.

Defective central B cell tolerance checkpoint in RA patients

Most polyreactive B cells in healthy donors are removed at a first checkpoint in the bone marrow between the early immature and immature B cell stage (13). Few low-polyreac-

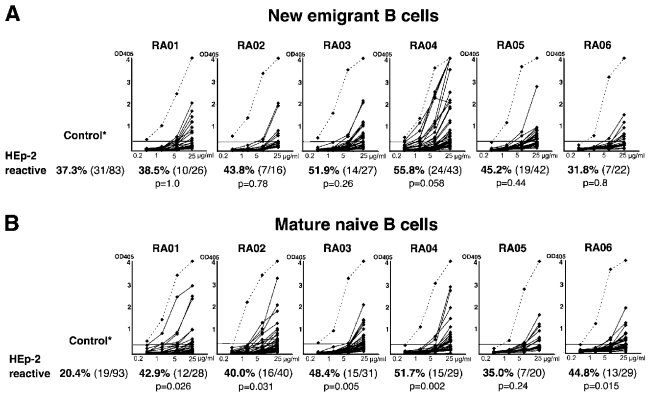

Figure 3. Increased frequency of HEp-2–reactive antibodies in mature naive B cells from RA patients. Data shown are from ELISAs for anti–HEp-2 cell reactivity of recombinant antibodies cloned from (A) new emigrant and (B) mature naive B cells from RA patients. Dotted lines show ED38 positive control (13, 17). The percentage of autoreactive clones for each fraction and their p-values for differences with controls are indicated. *, The controls for new emigrant and mature naive B cells isolated from HD01, HD02, HD03, and HD04 were previously reported (13, 14).

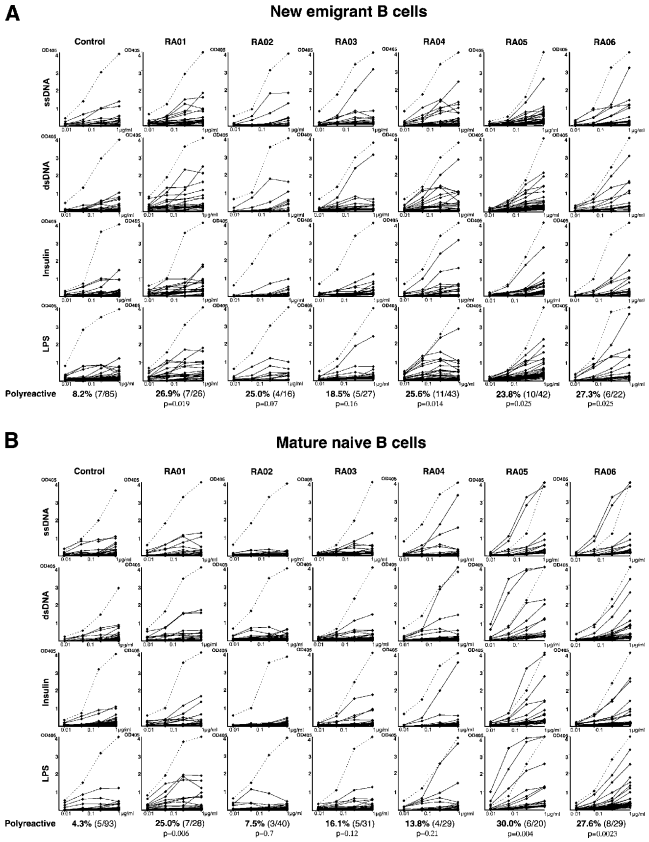

Figure 4. RA B cells express polyreactive antibodies. Antibodies from (A) new emigrant and (B) mature naive B cells from RA patients were tested by ELISAs for reactivity with single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), insulin, and lipopolysaccharide (LPS). Dotted lines show ED38-positive control (13, 17). Percentages represent frequency of polyreactive antibodies in each fraction. *, The controls for new emigrant and mature naive B cells isolated from HD01, HD02, HD03, and HD04 were previously reported (13, 14).

tive B cells escape this counterselection step and migrate to the periphery of healthy donors where they represent 7.4–9.7% of new emigrant and 4–5% of mature naive B cells (13, 14, 17). To determine whether bone marrow early B cell tolerance was established properly in RA patients we analyzed the frequency of polyreactive antibodies in new emigrant B cells. We found by ELISA that four out of six patients showed significantly increased proportions of polyreactive antibodies in new emigrant B cells (RA01: 26.9%, $P = 0.019$; RA04: 25.6%, $P = 0.014$; RA05: 23.8%, $P = 0.025$; RA06: 27.3%, $P = 0.025$; Fig. 4 A). RA02 and RA03 new emigrant B cells also expressed polyreactive antibodies at a higher frequency than control new emigrant B cells but without reaching statistical significance (RA02: 25.0%, $P = 0.07$ and RA03: 18.5%, $P = 0.16$; Fig. 4 A). The frequency of polyreactive antibodies was also increased in the mature naive B cell compartment of five out of six RA patients and reached significance for three of them (RA01: 25%, $P = 0.006$; RA05: 30%, $P = 0.004$; RA06: 27.6%, $P = 0.0023$; Fig. 4 B). Thus, most RA patients fail to efficiently remove polyreactive B cells and reveal defects at the early B cell tolerance checkpoint in the bone marrow.

