B Cell Depletion Delays Collagen-Induced Arthritis in Mice: Arthritis Induction Requires Synergy between Humoral and Cell-Mediated Immunity

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B Cell Depletion Delays Collagen-Induced Arthritis in Mice: Arthritis Induction Requires Synergy between Humoral and Cell-Mediated Immunity

Koichi Yanaba, Yasuhiro Hamaguchi, Guglielmo M. Venturi, Douglas A. Steeber, E. William St.Clair, and Thomas F. Tedder

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation of the joint capsule and synovial membrane, resulting in synoviocyte proliferation, cartilage injury, and bone erosion, with eventual joint destruction and deformity (1). Although the precise pathogenesis of RA remains unclear, T cells, macrophages, neutrophils, and synovial fibroblasts are central to the mechanisms of joint inflammation and disease progression. However, B cells may also play a critical role in RA pathogenesis in light of the frequent occurrence of autoantibodies in this disease. For example, rheumatoid factor (RF) and anti-cyclic citrullinated peptide (CCP) Abs are detected in ∼75% of patients with RA (2). Autoantibodies to type II collagen, a major constituent of articular cartilage, are also found in sera (30–70%) and synovial fluid from patients with RA (3–5). Autoantibodies are envisioned to initiate immune complex formation within the joint, leading to complement activation and inflammatory cell recruitment (1, 6). In fact, RA patients whose sera contain RF or anti-CCP Abs develop more severe articular disease, show a higher frequency of extra-articular manifestations, and are at risk for increased mortality and morbidity (7, 8). Some patients with RA (∼30%) also develop synovitis with lymphatic follicles containing T and B cells arranged around a network of follicular dendritic cells (9) with germinal center formation (10). When human synovial tissue is transferred into immunodeficient mice, human B cells are required for germinal center CD4⁺ T cell activation, but macrophages and dendritic cells are not needed (11). Thus, several lines of evidence implicate B cells in the induction of RA and its propagation.

Collagen-induced arthritis (CIA) is a well-established model for human RA that develops in susceptible rat or mouse strains immunized with heterologous type II collagen emulsified in CFA (12, 13). CIA is characterized by severe swelling of the paws, extensive synovial hyperplasia, cartilage damage, bone erosion, and joint ankylosis (12). CIA and RA susceptibility associate with a limited number of MHC class II haplotypes, suggesting immunological similarities between the two diseases (14, 15). The chronic inflammatory arthritis induced by collagen immunization consists of a T cell component, as reflected by the CD4⁺ T cells and macrophage-like cells that infiltrate the synovial membrane, and a B cell component as indicated by the production of collagen-specific IgG autoantibodies (12, 16, 17). The transfer of collagen-specific T cells alone induces synovitis, but not clinical arthritis (18, 19). T cell depletion in mice with CD4 and TCR-specific Abs also attenuates CIA, although treatment is more effective if conducted before collagen immunization (19–21). Likewise, B cell-deficient mice or xid mice with defective humoral immunity do not develop CIA (22, 23). In addition, IFN-γ-deficient B6 mice treated with CD22 mAbs conjugated with calicheamicin toxin do not develop CIA.
(24), implying that B cells play a pivotal role in the disease. Transferring collagen-specific autoantibodies alone leads to transient arthritis, with a histopathology that is somewhat different from that of CIA (19, 25–28). Thus, synergies between humoral and cell-mediated immunity appear critical for CIA, although the role(s) for B cells in CIA induction or pathogenesis remain poorly understood (26, 29).

B cell depletion using a chimeric CD20 mAb-based immunotherapy is an effective treatment for human RA (30, 31). CD20 is a B cell-specific cell-surface molecule involved in the regulation of transmembrane Ca \(^{2+}\) conductance and cell-cycle progression during human B cell activation (32). Unfortunately, however, the mechanisms by which B cell depletion affects disease in RA patients undergoing immunotherapy with CD20 mAbs remains unknown. Because human studies are primarily restricted to

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**FIGURE 1.** Reactivity of the MB20-11 and MB20-16 mAbs with spleen B cells from B6 or DBA mice. The histograms show the relative fluorescence intensities of B220\(^+\) cells stained with MB20-11, MB20-16, or isotype-matched control mAbs (10 \(\mu\)g/ml) in indirect immunofluorescence assays with flow cytometry analysis. Results represent those obtained in more than or equal to three experiments.

