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**Supplementary Material**

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Pathogenic Autoreactive B Cells Are Not Negatively Selected toward Matrix Protein Collagen II

Duojia Cao,*1 Ia Khmaladze,†1 Hongwei Jia,* Estelle Bajtner,* Kutty S. Nandakumar,*† Thomas Blom,* John A. Mo,*2 and Rikard Holmdahl*†

We have addressed the importance of B cell tolerance to collagen type II, a matrix protein, which is a target in rheumatoid arthritis (RA) and its mouse models. We generated a germline-encoded anti-collagen type II (CII) IgH replacement anti-C1 B cell mouse strain (ACB) to investigate how B cell tolerance to CII, a matrix protein, is subverted and to further understand pathogenesis of RA. Phenotypic analysis revealed that CII-specific B cells were surprisingly neither deleted nor anergized. Instead, they were readily detected in all lymphoid organs. Spontaneously produced autoantibodies could bind directly to cartilage surface without detectable pathology. However, exaggerated arthritis was seen after injection of anti-CII Abs specific for other epitopes. In addition, Abs from CII-specific hybridomas generated from ACB mice induced arthritis. Interestingly, IgH/L chain sequence data in B cell hybridomas revealed a lack of somatic mutations in autoreactive B cells. The ACB model provides the first possibility, to our knowledge, to study B cell tolerance to a matrix protein, and the observations made in the study could not be predicted from previous models. B cell-reactive epitopes on CII are largely shared between human RA and rodent CII-induced arthritis; this study, therefore, has important implications for further understanding of pathological processes in autoimmune diseases like RA. The Journal of Immunology, 2011, 187: 4451–4458.

Multiple mechanisms of tolerance induction on both T and B cell levels limit autoimmunity. Despite these strategies, autoimmune diseases do occur. Rheumatoid arthritis (RA) is a systemic human autoimmune disease, which primarily targets peripheral synovial joints. Recently, a pathogenic role of B cells in RA has been highlighted, mainly due to two reasons. Anti-CD20 therapy, which depletes B cells, was found effective in RA (1), and the anti-citrullinated protein Abs (ACPA) have high prevalence and specificity in RA patients (2–4). The level of ACPA is strongly linked to the MHC class II locus, indicating that they are involved in the pathogenesis of the disease (5). Among previously described ACPA, Abs against the citrullinated C1 epitope of collagen type II (CII) were detected in both serum and synovial fluid of RA patients (6, 7). In addition, Abs to the C1 epitope itself occur in ~15% of the patients with early RA, and the occurrence is also associated with the MHC class II region (8). Therefore, an Ab response to the C1 epitope is likely to play a pathogenic role in RA.

CII is the major matrix protein of cartilage in articular joints, the target of autoimmunity attack in RA. The C1 epitope is one of the six epitopes identified to date on CII triple-helical molecule in mice: C1, J1, U1, D3, F4, and E8 (9–11). The C1 epitope, corresponding to aa residues 358–369 of CII, was recognized as one of the major immunodominant epitopes (9, 10, 12). Interestingly, it was shown previously that the C1-specific hybridomas generated in mice were all germline encoded from a single pair of H chain (VH J558) and L chain (Vk 21 family) without any somatic mutations in V genes (13). However, to date it is obscure whether and how B cell tolerance to CII, a matrix protein, or any cartilage-specific Ag is broken.

It has been suggested that defective central/peripheral B cell tolerance checkpoints allow the accumulation of self-reactive B cells that may contribute to RA pathogenesis (14). Autoreactive B cell tolerance is established through multiple mechanisms, which have been previously described using different animal models (15, 16). In the central lymphoid organ, newly generated self-reactive B cells can be eliminated by clonal deletion (17, 18), or B cell autoreactivity can be revised by the secondary rearrangement of the new L chain, termed as receptor editing (19). To prevent self-antigen–specific responses, B cells can be inactivated; the process is dubbed as clonal anergy (20). Mechanisms of peripheral tolerance are also important; some self-reactive B cells escape central tolerance mechanisms, emerge into the periphery, produce autoantibodies, and contribute to autoimmune disease induction.

To analyze some of these B cell selection processes in the context of self-antigen–specific autoimmunity, we generated a variable, diversity, and joining gene segment (VDJ) replacement mouse, designated as anti-C1 B cell mouse strain (ACB), in which the J locus in H chain (JH) was replaced by rearranged germline-encoded H chain VDJ segments of an anti-C1 hybridoma, CB20. To our knowledge, this model allows for the first time the study of B cell tolerance to CII, a matrix protein. We demonstrated that C1-specific B cells are neither negatively selected nor functionally...
anergized, and C1-specific germline-encoded autoantibodies are capable of contributing to arthritis pathogenesis. Importantly, epitope-specific recognition of CII by Abs is largely shared between RA patients and rodents immunized with CII (8, 21). Thus, the results presented in this work will contribute to the increased understanding of the autoimmunity and pathogenesis of human RA.

Materials and Methods

Generation of ACB mice and SNP typing

CB20, a germline-encoded anti-C1 Ab, was chosen to generate a VpDpH4 knockin mouse strain. The rearranged VDJ region of IgH from CB20 was engineered to replace a germline JH locus in ACB mice by a homologous recombination, as shown in a schematic illustration in supporting Supplemental Fig. 1A and 1B. The correctly targeted embryonic stem (ES) cell clone was confirmed by Southern blot (Supplemental Fig. 1C). Injection of selected positive ES cells into blastocysts resulted in germline-transmitting chimeric animals. The positive offsprings were deleted for the NeoR fragment and backcrossed to B10.Q strain. After nine generations of backcross, a fragment of 15 mbp in chromosome 12 containing the targeted locus IgD4 was found. The genetic purity was confirmed using a 10k single nucleotide polymorphism chip (22) to scan genome of ACB and B10.Q strains.