Presence of naive B cells reactive to Ig and CCP in RA patients

Igs and CCP are the most common autoantigens in RA (2, 4, 5). Five out of six of our RA patients showed elevated anti-CCP antibody levels in their serum and only RA01 was rheumatoid factor negative (Table I). To determine if naive RA B cells can recognize IgG and CCP, we used ELISAs to analyze the rheumatoid factor and anti-CCP reactivity of recombinant antibodies from new emigrant and mature naive B cells. We found that new emigrant B cells from RA01 and RA04 expressed a higher proportion of rheumatoid factors (RA01: 34.6%, $P = 0.027$ and RA04: 30.2%, $P = 0.046$ vs. 9.7% in controls; Fig. 5 A). RA02, RA03, and RA05 new emigrant B cells also express rheumatoid factors at a higher frequency than control new emigrant B cells but without reaching statistical significance (RA02: 25.0%, $P = 0.21$; RA03: 18.5%, $P = 0.45$; and RA05: 21.4%, $P = 0.22$; Fig. 5 A). The percentage of B cells expressing rheumatoid factors was still found elevated in the naive B cell compartment of RA01, RA04, RA05, and RA06 (Fig. 5 B). Most of the antibodies that showed rheumatoid factor reactivity in either controls or patients were not specific for IgG but also recognized single-stranded DNA, double-stranded DNA, insulin, and LPS and were therefore polyreactive (Table S2 available at http://www.jem.org/cgi/content/full/jem.20042321/DC1). Similarly to rheumatoid factors, the proportion of anti-CCP antibodies was increased in RA new emigrant B cells, and the differences with controls reached significance

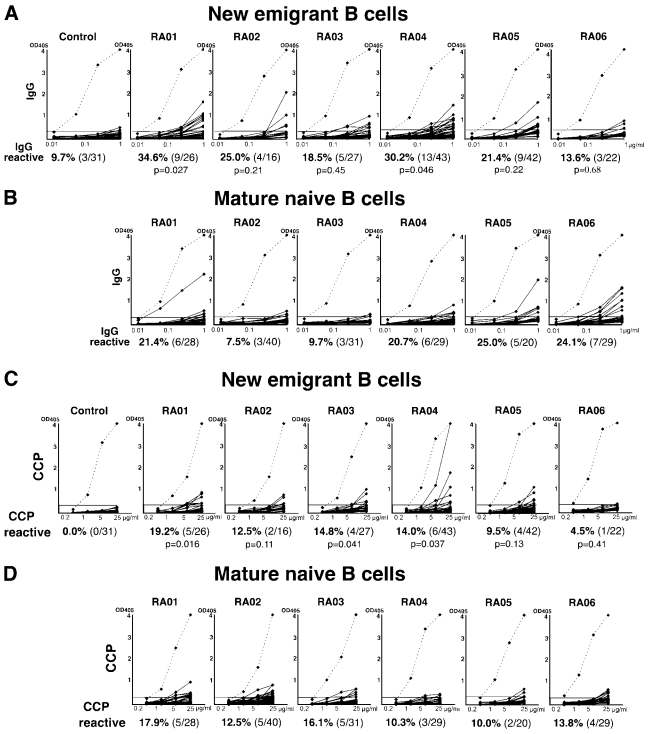

Figure 5. RA peripheral B cells contain rheumatoid factor and anti-CCP clones. Antibodies from new emigrant and mature naive B cells from RA patients were tested by ELISAs for reactivity against IgG (A and B) and CCP (C and D). Dotted lines show either ED38-positive control (A and B) or serum anti-CCP IgG-positive control (C and D). Percentages represent frequency of anti-CCP antibodies in each fraction. P-values are in comparison to control new emigrant B cells (13, 14).

for half of the patients (RA01: 19.2%, P = 0.016; RA03: 14.8%, P = 0.041; and RA04: 14.0%, P = 0.037; Fig. 5 C). The percentage of anti-CCP antibodies remained elevated and ranged from 10 to 17.9% in the mature naive B cell compartments of RA patients (Fig. 5 D). Anti-CCP antibodies showed low levels of reactivity when compared with serum anti-CCP IgG antibodies from positive controls (Fig. 5, C and D). Most of the antibodies that reacted to CCP were in fact polyreactive antibodies (Tables S2–S13 available at http://www.jem.org/cgi/content/full/jem.20042321/DC1). However, none of the polyreactive antibodies from healthy individuals recognized CCP (Fig. 5 C). We conclude that a fraction of new emigrant and mature naive B cells from RA patients express polyreactive antibodies that can recognize RA-specific antigens IgG and CCP with low affinity.