**FIGURE 2.** CD20 mAb-induced B cell depletion in DBA mice. Representative depletion of B cells from the BM (A), blood (B), spleen (C–E), peripheral lymph nodes (F), and peritoneal cavity (G and H) 7 days following MB20-16 or isotype-matched control mAb treatment (250 \(\mu\)g, more than or equal to five mice), as determined by immunofluorescence staining with flow cytometry analysis. Numbers indicate relative percentages of lymphocytes within the indicated gates. Bar graphs indicate mean numbers (±SEM) of blood (per milliliter) and tissue B cells following mAb treatment. Percentages indicated within the bar graphs represent the percentage of B cells of each phenotype found in CD20 mAb-treated mice relative to the numbers of B cells found in control mAb-treated littermates. Significant differences between mean results for MB20-16 or control mAb-treated mice are indicated; *, \(p < 0.05\); **, \(p < 0.01\).
measuring changes in blood B cells, which represent <2% of all B cells outside of the bone marrow (BM) (33), mechanistic studies often fail to take into account the possible changes in tissue B cells. Moreover, there is limited information about the effects of B cell depletion on the progression of articular damage in RA patients (34). Therefore, we have developed mouse anti-mouse CD20 mAbs (35) to provide an animal model for investigating the effects of CD20 mAb-mediated B cell depletion in vivo that is amenable to detailed tissue analysis (36–38). As with human CD20, mouse CD20 is also B cell specific, being first expressed during B cell maturation until plasma cell differentiation (39). This model system has allowed us to effectively deplete B cells from adult mice with fully developed immune systems to assess the role of B cells in the initiation and pathogenesis of a chronic inflammatory arthritis similar to human RA.

Materials and Methods

Abs and immunofluorescence analysis

Mouse CD20-specific mAbs MB20-11 and MB20-16 were used as described (35). Other mAbs included: B220 mAb RA3-6B2; Thy-1.2 mAb 53-7.3; CD5 (B6.H-129S1); and marginal zone (CD21<sup>high</sup>/CD14<sup>high</sup>CD1d<sup>−</sup>), immature B (IgM<sup>−</sup>B220<sup>−</sup>), mature B (IgM<sup>+</sup>B220<sup>−</sup>), and B1b (CD5<sup>−</sup>B220<sup>−</sup>) or B2 (CD5<sup>−</sup>IgM<sup>−</sup>B220<sup>−</sup>) mAbs were obtained from Southern Biotechnology Associates. Single-cell suspensions of BM (bilateral femurs), spleen, and peripheral lymph node (paired axillary and inguinal) lymphocytes were generated by gentle dissection. To isolate peritoneal cavity leukocytes, 10 ml of cold PBS was injected into the peritoneum of sacrificed mice followed by gentle massage of the abdomen. Viable cells were counted using a hemocytometer. Blood erythrocytes were lysed after immunofluorescence staining using FACS Lysing Solution (BD Biosciences). Single-cell leukemia suspensions were stained on ice using predetermined optimal concentrations of each Ab for 20–60 min, and fixed as described (40, 41). Cells with the light scatter properties of lymphocytes were analyzed by two- to four-color immunofluorescence staining and were analyzed using FACScan or FACSCalibur flow cytometers (BD Biosciences). Background staining was determined using unreactive isotype-matched control mAbs (Caltag Laboratories) with gates positioned to exclude ≥98% of unreactive cells.

Mice and immunotherapy

C57BL/6 (B6) and DBA/1J (DBA) mice were obtained from The Jackson Laboratory. Sterile anti-mouse CD20 and isotype control mAbs (250 µg) in 200 µl of PBS were injected through lateral tail veins. Mice were housed in a pathogen-free barrier facility and used at 8–12 wk of age. The Duke University Animal Care and Use Committee approved all studies.

CIA induction

CIA was induced in male DBA mice, which show a high penetrance of disease after heterologous collagen immunization (16). Chicken collagen (Chondrex) was dissolved in 10 mM acetic acid solution (5 mg/ml) overnight at 4°C. Dissolved collagen (100 µg) was emulsified with an equal volume of CFA containing 1 mg/ml heat-killed Mycobacterium tuberculosis (H37Ra; Difco), with 100 µl injected s.c. into the base of the tail. Mice were boosted s.c. with collagen (100 µg) emulsified in IFA on day 21.