Genotyping

ACB mice were genotyped routinely with specific PCR and microsatellite markers around the transgene. Genomic DNA was prepared by dissolving tail biopsies in 300 μl 50 mM NaOH at 95°C for 1–2 h, and subsequently neutralized with 30 μl 1 M Tris-HCl (pH 8). A 2 μl DNA sample (50 ng/μl) was used to perform a standard 25 μl ACB-specific PCR, and PCR products were visualized in a 2% agarose gel. Transgene-specific PCR primers used were as follows: forward primer, 5′-AGG GGG GGA GGC CAT GGA CTA-3′ and reverse, 5′-GCT GAT CCG TGG CAT CTA TT-3′. For genotyping using microsatellite markers, 1 μl genomic DNA sample was used for a standard 10 μl PCR with various microsatellite marker primers. Microsatellite markers used were D12Mit263, MUSI-GHAB2 (igh), MMGIVH16 (igh-V), and D12mit144. Marker variants primers used were as follows: forward primer, 5′-GAC GAG GGG GAA GAC-3′ and reverse, 5′-AGG AGA TC-3′.

ELISA

Rat CII at 10 μg/ml or C1 triple-helical peptides at 4 μg/ml were used to directly coat ELISA plates in PBS; biotin-labeled anti-κ Abs (1 μg/ml, clone 187.1) were used for total Ab level assessment. For isotype ELISA, biotin-labeled isotype-specific secondary Abs at 1 μg/ml were used (Southern Biotechnology Associates).

In vitro B cell stimulation

One million naïve spleen (SP) cells and CII-immunized lymph node (LN) cells from ACB (n = 5) and BQ (n = 5) mice were stimulated in vitro with TLR2/6, TLR4, and TLR9 agonists (FSL1, LPS, ODN1826). Cells were cultured for 48 h for LN cells and 96 h for SP cells. C1-specific Ab levels present in these supernatants were measured by ELISA.

Immunization with CII

Sex- and age-matched ACB+/+ and ACB–/– mice were immunized with 100 μg rat CII emulsified with CFA at the base of the tail. Mice were boosted with 50 μg CII emulsified with IFA at day 35. All of the mice were bled at different time points, and serum samples were analyzed for total anti-CII Ab content. SP taken at different time points were also used for immunofluorescence staining and flow cytometric analysis.

Collagen Ab-induced arthritis and clinical evaluation

Collagen Ab-induced arthritis (CAIA) was performed by injecting a mixture of three affinity-purified mAbs (M2139 [IgG2b], CIIC2 [IgG2a], and UL1 [IgG2b]) i.v. at 2 mg each to ACB mice. These Abs have been characterized in detail previously (25). The mixture was prepared by mixing equal concentration of each sterile Ab solution, 2 mg each, into 500 μl total volume. On day 7, 25 μg LPS was injected i.p. Arthritis development was monitored daily and blindly for 22 d.

Clinical arthritis is defined as swelling and redness in the joint and was scored as follows: 1 point for an inflamed knuckle or toe and 5 points for an inflamed ankle or wrist, resulting in a score of 0–15 points for each paw and 0 points for each mouse.

Immunohistochemical analysis

The knee joint was dissected from six ACB+/+, four ACB–/–, and five BQ male naïve mice of 8–10 wk old. As positive control, three sex- and age-matched BQ mice were injected i.v. with 1 mg purified CII at 24 h prior to knee joint dissection. Without any decalcification, frozen knee joints were cryosectioned, and sections of 7 μm from each mouse were subjected to immunohistochemical staining with biotinylated anti-κ (clone 187.1) Abs at concentration of 5 μg/ml for 40 min. Extra avidin peroxidase and diaminobenzidine were used as detecting system. Stained sections were blindly scored with a scoring system of 0–2, with 0 for no staining, 1 for weak staining, and 2 for maximum staining.

The hind paws of 2-mo-old naïve ACB+/+ and BQ male mice were dissected, fixed, decalcified, dehydrated, and embedded in paraffin. Ankle joint sections of 5 μm were stained with toluidine blue to identify proteoglycan depletion.

Statistics

Mann–Whitney was performed for comparison of two unpaired non-parametric sample groups. Kruskal–Wallis was used for comparison of two paired nonparametric sample groups. A p value <0.05 was considered as significant.

Results

Generation of ACB mice

CB20, a germline-encoded anti-C1 Ab, was chosen to generate ACB, a VpDpH4 knock-in mouse strain on the B10.Q background.

Flow cytometry analysis

Triple-helical C1 peptides were biotinylated, as described (24), and used in cell surface staining at 1 μg/ml and detected by streptavidin PE or PerCP complex. Single-cell suspensions (10⁶/sample) were prepared from peripheral blood (PB), peritoneum, and lymphoid tissues, and then surface labeled with predetermined optimal concentration of following mAbs and analyzed by flow cytometry: anti-CD45R/B220 allophycocyanin (RA3-6B2), anti-CD21 FITC (7G6), anti-CD23 PE (B34), anti-CD24-bio (M1/69), anti-CD19 allophycocyanin (1D3), anti-IgMa FITC (DS-1), anti-IgMb PE (217-170), anti-CD3 FITC (YCD3-1), anti-CD4 PE (H129.19), anti-CD25 allophycocyanin (AMT13), anti-CD80 PE (16-10A1), anti-CD86 PE (GL1), anti-MHC II (A9°), FITC (PCQ6), anti-CD5 FITC (53-7.3), anti-CD43 PE (S7), anti-CD11b PE (M1/70), anti-IgGc FITC (187.1), and anti-IgA PE (LSI36) from BD Pharmingen; anti-IgG PE (11-26c.2a) from BioLegend (San Diego, CA); and anti-IgM PE-Cy7 (H/4/1) from eBioscience (San Diego, CA).
The upper and lower borders of the 129-derived fragment were in between the microsatellite markers D12Mit120b and D12Mit100, and between D12Mit150b and D12Mit144, respectively (Fig. 1A). No other genetic contamination was found.