RA antibodies with unusual ≥11–amino acid–long Igκ CDR3s are frequently self-reactive

The proportion of B cells expressing antibodies with unusual ≥11–amino acid–long Igκ CDR3s is increased in most RA patients (11, 12). We analyzed the reactivity of those unusual clones and found that most antibodies containing ≥11–amino acid–long CDR3 Igκ chains were either HEp-2–reactive and/or polyreactive (77.8%, 14 out of 18; Table II). The frequency of autoreactivity for RA antibodies with shorter Igκ CDR3s was lower and averaged 48.8% (126/258; Tables S2–S13 available at http://www.jem.org/cgi/content/full/jem.20042321/DC1). In addition, B cells that express ≥11–amino acid–long Igκ chains preferentially used Vκ3-11–variable gene that was found in almost half (8/18) of these unusually long Igκ chains whereas Vκ3-11–variable gene only accounted for 0–11.1% and 0–17.4% of control and RA Igκ chains with shorter CDR3s, respectively (Table II and Tables S2–S20 available at http://www.jem.org/cgi/content/full/jem.20042321/DC1). Thus, a majority of peripheral B cells expressing unusual antibodies with ≥11–amino acid–long Igκ CDR3s are autoreactive and contribute to the enlarged compartment of peripheral self-reactive B cells in some RA patients.

DISCUSSION

Autoreactive B cells generated during bone marrow B cell development fail to be counterselected in RA patients. Despite the broad age range (28–64 yr old) and duration of disease (1–13 yr), all six patients show similarly elevated percentages of autoreactive mature naive B cells that differ from those of six adult control donors previous reported (23–40 yr old; 13, 14, 17). The first B cell tolerance checkpoint that normally removes most polyreactive B cells in the bone marrow is defective in RA patients, as reflected by the increased percentage of polyreactive B cells in their new emigrant B cell compartment (13). Most polyreactive antibodies enriched in RA peripheral B cells are also reactive with RA-specific antigens and show low rheumatoid factor and anti-CCP reactivity. This early break in B cell tolerance may explain why RA patients relapse after successful treatment with anti-CD20 monoclonal antibodies that target immature and mature B cells but not their precursors (10). The second peripheral checkpoint that accounts for additional removal of autoreactive B cells in the periphery between the new emigrant and the mature naive B cell stage is also defective, and allows the accumulation of a large number of self-reactive B cells in the mature naive B cell compartment of all RA patients.

What are the defective mechanisms that may be responsible for the early break in B cell tolerance in RA patients? The biased repertoires observed in our RA patients were found in new emigrant B cells and may reflect abnormal bone marrow B cell development. In the mouse, three central mechanisms are responsible for self-reactive antibody silencing: deletion, anergy, and receptor editing (18–22). Light chain antibody sequences from RA01, RA02, and RA08 patients showed an increase in downstream Vκ genes associated with the most upstream Jκ, Jκ1, suggesting inefficient secondary recombination and therefore a potential defect in receptor editing. Consistent with our data, defects in secondary recombination have also been reported in other rheumatic diseases such as systemic lupus erythematosus

Table II. RA antibodies with 11- and 12-amino acid-long Igκ CDR3s are frequently autoreactive

	Heavy						Light				Reactivity						
Ig	VH	D	RF	JH	CDR3 (aa)	Length	Vκ	Jκ	CDR3 (aa)	Length	ssDNA	dsDNA	Insulin	LPS	IgG	CCP	HEp-2
RA01 10+ 180	3-74	5-12	2	4	ARDPPEALSGYDW DPYYFDY	20	3-11	2	QQRSNWPPGYT	11	−	−	−	−	+	−	−
RA02 10+ 08	3-43	/	/	4	AKD-DDDRYPSWGLLDY	16	3-11	3	QQRSNWPPPFT	11	−	−	−	−	+	−	−
RA02 10+ 21	3-30	3-22	2	4	ARERDYYDSSG-FDY	14	1-9	2	QQLNSYPYMYT	11	−	−	−	−	−	−	+
RA03 10+ 01	3-21	/	/	4	ARSRGITVDDY	11	1-5	4	QQYNSYSPSFT	11	+	+	+	+	+	−	+
RA03 10+ 27	3-66	3-3	2	4	ARGSGENYDFWS-VPFDY	17	3-11	1	QQRSNWPPTWT	11	−	−	−	−	−	−	+
RA03 10+ 56κ	3-7	3-9	1	4	ARDGVLRYFD-WLFHRFDY	18	3-11	2	QQRSNWPPY-MYT	12	+	+	+	+	+	−	+
RA03 10+ 67	3-11	/	/	4	ARGDLAHPTDY	11	3-15	2	QQYNNWPRMYT	11	−	−	−	−	−	−	−
RA03 10+ 77	3-73	4-17	2	6	TRRLDDYGDYGV-VSHYYYYGMDV	23	1-33	3	QQYDNLPPIFT	11	+	+	+	+	−	−	−
RA04 10+ 21	3-23	2-15	2	4	ARFLVLGYC-SGGSCYTFDY	19	1-5	1	QQYNSYSRAWT	11	−	−	−	−	−	−	+
RA04 10+ 53	3-53	2-2	3	6	ARGRDIV-VVPAAIVYYGMDV	20	1-5	2	QQYNSYSPMCS	11	−	+	−	+	−	−	+
RA04 10+ 83	3-15	5-5	2	6	TTAGEPGYSY-LYYYYGMDV	19	3-11	4	QQRSNWPPGLT	11	−	−	−	−	+	+	+
RA05 10+ 39	1-69	1-7	3	4	ASITGTLN	8	1-5	1	QQYNSYSPTWT	11	−	−	−	−	−	−	−
RA05 10+ 57	1-46	2-15	2	4	ARDFSERYC-SGGSCYSEFDY	20	3-11	4	QQRSNWPLALT	11	−	−	−	−	−	−	−
RA06 10+ 70	3-21	3-16	2	3	ARGSAQRSEY-DYVWGSYR-PLDAFDI	25	3-11	5	QQRSNWPPEVT	11	+	+	+	+	−	−	+
RA06 10+ 181	3-11	3-22	3	5	ARSMIVVVPPPG-FDP	15	1-33	2	QQYDNLPPLYT	11	+	+	+	+	−	−	−
RA01 10− 60	1-69	5-24	2	4	ARGDGGGDH-PFFDY	14	3-15	2	QQYNNWPPLYT	11	+	+	+	+	+	+	+
RA06 10− 92	3-53	/	/	4	AREPLGSPGFDY	12	1-9	3	QQLNSYPPQFT	11	−	+	−	+	−	+	+
RA06 10− 95	3-53	2-21	2	4	AHGDCYFDY	9	3-11	3	QQRSNWPPLFT	11	−	−	−	−	−	−	−