ACB mice heterozygous for transgene were intercrossed to generate homozygous, heterozygous, and negative littermates, designated as ACB+/+, ACB+, and ACB−/−, respectively. The expression efficiency of target allele was determined (Fig. 1B), as follows: 23–36% of the B220+ cells in bone marrow (BM) of ACB+/+ and ACB+/− mice expressed IgMa, the allotype of knock-in H chain, whereas 20–38% in ACB−/− expressed IgMb, the endogenous allele. In SP, 82–93% of B220+ B cells in ACB+/+ and ACB−/− mice expressed IgMb. These data largely confirm allelic exclusion.

Frequency (mean ± SD) of proper B cells (B220+IgM+), pre-B cells (B220+IgM-CD43+), immature B cells (B220+IgM+IgD-), and mature B cells (B220+IgM+IgD+) in B220+ B cells in BM. Total number of mononuclear cells was comparable between different genotypes of ACB mice in all lymphoid organs investigated (Supplemental Fig. 1D). In BM, a reduced frequency of pre-B cells (B220+IgM-CD43+) was observed in ACB-positive mice as compared with ACB−/− mice. Interestingly, such reduction was accompanied by a tendency of increased frequency of both immature and mature/recirculating B cells (B220+IgM-IgD−) and B220+IgM+IgD+, respectively (Fig. 1C). It indicates that B cells are not developmentally arrested in BM; instead, they are preferentially selected to go through all developmental processes and become mature B cells. This could be the consequence of the already recombined transgene in the IgH locus increasing the avidity of BCR to self-antigen, that is, CII.

Increased numbers of marginal zone B cells are present in ACB mice SP

Upon emigration from BM, B cells pass through further developmental checkpoints in SP. Although the number of splenic B220+ B cells was comparable, the frequency of total marginal zone (MZ) B cells, which contain MZ precursor (MZP; B220+CD21lowCD23hi), B220+CD21hiCD23hi, as defined earlier (26), and MZ (B220+CD21hiCD23low) B cells, was significantly increased in ACB-positive mice compared with littermate controls (Fig. 1D). Such increase is accompanied by a significant decrease of follicular B cells (B220+CD21lowCD23hi), which indicates that some of the autoreactive B cells preferably reside in MZ. In peritoneum, frequency of B1, B1a, and B1b B cells, identified as CD19+CD11b+, CD19+CD11b+CD5−, and CD19+CD11b+CD5+, respectively, did not differ significantly between the three genotypes (Supplemental Fig. 1E). Together, these data indicate that B cells with expression of IgH transgene were not clonally deleted or arrested; instead, they were allowed to populate in each B cell compartment with a skewing toward more MZ B cells.

Three distinct C1-binding B cell populations in ACB mice

To identify CII-specific B cells and track their fate, B cells expressing CII-specific BCR were detected by cell surface staining with biotin-labeled triple-helical C1 peptide. Interestingly, C1-binding B cells could be detected in all lymphoid organs of ACB mice. In SP, two distinct C1-binding populations were detected, designated as C1++ and C1+ (Fig. 2A). C1++ showed a higher C1-binding intensity and consisted of 1–3% of B220+ B cells, whereas C1+ consisted of 2–2.5% of B220+ B cells in ACB-positive mice. The C1-binding B cells can also be readily detected in LN, BM, PB, and peritoneum (Fig. 2B). The frequency of C1++ and C1+ populations was comparable between ACB+/+ and ACB−/− mice, and they were both significantly higher than that in ACB−/− controls. Furthermore, it is noteworthy that a C1-reactive population with a staining intensity even higher than that of C1++, designated as C1+++ was detected in peritoneum (Fig. 2A) and represented 1–3% of CD19+ B cells. The percentage of C1+++ population in peritoneum presented in Fig. 2B contains both C1++ and C1+++ populations, and together they represent 7–13% of CD19+ B cells. The cutoff for C1+/CD19+ B cells is set in accordance to ACB−/− mice staining level (Supplemental Fig. 2A). C1-binding B cells are comprised of more B1a B cells and are generally bigger in size, which may indicate the activation status of these cells (Supplemental Fig. 2B).

Autoreactive B cells in ACB mice are not arrested in BM

Previously, it was shown that clonal deletion of B cells expressing high-affinity BCR to autoantigen leads to the developmental arrestment of B cells. We next addressed whether the transgene alters development and selection of B lymphocytes in ACB mice. Cells from various lymphoid organs of ACB mice were analyzed by flow cytometry. Total number of mononuclear cells was comparable between different genotypes of ACB mice in all lymphoid organs investigated (Supplemental Fig. 1D). In BM, a reduced frequency of pre-B cells (B220+IgM−CD43+) was observed in ACB-positive mice as compared with ACB−/− mice. Interestingly, such reduction was accompanied by a tendency of increased frequency of both immature and mature/recirculating B cells (B220+IgM−IgD−) and B220+IgM+IgD+, respectively (Fig. 1C). It indicates that B cells are not developmentally arrested in BM; instead, they are preferentially selected to go through all developmental processes and become mature B cells. This could be the consequence of the already recombined transgene in the IgH locus increasing the avidity of BCR to self-antigen, that is, CII.

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B220+ B cells in indicated organ of ACB+/+ (E) (Fig. 2 and C1++ a comparable level to non-C1–binding (C1–) intensity in B220+ splenic B cells. Dotted line represents the cutting off level representative FACS plot to show C1 binding in relation to CD21-staining expression intensity (mean C1++ and C1+++ in CD19+ B cells. *p < 0.05, **p < 0.01, C. A representative FACS plot to show C1 binding in relation to CD21-staining intensity in B220+ splenic B cells. Dotted line represents the cutting off level of CD21high. CD23 (D) and IgMa, CD80, CD86, and MHCIId (E) surface expression intensity (mean ± SD) was compared between C1++, C1+, and C1− B cell fraction, slightly increased expression levels of CD86 and CD80 were observed in comparison with C1− B cells. Importantly, both C1-binding populations expressed a high level of MHCIId (A), as shown in Fig. 2E. Together, the data indicate an Ag-experienced phenotype in C1-reactive B cells, but that no classical anergy induction appears to be operating during this process.

**Autoreactive B cells have omitted somatic hypermutations**

Ab-producing hybridomas were generated from CII-immunized ACB mice. All C1-specific hybridoma clones generated from various lymphoid organs of ACB mice used germline sequence with no V gene somatic mutation either in H or L chain (Table I). All six C1-specific clones, CB21-23 and CB26-28, irrespective of their isotype and L chain usage, have 100% homology to CB20 H chain (high similarity with J558-42) and Vx (high similarity with Vc21-4). In addition, clones CB22 and 23 could use a different L chain (Vx21-7) from CB20 L chain (Vc21-4), but still formed C1 specificity.

**Spontaneous anti-CII Ab production in ACB mice is T cell independent**

Because CII-reactive B cells can be readily detected in ACB mice, we investigated whether anti-CII autoantibodies could be spontaneously produced. CII-specific ELISA data showed that both ACB+/+ and ACB−/− mice could produce significant levels of anti-CII Abs from 30 d of age, and the level stabilized by 50 d onward (Fig. 3A). Interestingly, we observed a large difference in Ab titers between ACB+/+ and ACB−/−, which reached 200 and 30 μg/ml in the circulation at day 50, respectively (Fig. 3A). Possibly, the presence of transgene in both alleles increased the avidity to Ag due to higher density in ACB+/+. ACB−/− littermate controls did not produce any detectable level of Abs at any of the time points. In addition, when the sera samples from mature mice were subjected for anti-CII and anti-CI epitope ELISA in parallel, it showed that the anti-CII Abs in ACB mice consisted mainly of anti-C1 Abs from 30 d of age, and the level stabilized by 50 d onward (Fig. 3A). Importantly, both C1-binding populations expressed a high level of MHCIId and CD80 were observed in comparison with C1− B cells. Together, the data indicate an Ag-experienced phenotype in C1-reactive B cells, but that no classical anergy induction appears to be operating during this process.

**FIGURE 2.** Characterization of C1-reactive B cells. A, Identification of C1−, C1+, and C1+++ populations in B220+ splenocytes (SP) or CD19+ peritoneum (Peri) cells. B, Frequency (mean ± SD) of C1+ and C1++ in B220+ B cells in indicated organ of ACB+/+ (n = 4), ACB−/− (n = 7), and ACB+/− (n = 4). Frequency of C1++ in peritoneum represents the sum of C1+++ and C1+++. In CD19+ B cells, *p < 0.05, **p < 0.01, C. A representative FACS plot to show C1 binding in relation to CD21-staining intensity in B220+ splenic B cells. Dotted line represents the cutting off level of CD21high. CD23 (D) and IgMa, CD80, CD86, and MHCIId (E) surface expression intensity (mean ± SD) was compared between C1++, C1+, and C1− B cell fraction, slightly increased expression levels of CD86 and CD80 were observed in comparison with C1− B cells. Importantly, both C1-binding populations expressed a high level of MHCIId (A), as shown in Fig. 2E. Together, the data indicate an Ag-experienced phenotype in C1-reactive B cells, but that no classical anergy induction appears to be operating during this process.

Collectively, it shows that autoreactive B cells with a potential to recognize CII peptide could successfully differentiate into MZ, FO, and B1 B cell subset. Probably the interaction strength between BCR and Ag decides their physical location and cell fate.

The C1-binding B cells showed an Ag-experienced, but not anergic phenotype

Anergy, a state of unresponsiveness to Ag stimulation, is one of the major B cell tolerance mechanisms. A hallmark of B cell anergy is the downregulated surface expression of IgM. However, in ACB mice, C1+ population showed a higher surface IgMa expression, and C1+++ a comparable level to non-C1−-binding (C1−) population (Fig. 2E). Furthermore, it was previously reported that the antigened expression of costimulatory molecules is important in keeping anergic status (27, 28). Yet, in C1+ population, elevated levels of surface expression of CD86 and CD80 were observed. It is in agreement with their physical locations, as MZ B cells were reported as partially activated, possibly as a consequence of encountering with self-antigen during positive selection (29). As for C1+++ B cell fraction, slightly increased expression levels of CD86 and CD80 were observed in comparison with C1− B cells. Importantly, both C1-binding populations expressed a high level of MHCIId (A), as shown in Fig. 2E. Together, the data indicate an Ag-experienced phenotype in C1-reactive B cells, but that no classical anergy induction appears to be operating during this process.

**Table I.** Characteristics of hybridoma clones generated from ACB mice in comparison with CB20

<table>
<thead>
<tr>
<th>Clone</th>
<th>Origin</th>
<th>Organ</th>
<th>Isotype</th>
<th>Epitope</th>
<th>H Chain (%)</th>
<th>L Chain (%)</th>
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<tr>
<td>CB20</td>
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<td>SP</td>
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<td>CII/c</td>
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<td>97.6 ai-4</td>
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<td>CII/c</td>
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<td>CII/c</td>
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</table>

*H chain sequence homology to CB20 H chain J558.