Sequence features and reactivity of antibodies with 11– and 12–amino acid–long Igk CDR3s from new emigrant (10+) and mature naive (10−) B cells from RA patients.
RF, reading frame.

(SLE) and may therefore account for the increased frequency of polyreactive and autoreactive B cells in the periphery (23, 24). In contrast, Igκ chains from RA06 new emigrant B cells showed an increase in upstream Vκ genes associated with downstream Jκs suggesting extensive secondary recombination in those cells. Interestingly, extensive secondary recombination has also been reported in B cells from an untreated SLE patient and suggests that it can be an abnormal mechanism shared by different rheumatic diseases (25). BCR signaling plays an important role in controlling receptor editing by regulating recombination activating genes in mice and in humans (14, 26–28). Defects in BCR signaling may result either in a failure to induce receptor editing or in a premature termination of secondary recombination at the immature B cell stage. Alternatively, other BCR signaling defects may fail to terminate secondary recombination. X-linked agammaglobulinemia (XLA) patients suffer from defective *BTK* genes, which encode a cytoplasmic tyrosine kinase that plays an essential role in mediating BCR signaling (29, 30). We recently reported that XLA B cells showed extensive secondary recombination events and expressed autoreactive BCRs thereby demonstrating that proper BCR signaling is essential in regulating thresholds for human B cell tolerance (14). Little is known about BCR signaling defects in RA B cells, but abnormal BCR signaling has been reported in SLE B cells that also suffer from defective B cell tolerance (31, 32). More recently, a missense polymorphism in the protein tyrosine phosphatase *PTPN22* gene have been shown to segregate with RA and SLE patients (33, 34). This missense polymorphism in PTPN22 disrupts its interaction with Csk tyrosine kinase and potentially alters these proteins' normal function as regulators of BCR signaling (33).

Patients RA03, RA04, RA05, RA07, and RA09 do not show any significant evidence of defective secondary recom-

bination, illustrating the heterogeneity of rheumatic diseases. However, those RA patients like others showed an increased proportion of new emigrant B cells expressing ≥11–amino acid–long CDR3 Igκ chains (11, 12). Interestingly, ≥11–amino acid–long CDR3 Igκs were enriched in new emigrant B cells but not in mature naive B cells. The disappearance of those unusual clones from the mature naive B compartment in the peripheral blood may reflect a counterselection step still functional in RA patients. Alternatively, B cells expressing ≥11–amino acid–long CDR3 Igκs may not be counterselected but migrate to the joints of RA patients where they accumulate (12). In line with this hypothesis, antibodies with ≥11–amino acid–long CDR3 Igκs were frequently found to be self-reactive and may recognize joint antigens. The production of 11–amino acid–long CDR3 Igκ chains mostly requires the addition of nontemplate nucleotides to V–J junctions by the terminal deoxynucleotidyl transferase (TdT) whose expression is normally down-regulated by preBCR expression at the preB cell stage (35, 36). Abnormal BCR signaling may fail to down-regulate TdT expression and may therefore result in the generation of those 11–amino acid–long CDR3 Igκ chains as reported in some XLA patients (14, 37). Alternatively, light chain gene rearrangements at the pro-B cell stage when TdT is expressed may generate 11–amino acid–long CDR3 Igκ chains found in RA patients (38).

Autoreactive B cells that escape central B cell tolerance are normally counterselected in the periphery and fail to enter the long-lived mature naive B cell pool (39, 40). The appearance of autoreactive B cells in the periphery of RA patients may result from either defective negative or abnormal positive selection processes that are believed to control peripheral B cell tolerance (41–43). Interestingly, serum BAFF levels have been recently shown to regulate autoreactive B cell deletion by rescuing self-reactive B cells from cell death (44, 45). Patients with rheumatic diseases including RA have been reported to display elevated BAFF serum levels that may therefore result in the survival of unusual autoreactive B cell clones in the blood of RA patients (46).