†Unknown epitope specificity.

Peri, peritoneum.
IgG2a, IgG2b, IgG1, and IgM isotypes in ACB+/+ mice, whereas IgG1 and IgM were predominant in ACB+/− (Supplemental Fig. 3C).

To understand whether T cells play a role in such autoimmunity, we crossed ACB+/+ mice with T cell-deficient mice, in which TCR tcrb gene was targeted, resulting in absence of functional αβ T cells (designated as ACB+/+ × TCR−/−). The ACB+/+ × TCR−/− mice could produce CII-specific Abs to a level comparable to TCR-sufficient littermate group (ACB+/+ × TCR−/−) (Fig. 3C). Thus, B cells against a self-matrix protein differentiate to plasma cells and produce potentially pathogenic autoantibodies independently of T cells. T cell-independent, CII-specific Ab production could be induced ex vivo by targeting TLR2/6 and TLR9 with corresponding agonists: FSL1; ODN1826. *p < 0.05, **p < 0.01. In vitro recall response depicted as OD values from CIU/CFA-immunized ACB+/+ (n = 5) and B10.Q (n = 5) mice. LN cells by LPS (from Escherichia coli). *p < 0.01, ***p < 0.001.

**FIGURE 3.** Anti-CII autoantibodies could be readily detected in ACB mice. A, Total anti-CII Ab levels (μg/ml, mean ± SD) were monitored over time in sera of ACB+/+, ACB+/−, and ACB−/− (n = 12, 16, and 9, respectively) with a polyclonal serum serving as a standard. The Ab levels in ACB−/− mice were undetectable. Both ACB+/+ and ACB+/− mice had significantly higher Ab concentrations than ACB−/− mice from day 30 onward with ***p < 0.0001 (a). b, Significant difference between ACB+/+ and ACB+/− groups from day 40 onward. B, Fluorescent counts (europium counts) of total anti-CII (left) and anti-C1 (right) Ab levels in sera of ACB+/+ (n = 5), ACB+/− (n = 5), and ACB−/− (n = 6) mice. C, OD of total anti-CII Ab levels in naive ACB+/+ × TCR−/− (n = 4) and ACB+/+ × TCR−/− (n = 4) mice. D, Anti-CII Ab units in ACB+/+ × TCR−/− (n = 6) and ACB+/+ × TCR−/− mice (n = 6) at day 0 (preimmunization) and days 35 and 80 after CII/CFA challenge. *p < 0.05, **p < 0.01, significant difference between ACB+/+ × TCR−/− and ACB+/+ × TCR−/− mice groups. E, Total anti-CII Ab levels in naive (day 0) and CII-immunized ACB+/+, ACB+/−, and ACB−/− mice (n = 5) at indicated time points. A polyclonal serum was used as standard to determine Ab concentration, presented in log10 scale. *p < 0.05, ***p < 0.001 represents the significant difference between ACB+/+, and BQ mouse groups. F, Ex vivo anti-CII Ab production (europium counts) from naive splenocytes of ACB+/+ (n = 3) and B10.Q (n = 4) mice by targeting TLR2/6 and TLR9 with corresponding agonists: FSL1, ODN1826. *p < 0.05, **p < 0.01. G, In vitro recall response depicted as OD values from CIU/CFA-immunized ACB+/+ (n = 5) and B10.Q (n = 5) mice. LN cells by LPS (from Escherichia coli).

ACB mice develop more severe arthritis due to endogenous autoreactive Ab binding to the cartilage in vivo

The next apparent question was whether autoantibodies in ACB mice contribute to arthritis development. We first investigated whether autoantibodies are able to bind to cartilage surface in natural condition. Frozen sections of knee joint of naive mature ACB+/+, ACB+/−, and B10.Q mice were directly stained with biotinylated anti-κ Abs. As positive control, a group of B10.Q mice was injected with 1 mg purified CB20 Abs 1 h prior to sample collection (designated as BQ’ CB20). As shown in Fig. 4A, the bindings of Abs on cartilage surface in both ACB+/+ and ACB+/− mice were readily detected, with a staining intensity comparable to positive control (Fig. 4B), whereas in naive B10.Q mice it remained negative. However, we did not observe any proteoglycan depletion (Fig. 4C), which is normally observed after injection of Abs to the C1 epitope (30).

However, we observed that, despite having Abs bound to joint cartilage, ACB mice did not develop spontaneous arthritis within 1.5 y of age. Possibly, several epitopes on CII need to be targeted...
to trigger arthritis, whereas in ACB mice C1-specific Abs were dominant (Fig. 4D). Therefore, a mixture of Abs containing CIC2 (recognizing D3 epitope), M2139 (J1 epitope), and UL1 (U1 epitope) (25) at 2 mg each was passively transferred to ACB mice. As expected, ACB5/5 group developed most severe arthritis and ACB7/7 intermediate arthritis with mean incidence and maximum score of 100%, 38.6 ± 5.3 and 100%, 21.2 ± 4.2 (mean ± SEM), respectively (Fig. 4D). In both groups, disease was more severe than that of ACB7/7 group, which had 64% of incidence and a score of 14.3 ± 4.3. Interestingly, the disease pattern was in agreement with their endogenous anti-CII Ab titers, suggesting that the anti-CII autoantibodies in ACB mice render them more susceptible to disease.

Anti-C1-specific mAbs are pathogenic in CAIA model of arthritis

Pathogenicity of affinity-purified C1-specific mAbs generated in ACB mice, CB21 (IgM), CB26 (IgG2a), and CB28 (IgG2b) (Table I) was tested by passive transfer at 4.5 mg each in combination with M2139 at 2.25 mg (subarthritogenic dosage) to B10.RIII mice. As shown in Fig. 4E, all three mAbs induced severe arthritis and significantly increased both disease incidence and severity as compared with M2139 alone. This observation clearly demonstrates the pathogenic capacity of C1-specific autoantibodies from ACB mice.

Discussion

The occurrence of Abs to CII in RA, as well as their pathogenicity and binding to the target tissue in vivo, highlights the importance of understanding the roles that CII, a cartilage-specific matrix protein, plays in autoimmunity and arthritis. Through the establishment of a CII-specific IgH VDJ replacement mouse strain, we could now show that B cells autoreactive to a matrix protein do not appear to be either negatively selected or tolerated. Instead, these autoreactive B cells were readily detected in all lymphoid organs and spontaneously produced high levels of autoantibodies. This transgenic mouse model represents the first possibility, to our knowledge, to study the B cell response to an endogenous matrix Ag. Our study also provides some of the first insights into the mechanisms of B cell tolerance to a matrix protein.

When soluble and membrane-bound Ags are in focus, clonal deletion, receptor editing, and anergy have been shown to be major B cell tolerance mechanisms (17, 31–33). Clonal deletion leads to B cell developmental arrest at one or more stages as a consequence of negative selection and elimination of B cells expressing autoreactive high-affinity BCR. The process of receptor editing often results in coexpression of dual L chains to avoid autoimmune (reviewed in Ref. 31). Moreover, autoreactive B cells that fail in deletion or receptor editing may emerge to periphery and be subjected to anergy, a functionally inactive state (34). However, in the current study, when CII, an endogenous matrix protein, is in concern, we could demonstrate that B cells could normally populate in different B cell compartments in lymphoid organs. No evidence for developmental arrest was observed. However, more experiments are needed to address the receptor-editing mechanism in detail, but we could clearly show that autoreactive B cells were not functionally anergic, as they could spontaneously produce autoantibodies and could properly respond to self-matrix protein.

**FIGURE 4.** Autoantibodies bound to cartilage surface in vivo in naive ACB mice and enhanced arthritis development. A, Cryosections from knee joints of naive mature ACB5/5 (n = 6), ACB7/7 (n = 4), and BQ (n = 5) mice were stained with biotinylated anti-κ Abs or PBS (blank). As positive control, BQ mice (n = 3) were injected i.v. with 1 mg purified CB20 mAbs 24 h prior to sacrifice (BQ+CB20). Arrowheads identify cartilage surface (original magnification ×20). Stained section from each group was blind scored with a scoring system of 0–2, with 0 for no staining, 1 for intermediate, and 2 for maximum staining. Scores (mean ± SD) were summarized in B. Positive staining on synovial tissue was ignored. *p < 0.05, **p < 0.01. C, Representative image of toluidine blue-stained ankle joint sections from naive, 2-mo-old ACB+/+ and BQ mice (right). As positive control, BQ mice (blank). As positive control, BQ mice (blank). Representative image of toluidine blue-stained ankle joint sections from naive, 2-mo-old ACB5/5 and BQ mice (n = 3/group). D, CAIA was induced by injection of a mixture of Abs containing M2139, CIC2, and UL-1 at 2 mg per Ab per mouse to mature ACB5/5 (n = 11), ACB7/7 (n = 11), and ACB+-/- mice (n = 12). LPS at 25 μg/mouse was given i.p. at day 7. The mean maximum disease score (± SD) (left) and incidence (right) are presented. *p < 0.05, **p < 0.01. E, Arthritis was induced in 3- to 4-mo-old male B10.RIII mice by i.v. injection of purified CB20 (n = 8) and mAbs generated in ACB mice CB21 (n = 9), CB26 (n = 5), and CB28 (n = 9) at 4.5 mg in combination with M2139 at 2.25 mg per mouse. M2139 (n = 11) alone at 2.25 mg per mouse was also injected in parallel. LPS injection at 25 μg/mouse followed at day 7. Mice were scored for 18 d. *p < 0.05.
stimulation in vivo and in vitro system. Using rheumatoid factor transgenic mouse, similar questions were addressed previously by Shlomchik and colleagues (15), and they showed that autoreactive rheumatic factor B cells could generate normal primary immune responses and did not appear to be anergic. Our findings are in agreement with their observations.

The influence of the nature of autoantigen in tolerance induction can be multiplex. First of all, Ag accessibility differs. Soluble Ags are systemically available for positive and negative selection of B cells. In contrast, the accessibility of a matrix protein for B selection process is unknown. In the ACB model, we noticed that pre-B cells were preferentially selected toward immature and mature B cell pool in BM, possibly due to the transgene in IgH locus increasing the avidity of BCR toward self-antigen. It suggests the presentation of CII in BM. Whether or not CII is processed and presented to B cells in either BM or peripheral, and where and how this happens are essential questions. It is noteworthy to mention that the frequency of CII-specific B cells in ACB mice reaches to 5–10%, which is surprisingly high when knowing that the Abs binding the C1 epitope, like the CB20 hybridoma, could use only one κ V gene. In fact, it approaches the frequency of specific B cells in mouse strains made with both H and L chain. For example, in the GPI-reactive H/L chain transgenic mouse model, 15–25% of the B cells were GPI reactive (35), and in the hen egg lysozyme (HEL) transgenic mouse model, 5–6% of B cells could be shown to exhibit saturable binding of HEL at the highest HEL concentration tested (36). The high frequency in our model suggests the possibility of positive selection of CII-reactive B cells. Clarke and colleagues (37) showed that Sm-specific B cells seemed to undergo a positive selection and differentiated into peritoneal B1 B cells by using an anti-Sm transgenic mouse model. Indeed, the highest frequency of C1-specific B cells that we observed was in peritoneum, and they were also B1 B cells.