How could mature naive autoreactive B cells contribute to RA? A large number of mature naive B cells from RA patients express autoreactive and polyreactive antibodies that can recognize classical RA autoantigens with low affinity. A recent report showed that similar autoreactive/polyreactive mature naive B cells accumulated in the periphery of SLE patients (32). Genetic and environmental factors may select and activate those autoreactive/polyreactive mature naive B cells in a differential manner in RA and SLE patients. This would allow the development of B cells secreting isotype-switched antibodies with high affinity to self-antigens specific for either RA or SLE. For instance, polyreactive B cells that recognize Igs may bind IgG–chromatin immune complexes in the joints of RA patients. Dual engagement of BCR and toll-like receptors such as TLR9 may activate such B cells and induce their development into rheumatoid factor–producing B cells (47, 48). Polyreactive RA B cells that recognize CCP may bind and internalize citrullinated peptides that can interact with RA-associated HLA-DRB1*0401 MHC class II molecules and activate specific autoreactive T cells (49). In return, those T cells would provide activation signals to CCP-reactive B cells and induce their proliferation and eventually the formation of ectopic germinal centers, resulting in the production of specific high affinity anti-CCP autoantibodies (50, 51).

In conclusion, the antibody repertoire heterogeneity of our RA patients reflects different defective mechanisms of B cell tolerance resulting from potentially diverse RA genetic polymorphisms. Yet all six patients showed defective tolerance checkpoints and the presence of a reservoir of self-reactive mature naive B cells, likely contributing to RA pathogenesis.

MATERIALS AND METHODS

Patients. Patient 1 (RA01) is a 60-yr-old black woman who was diagnosed with RA in 1990 with symmetric polyarticular symptoms and severe morning stiffness. We first met her after 13 yr of intermittent treatment with steroids, hydroxychloroquine, and methotrexate but she had no treatments in the previous 5 mo (Table I). Initial data included an erythrocyte sedimentation rate of 127, a negative rheumatoid factor (<20), a slight elevation in anti-CCP titer (32), and multiple erosions on radiographs. Patient 2 (RA02) is a 62-yr-old Asian man who presented with 2 yr of increasing right shoulder, left elbow and bilateral wrist/hand pains, and years of increasing right knee pain. His medications at presentation were celecoxib, atenolol, and omeprazole. Data were significant for an erythrocyte sedimentation rate of 14 but elevated levels of rheumatoid factor (848) and anti-CCP antibodies (88), and radiographs with erosions and an effusion in the left elbow, and erosions in the right wrist and left IP joints. Patient 3 (RA03) is a 62-yr-old Hispanic woman who presented 3 yr prior with symmetrical polyarticular joint swelling/pain and morning stiffness, and a diagnostic work up consistent with RA. Treated intermittently with methotrexate and steroids, she stopped all of her medications 4 mo before the most recent flare because she was feeling well and presented with severe pain and swelling in both her upper and lower extremities. Medications on presentation included Prevacid and naproxen. Significant data included high titers of rheumatoid factor (1,110) and anti-CCP antibodies (240), and normal radiographs. Patient 4 (RA04) is a 36-yr-old white woman who presented with 1 yr of increasing pain and morning stiffness in her hands and elbows, but had refused all treatment. Workup included elevated levels of rheumatoid factor (55) and anti-CCP antibodies (63) but no radiographic erosions. Patient 5 (RA05) is a 30-yr-old white woman who presented with 2 yr of increasing left wrist and ankle pain and swelling, but had not taken any medication. Data included an elevated rheumatoid factor (48) but an anti-CCP antibody titer <20, with erosions (only seen with MRI) in both joints. Patient 6 (RA06) is a 64-yr-old black woman who presented with 2 yr of increasing pain and swelling in the right hand and shoulder, and polyarticular arthralgias, but had not been on any medication. Data included elevated titers of rheumatoid factor (474) and anti-CCP antibodies (>250), but no radiographic erosions. Patient 7 (RA07) is a 44-yr-old black woman who presented with 2 yr of increasing pain and swelling in her hands and knees, and had not improved while on hydroxychloroquine for 2 mo. Data included elevated titers of rheumatoid factor (267) and anti-CCP antibodies (218), but no radiographic erosions. Patient 8 (RA08) is a 28-yr-old Hispanic male who first presented 2 yr prior with morning stiffness and symmetrical swelling of the hands, wrists, knees and ankles, and responded well to steroids, methotrexate, and hydroxychloroquine. He stopped the medication 10 mo before representing with 2 wk of polyarticular arthritis more severe than on initial presentation, with elevated titers of rheumatoid factor (258) and anti-CCP antibodies (80) but no radiographic erosions. Patient 9

(RA09) is a 52-yr-old Hispanic female who presented with 1 yr of pain and swelling in her hands, wrists, knees, and ankles, but had received no medication. Data included elevated titers of rheumatoid factor (36) and anti-CCP antibodies (150), and erosions of her MCPs by ultrasound.

We analyzed three additional healthy donors (HD05, 57; HD06, 60; and HD07, 48) and further investigated the four previously reported control healthy donors (HD01 [GO] 23; HD02 [JB] 24; HD03 [JH] 24, and HD04 [PE] 31 yr old; Table S1; 13, 14). All samples were collected after patients signed informed consent in accordance with IRB-reviewed protocols.