In addition, accessibility of BCR toward autoantigens could differ between soluble and matrix proteins. Affinity was believed to play an essential role in regulating autoreactive B cell developmental fate in soluble autoantigen models [reviewed by Su et al. (38)]. Indeed, in the ACB model, we could identify three C1-binding B cell populations with different binding affinity and segregating in different physical locations, as follows: the C1* population in the MZ of SP; the C1** in follicles and peritoneum; and the C1*** in peritoneum only. The segregation of nontolerant autoantigen-binding B cells in MZ and follicular cell compartments in SP was also described by Mandik-Nayak et al. (39) by using a low-affinity anti-GPI H chain transgenic mice VH147. However, the anti-CII Abs cannot readily be classified into low- or high-affinity natural Abs, because that affinity is relevant only in vivo, in particular for matrix Ags. The anti-CII CB20 Abs could bind cartilage in vivo with even stronger staining in immunohistochemical analysis than most of the Abs that had higher affinity values in vitro (23, 40). An important question is whether autoreactive receptor affinity shapes tolerance induction in a similar way when nonsoluble matrix autoantigens are concerned.

Previously, it was shown that those B cells that escape tolerance induction can pose a potential danger, as they can somatically mutate and affinity mature in the periphery (41). However, a striking finding in ACB model is that somatic mutation did not occur in any of the C1-specific B cell clones investigated. Together with the CAIA data, showing that these C1-specific clones were potentially pathogenic, it poses a very important possibility of the prevalence of a physiological mechanism that prevents the occurrence of high-affinity autoimmunity. Yet, the reason behind the lack of somatic mutation is unclear. Could that be due to the lack of T cell help? Somatic mutation normally takes place in germlinal center when sufficient T cell help is obtainable. However, our data demonstrated that deleting T cells did not affect a naive autoantibody phenotype in ACB mice. Apparently, spontaneous activation of autoreactive B cells and thereafter formation of plasma B cells can occur also in a T cell-independent manner in ACB mice, but, to respond to CII self-matrix protein stimulation, T cell help is crucial.

Abs are likely to play an essential role in triggering inflammation in a number of autoimmune diseases, including RA. In this study, a unique finding is that we could visualize the natural autoantibodies specifically binding to cartilage in vivo in naive ACB mice. However, the binding per se is not sufficient to induce a clinically visible pathogenic inflammation. An interesting feature of the C1 epitope is that it is recognized predominantly by germline-encoded Abs (13, 23). Hence, the recognition of the C1 epitope might be limited to germline-encoded Abs, but this does not seem to relate to pathogenicity, as the CIIC1 Abs have been shown to be able to induce arthritis (42) and the binding of this Ab is depending on its germline-encoded amino acids rather than somatic mutations (43). However, it is also quite apparent that the occurrence of Abs to more than one C1II epitope dramatically enhances that arthritogenicity. Indeed, when Abs against other C1II epitopes were transferred, ACB mice developed severe arthritis and autoantibodies in the ACB mice showed potential pathogenicity by enhancing CAIA disease. Taken together, these data emphasize that germline-encoded autoantibodies can also pose a pathogenic potential when quantity and/or inflammation milieu allow.

In closing, this germline-encoded CII IgH transgenic mouse model allowed us to demonstrate that CII-specific autoantibody generation in ACB mouse is regulated differently and is not dependent on either central tolerance mechanisms or on peripheral anergy. The present study thus promotes the possibility that such potentially pathogenic B cells occur normally in humans as well, and they could be dangerous in a situation in which B and T cells reactive with several epitopes on the same molecule are activated.

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Disclosures
The authors have no financial conflicts of interest.

References


Corrections


There were several errors in the legend for Fig. 3. In the legend, the description of E corresponds to G, the description of F corresponds to E, and the description of G corresponds to F. The corrected figure legend is below. Fig. 3 was correct as published and is shown below for reference.

**FIGURE 3.** Anti-CII autoantibodies could be readily detected in ACB mice. A, Total anti-CII Ab levels (μg/ml, mean ± SD) were monitored over time in sera of ACB^+/+, ACB^+/−, and ACB^−/− (n = 12, 16, and 9, respectively) with a polyclonal serum serving as a standard. The Ab levels in ACB^−/− mice were undetectable. Both ACB^+/+ and ACB^−/− mice had significantly higher Ab concentrations than ACB^−/− mice from day 30 onward with ***p < 0.001 (a). B, Fluorescent counts (europium counts) of total anti-CII (left) and anti-C1 (right) Ab levels in sera of ACB^+/+ (n = 5), ACB^+/− (n = 5), and ACB^−/− (n = 6) mice. C, OD of total anti-CII Ab levels in naive ACB^+/+ × TCR^−/− (n = 4) and ACB^+/+ × TLR^+/− (n = 4) mice. D, Anti-CII Ab units in ACB^+/+ × TCR^−/− (n = 6) and ACB^+/+ × TLR^+/− mice (n = 6) at day 0 (pre-immunization) and days 35 and 80 after CII/CFA challenge. *p < 0.05, **p < 0.01, ***p < 0.001 represent the significant difference between ACB^+/+ and ACB^−/− groups. E, Ex vivo anti-CII Ab production (europium counts) from naive splenocytes of ACB^+/+ (n = 3) and B10.Q (n = 4) mice by targeting TLR2/6 and TLR9 with corresponding agonists: FSL1; ODN1826. *p < 0.05, **p < 0.001 represent the significant difference between ACB^+/+ and BQ mouse groups. F, In vitro recall response depicted as OD values from CII/CFA-immunized ACB^+/+ × TCR^−/− (n = 5) and B10.Q (n = 5) mice LN cells by LPS (from Escherichia coli). *p < 0.05, **p < 0.01. G, Total anti-CII Ab levels in naive (day 0) and CII-immunized ACB^+/+, ACB^+/−, ACB^−/−, and ACB^+/− mice (n = 5) at indicated time points. A polyclonal serum was used as standard to determine Ab concentration, presented in log10 scale. *p < 0.01, ***p < 0.001.