Single cell sorting. Peripheral B cells were purified from the blood of RA patients by negative selection using RosetteSep™ procedure (StemCell Technologies Inc.). New emigrant B cells were further enriched by positive selection with anti-PE magnetic microbeads (Miltenyi Biotech) after PE anti–human CD10 staining (BD Biosciences). CD10-enriched and CD10-depleted peripheral B cells from RA patients were stained with FITC anti–human CD27, anti–human IgM-Biotin, and APC anti–human CD19 (BD Biosciences). Biotinylated antibodies were revealed using PE-cy7–conjugated streptavidin (BD Biosciences). Single $CD19^+CD10^+IgM^+CD27^-$ new emigrant and $CD19^+CD10^-IgM^+CD27^-$ mature naive B cells were sorted on a FACSVantage (Becton Dickinson) into 96-well PCR plates containing 4 μl lysis solution (0.5× PBS containing 10 mM DTT, 8 U RNAsin (Promega), 0.4 U 5′–3′ RNase inhibitor (Eppendorf), and immediately frozen on dry ice. All samples were stored at –70°C.

cDNA, RT-PCR, antibody production, and purification. RNA from single cells was reverse transcribed in the original 96-well plate in 12.5 μl reactions containing 100 U of Superscript II RT (GIBCO BRL) for 45 min at 37°C. RT-PCR reactions, primer sequences, cloning strategy, expression vectors, antibody expression, and purification were as described (13). Ig sequences were analyzed by Ig BLAST comparison with GenBank/EMBL/DDBJ. Heavy chain CDR3 was defined as the interval between the conserved cysteine at position 92 in the V_H framework 3 and the conserved tryptophan at position 103 in J_H segments.

ELISAs and IFAs. Antibody concentration, reactivity against specific antigens, and IFAs were as described previously (13). High (polyreactive ED38) and weak (mGO186) HEp2-reactive and -nonreactive mGO53 and iGO13 were used as positive and negative controls in HEp2-reactivity and polyreactivity ELISAs (13, 17). Those control antibodies established that recombinant antibodies with absorbance values above 0.4 could be considered reactive with the coated antigen. Antibodies were polyreactive when they recognized at least two and usually all of the four analyzed antigens that include single-stranded DNA, double-stranded DNA, insulin, and LPS. Rheumatoid factor reactivity was determined by ELISA using rabbit whole IgG as antigens and recombinant antibody binding was revealed using peroxidase-conjugated rabbit anti–human IgG (Jackson ImmunoResearch Laboratories, Inc.). Anti-CCP reactivity was determined using QUANTA Lite™ CCP ELISA plates (INOVA Diagnostics, Inc.).

Statistical analysis. Fisher exact tests were used to analyze dichotomized data. Jκs were dichotomized into Jκ1, the most upstream of all Jκs used in primary recombination events, versus all the other Jκs whose usage may involve secondary recombination events. Student's *t* tests were used to analyze the differences in proportions of ≥11–amino acid–long Igκ CDR3s between healthy donors and RA patients. All statistical tests were considered two-tailed and probability values of $P < 0.05$ were considered to be statistically significant.

Online supplemental material. Healthy donor characteristics are shown in Table S1. Antibody characteristics from RA patients and control B cells are presented in Tables S2–S20. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20042321/DC1.

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REFERENCES

1. Feldmann, M., F.M. Brennan, and R.N. Maini. 1996. Rheumatoid arthritis. *Cell*. 85:307–310.
2. Leslie, D., P. Lipsky, and A.L. Notkins. 2001. Autoantibodies as predictors of disease. *J. Clin. Invest*. 108:1417–1422.
3. Smolen, J.S., and G. Steiner. 2001. Rheumatoid arthritis is more than cytokines: autoimmunity and rheumatoid arthritis. *Arthritis Rheum*. 44: 2218–2220.
4. Sebbag, M., M. Simon, C. Vincent, C. Masson-Bessiere, E. Girbal, D. Jean-Jacques, and E. Serre. 1995. The anti-perinuclear factor and the so-called anti-keratin antibodies are the same rheumatoid arthritis-specific autoantibodies. *J. Clin. Invest*. 95:2672–2679.
5. Schellekens, G.A., B.A.W. de Jong, F.H.J. van den Hoogen, L.B.A. van de Putte, and W.J. van Venrooij. 1998. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. *J. Clin. Invest*. 101:273–281.
6. Rantapaa-Dahlqvist, S., B.A.W. de Jong, E. Berglin, G. Hallmans, G. Wadell, H. Stenlund, U. Sundin, and W.J. van Venrooij. 2003. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheum*. 48: 2741–2749.
7. Kouskoff, V., A.S. Korganow, V. Duchatelle, C. Degott, C. Benoist, and D. Mathis. 1996. Organ-specific disease provoked by systemic autoimmunity. *Cell*. 87:811–822.
8. Sakaguchi, N., T. Takahashi, H. Hata, T. Nomura, T. Tagami, S. Yamazaki, T. Sakihama, T. Matsutani, I. Negishi, S. Nakatsuru, and S. Sakaguchi. 2003. Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. *Nature*. 426: 454–460.
9. Silverman, G.J., and S. Weisman. 2003. Rituximab therapy and autoimmune disorders: prospects for anti-B cell therapy. *Arthritis Rheum*. 48:1484–1492.
10. Edwards, J.C.W., L. Szczepanski, J. Szechinski, A. Filipowicz-Sosnowska, P. Emery, D.R. Close, R.M. Stevens, and T. Shaw. 2004. Efficacy of B cell-targeted therapy with Rituximab in patients with rheumatoid arthritis. *N. Engl. J. Med*. 350:2572–2581.
11. Victor, K.D., I. Randen, K. Thompson, O. Forre, J.B. Natvig, S.M. Fu, and J.D. Capra. 1991. Rheumatoid factors isolated from patients with autoimmune disorders are derived from germline genes distinct from those encoding the Wa, Po, and Bla cross-reactive idiotypes. *J. Clin. Invest*. 87:1603–1613.
12. Bridges, S.L., Jr., S.K. Lee, M.L. Johnson, J.C. Lavelle, P.G. Fowler, W.J. Koopman, and H.W. Schroeder Jr. 1995. Somatic mutation and CDR3 lengths of immunoglobulin kappa light chains expressed in patients with rheumatoid arthritis and in normal individuals. *J. Clin. Invest*. 96:831–841.
13. Wardemann, H., S. Yurasov, A. Schaefer, J.W. Young, E. Meffre, and M.C. Nussenzweig. 2003. Predominant autoantibody production by early human B cell precursors. *Science*. 301:1374–1377.
14. Ng, Y.-S., H. Wardemann, J. Chelnis, C. Cunningham-Rundles, and E. Meffre. 2004. Bruton's tyrosine kinase (Btk) is essential for human B cell tolerance. *J. Exp. Med*. 200:927–934.
15. Sims, G.P., R. Ettinger, Y. Shirota, C.H. Yarboro, G.G. Illei, and P. Lipsky. 2005. Identification and characterization of circulating human transitional B cells. *Blood*. 10.1182/blood-2004-11-4284.
16. Prak, E.L., and M. Weigert. 1995. Light chain replacement: a new model for antibody gene rearrangement. *J. Exp. Med*. 182:541–548.
17. Meffre, E., A. Schaefer, H. Wardemann, P. Wilson, E. Davis, and M.C. Nussenzweig. 2004. Surrogate light chain expressing human peripheral B

cells produce self-reactive antibodies. *J. Exp. Med.* 199:145–150.
18. Nemazee, D.A., and K. Burki. 1989. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature.* 337:562–566.
19. Goodnow, C.C., J. Crosbie, S. Adelstein, T.B. Lavoie, S.J. Smith-Gill, R.A. Brink, H. Pritchard-Briscoe, J.S. Wotherspoon, R.H. Loblay, K. Raphael, et al. 1988. Altered immunoglobulin expression and functional silencing of self- reactive B lymphocytes in transgenic mice. *Nature.* 334:676–682.
20. Gay, D., T. Saunders, S. Camper, and M. Weigert. 1993. Receptor editing: an approach by autoreactive B cells to escape tolerance. *J. Exp. Med.* 177:999–1008.
21. Tiegs, S.L., D.M. Russell, and D. Nemazee. 1993. Receptor editing in self-reactive bone marrow B cells. *J. Exp. Med.* 177:1009–1020.
22. Radic, M.Z., J. Erickson, S. Litwin, and M. Weigert. 1993. B lymphocytes may escape tolerance by revising their antigen receptors. *J. Exp. Med.* 177:1165–1173.
23. Bensimon, C., P. Chastagner, and M. Zouali. 1994. Human lupus anti-DNA autoantibodies undergo essentially primary V kappa gene rearrangements. *EMBO J.* 13:2951–2962.
24. Suzuki, N., T. Harada, S. Mihara, and T. Sakane. 1996. Characterization of a germline Vk gene encoding cationic anti-DNA antibody and role of receptor editing for development of the autoantibody in patients with systemic lupus erythematosus. *J. Clin. Invest.* 98:1843–1850.
25. Dorner, T., S.J. Foster, N.L. Farner, and P.E. Lipsky. 1998. Immunoglobulin kappa chain receptor editing in systemic lupus erythematosus. *J. Clin. Invest.* 102:688–694.
26. Melamed, D., and D. Nemazee. 1997. Self-antigen does not accelerate immature B cell apoptosis, but stimulates receptor editing as a consequence of developmental arrest. *Proc. Natl. Acad. Sci. USA.* 94:9267–9272.
27. Hertz, M., and D. Nemazee. 1997. BCR ligation induces receptor editing in IgM+IgD- bone marrow B cells in vitro. *Immunity.* 6:429–436.
28. Tze, L.E., K.L. Hippen, and T.W. Behrens. 2003. Late immature B cells (IgM(high)IgD(neg)) undergo a light chain receptor editing response to soluble self-antigen. *J. Immunol.* 171:678–682.
29. de Weers, M., G.S. Brouns, S. Hinshelwood, C. Kinnon, R.K. Schuurman, R.W. Hendriks, and J. Borst. 1994. B-cell antigen receptor stimulation activates the human Bruton's tyrosine kinase, which is deficient in X-linked agammaglobulinemia. *J. Biol. Chem.* 269:23857–23860.
30. Kurosaki, T., and S. Tsukada. 2000. BLNK: connecting Syk and Btk to calcium signals. *Immunity.* 12:1–5.
31. Liossis, S.N., B. Kovacs, G. Dennis, G.M. Kammer, and G.C. Tsokos. 1996. B cells from patients with systemic lupus erythematosus display abnormal antigen receptor-mediated early signal transduction events. *J. Clin. Invest.* 98:2549–2557.
32. Yurasov, S., H. Wardemann, J. Hammersen, M. Tsuiji, E. Meffre, V. Pascual, and M.C. Nussenzweig. 2005. Defective B cell tolerance checkpoints in systemic lupus erythematosus. *J. Exp. Med.* 201:703–711.
33. Begovich, A.B., V.E.H. Carlton, L.A. Honigberg, S.J. Schrodi, A.P. Chokkalingam, H.C. Alexander, K.G. Ardlie, Q. Huang, A.M. Smith, J.M. Spoerke, et al. 2004. A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis. *Am. J. Hum. Genet.* 75:330–337.
34. Kyogoku, C., C.D. Langfeld, W.A. Ortmann, A. Lee, S. Selby, V.E.H. Carlton, M. Chang, P. Ramos, E.C. Baechler, F.M. Batliwalla, et al. 2004. Genetic association of the R620W polymorphism of protein tyrosine phosphatase PTPN22 with human SLE. *Am. J. Hum. Genet.* 75: 504–507.