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Supplemental figure S1: Generation of transgenic mice
A) Organization of the 16 kb targeting vector piV_{H} L2 neoR for insertion of a rearranged CB20 VDJ region replacing the endogenous J_{H} locus in the E14 ES cell germline DNA. B) Recombination of endogenous locus and targeting vector leads to alteration of endogenous IgH locus. The 0.7 kb 32P-labeled external probe detects a Hind III (H) - Hind III 3.7 kb fragment from the CB20 VDJ gene replacement allele and 2.3 kb from endogenous allele in Southern Blot analysis in C). D) Total cell number in spleen (SP), popliteal / inguinal lymph nodes (LN), bone marrow (BM) and peritoneum (Peri) of a naïve ACB+/+ (n = 5), ACB+/− (n = 5) and ACB−/− (n = 5) mice.
E) The frequency of B1 (CD19+CD11b+) in CD19+ B cells, and the frequency of B1a (CD19+CD11b+CD5+) and B1b (CD19+CD11b+CD5-) among B1 B cells in peritoneum.

The construction strategy is outlined in (A, B). piVH L2 neoR is a 16 kb universal vector for targeted insertion of a rearranged VDJ region replacing the endogenous JH locus in the embryonic stem (ES) cell germline DNA (received from Dr W Müller, University of Cologne, Germany). From the 5’ end, the piVH L2 neoR vector consists of a pBSII backbone followed by HSV-TK gene, a 10 kb long homologous DNA region representing the genomic DNA region from BALB/c mouse upstream of the JH genes. Next followed the NeoR cassette flanked by LoxP sequences for later removal by Cre enzyme. Immediately after the NeoR cassette, Xho I and Cla I sites are located where the rearranged non-homologous VDJ region was inserted. The inserted VDJ region (B) is followed by a homologous 129 gDNA J-C intron terminated by a Not I site, where the construct was linearized before transfected into the ES cells.

A 2 kb DNA fragment containing the 1.3 kb 5’ upstream part of the V47 gene (in a pBSII plasmid) the B1-8 VDJ gene and parts of the downstream 3’ JH intron were received from Dr Müller, University of Cologne. The vector was digested with PstI releasing a region from the PstI site in the upper framework region 1 (FR1) V region to the 3’ PstI site in the MCS part of the pBSII plasmid. The remaining part was then re-ligated, opened up by digesting with Cla I, close to the 5’ Xho I site representing the boundary of the genomic upstream, 1.3 kb part of the V region. The Cla I site was filled in using Klenow enzyme, to eliminate the internal Cla I site. This Cla I neutralized construct was re-ligated with the original Pst I / Pst I V47 B1-8 VDJ region. This construct was digested at the Nco I site in the signal peptide V region and Cla I at the boundary of the JH intron and the vector. The resulting Nco I/Cla I fragment was ligated into a pSL1180 plasmid. This vector was digested with Pst I located in the FR1 region and Bsu 36I at the junction between the JH region and the JH intron, releasing the B 1-8 VDJ region, to be replaced by a CB20 CII specific region. CB20 was PCR amplified from M13 ssDNA, sequenced and partially digested with Pst I and digested to completion with Bsu 36I. The complete VDJ region of CB20 was ligated into the pSL1180 producing a complete VDJ construct from the signal peptide Nco I to the 3’ JH intron Cla I site. The Nco I to Cla I fragment was cleaved out and ligated into the pBSII V47 vector with the remaining 1.3 kb 5’ immunoglobulin DNA region, altogether constituting the 2 kb complete rearranged VDJ region. The 2 kb Xho I / Cla I cut VDJ CB20 DNA construct was ligated with the Xho I / Cla I cut 16 kb piVH L2 neoR giving the final gene replacement construct, which was Not I-linearized before transfection into E14 ES cells from the 129/Ola mouse strain.

ES cells of the E14 129/Ola line were transfected with the Not I-linearized targeting vector and selected with G418. Southern blot analysis was performed with Hind III-digested ES cell DNA and hybridized to a 0.7-kb 32P-labeled external probe to detect homologous recombinant clones. In addition, PCR was performed to confirm the homologous recombination. A positive clone denoted ACB (Anti-C1 B cell epitope) was injected into C57Bl/6 blastocysts and re-implanted into pseudo-pregnant hosts. The clone yielded germline-transmitting chimeric animals. The offsprings, which were positive for transgene were backcrossed with B10.Q mice. B10.Q mice were
from our inbred colony and have an arthritis-susceptible A\textsuperscript{q} class II congenic fragment on the C57B1/10 (B10.Q) background.
Supplemental figure S2: A) The cut off for C1+/CD19+ B cells, in accordance to ACB-/- mice staining level. B) C1 specific cell size in ACB-/-, ACB+/+ and ACB++/+ mice.
Supplemental figure S3: A) Frequency (mean ± SD) of C1++ and C1+ in B220+ B cells in peripheral blood (PB) of ACB+/+ (n = 14), ACB+/- (n = 18), and ACB-/- (n = 10) were monitored from 20 days after birth to 100 days. B) Frequency of (mean±SD) of IgMa+, IgMb+ in B220+ B cells in the spleen (n = 5 mice/group). C) Isotype distribution of total anti-CII Abs in sera of mature ACB+/+ (n = 12), ACB+/- (n = 16), and ACB-/- (n = 9). Optical density was presented.