35. Wasserman, R., Y.S. Li, and R.R. Hardy. 1997. Down-regulation of terminal deoxynucleotidyl transferase by Ig heavy chain in B lineage cells. *J. Immunol.* 158:1133–1138.
36. Ghia, P., E. ten Boekel, E. Sanz, A. de la Hera, A. Rolink, and F. Melchers. 1996. Ordering of human bone marrow B lymphocyte precursors by single-cell polymerase chain reaction analyses of the rearrangement status of the immunoglobulin H and L chain loci. *J. Exp. Med.* 184:2217–2229.
37. Campana, D., J. Farrant, N. Inamdar, A.D. Webster, and G. Janossy. 1990. Phenotypic features and proliferative activity of B cell progenitors in X-linked agammaglobulinemia. *J. Immunol.* 145:1675–1680.
38. Meffre, E., M. Milili, C. Blanco-Betancourt, H. Antunes, M.C. Nussenzweig, and C. Schiff. 2001. Immunoglobulin heavy chain expression shapes the B cell receptor repertoire in human B cell development. *J. Clin. Invest.* 108:879–886.
39. Cyster, J.G., S.B. Hartley, and C.C. Goodnow. 1994. Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire. *Nature.* 371:389–395.
40. Cyster, J.G., and C.C. Goodnow. 1995. Antigen-induced exclusion from follicles and anergy are separate and complementary processes that influence peripheral B cell fate. *Immunity.* 3:691–701.
41. Russell, D.M., Z. Dembic, G. Morahan, J.F.A.P. Miller, K. Bürki, and D. Nemazee. 1991. Peripheral deletion of self-reactive B cells. *Nature.* 354:308–311.
42. Gu, H., D. Tarlinton, W. Muller, K. Rajewsky, and I. Forster. 1991. Most peripheral B cells in mice are ligand selected. *J. Exp. Med.* 173: 1357–1371.
43. Levine, M.H., A.M. Haberman, D.B. Sant'Angelo, L.G. Hannum, M.P. Cancro, C.A. Janeway Jr., and M.J. Shlomchik. 2000. A B-cell receptor-specific selection step governs immature to mature B cell differentiation. *Proc. Natl. Acad. Sci. USA.* 97:2743–2748.
44. Lesley, R., Y. Xu, S.L. Kalled, D.M. Hess, S.R. Schwab, H.-B. Shu, and J.G. Cyster. 2004. Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF. *Immunity.* 20: 441–453.
45. Thien, M., T.G. Phan, S. Gardam, M. Amesbury, A. Basten, F. Mackay, and R. Brink. 2004. Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches. *Immunity.* 20:785–798.
46. Groom, J., S.L. Kalled, A.H. Cutler, C. Olson, S.A. Woodcock, P. Schneider, J. Tschopp, T.G. Cachero, M. Batten, J. Wheway, et al. 2002. Association of BAFF/BLys overexpression and altered B cell differentiation with Sjogren's syndrome. *J. Clin. Invest.* 109:59–68.
47. Leadbetter, E.A., I.R. Rifkin, A.M. Hohlbaum, B.C. Beaudette, M.J. Shlomchik, and A. Marshak-Rothstein. 2002. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature.* 416:603–607.
48. Viglianti, G.A., C.M. Lau, T.M. Hanley, B.A. Miko, M.J. Shlomchik, and A. Marshak-Rothstein. 2003. Activation of autoreactive B cells by CpG dsDNA. *Immunity.* 19:837–847.
49. Hill, J.A., S. Southwood, A. Sette, A.M. Jevnikar, D.A. Bell, and E. Cairns. 2003. Cutting edge: the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. *J. Immunol.* 171:538–541.
50. Shlomchik, M.J., A. Marshak-Rothstein, C.B. Wolfowicz, T.L. Rothstein, and M.G. Weigert. 1987. The role of clonal selection and somatic mutation in autoimmunity. *Nature.* 328:805–811.
51. Weyand, C.M., and J.J. Goronzy. 2003. Ectopic germinal center formation in rheumatoid synovitis. *Ann. NY Acad. Sci.* 987:140–149.