Clinical monitoring of arthritis

Thirty days after primary immunizations, mice were monitored three times weekly for arthritis severity. Arthritis clinical severity was evaluated by examining the appearance of each joint on both front and hind paws by blinded investigators. Mice were monitored for signs of clinical arthritis and given a “consensus clinical score” as follows: 0, normal; 1, mild; 2, moderate; 3, severe. Swollen digits were noted but paws were only considered arthritic when the entire paw was not used as a result of paw inflammation. During the chronic phase of arthritis, paws and digits were inspected for distortion and manipulated to identify loss of flexion (ankylosis). Paw distortion was considered as a persistent deviation from the normal shape, alignment, positioning, or size of each paw that did not change during the examination of each mouse. Both distortion and ankylosis were evaluated by a semiquantitative scoring system: 0, absent; 3, present. Total clinical scores were calculated by summing each parameter score for inflammation, distortion, and ankylosis. Total scores for inflammation, distortion, and ankylosis were also reported individually.

Histological analysis

Whole ankle and ankle joints were fixed for 3 days in 10% formalin. After decalcification for 18 days in Cal-Ex II (Fisher Scientific), the specimens were processed for paraffin embedding. Tissue sections (5 µm) were stained with H&E for microscopic evaluation. Degrees of synovial hyperplasia, cartilage damage, bone erosion, and ankylosis were each assessed in a blinded fashion with the severity of each disease parameter graded in

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Table I. Tissue B cell depletion following CD20 mAb treatment

<table>
<thead>
<tr>
<th>Tissue</th>
<th>B Subset&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Control mAb&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CD20 mAb</th>
<th>% Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM: Pro/pre</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Immature</td>
<td>0.42 ± 0.01</td>
<td>0.33 ± 0.03</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td>Mature</td>
<td>1.1 ± 0.1</td>
<td>0.13 ± 0.02</td>
<td></td>
<td>88**</td>
</tr>
<tr>
<td>Blood: B220&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.4 ± 0.3</td>
<td>0.03 ± 0.01</td>
<td></td>
<td>98**</td>
</tr>
<tr>
<td>Spleen: B220&lt;sup&gt;+&lt;/sup&gt;</td>
<td>51.2 ± 1.6</td>
<td>1.9 ± 0.3</td>
<td></td>
<td>96**</td>
</tr>
<tr>
<td>Mature</td>
<td>32.4 ± 1.7</td>
<td>1.2 ± 0.2</td>
<td></td>
<td>96**</td>
</tr>
<tr>
<td>T1</td>
<td>7.8 ± 0.1</td>
<td>0.38 ± 0.04</td>
<td></td>
<td>87**</td>
</tr>
<tr>
<td>T2</td>
<td>8.8 ± 1.1</td>
<td>0.03 ± 0.01</td>
<td></td>
<td>&gt;99**</td>
</tr>
<tr>
<td>Marginal zone</td>
<td>3.6 ± 0.3</td>
<td>0.03 ± 0.02</td>
<td></td>
<td>&gt;99**</td>
</tr>
<tr>
<td>Peripheral lymph node:</td>
<td>B220&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.56 ± 0.13</td>
<td>0.05 ± 0.02</td>
<td>91*</td>
</tr>
<tr>
<td>Peritoneum: B220&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.11 ± 0.05</td>
<td>1.09 ± 0.11</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>B1a</td>
<td>0.29 ± 0.02</td>
<td>0.19 ± 0.03</td>
<td></td>
<td>34</td>
</tr>
<tr>
<td>B1b</td>
<td>0.48 ± 0.02</td>
<td>0.61 ± 0.07</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>B2</td>
<td>0.38 ± 0.06</td>
<td>0.33 ± 0.06</td>
<td></td>
<td>13</td>
</tr>
</tbody>
</table>

<sup>a</sup> B cell subsets were: BM pro/pre-B (IgM<sup>+</sup>B220<sup>−</sup>), immature B (IgM<sup>−</sup>B220<sup>−</sup>), mature B (IgM<sup>+</sup>B220<sup>−</sup>), and marginal zone (CD21<sup>high</sup>/CD14<sup>high</sup>CD1d<sup>−</sup>), T1 (CD4<sup>+</sup>CD21<sup>−</sup>B220<sup>−</sup>), T2 (CD4<sup>−</sup>CD21<sup>−</sup>B220<sup>−</sup>), and marginal zone (CD21<sup>high</sup>/CD14<sup>high</sup>CD1d<sup>−</sup>); peritoneal B1a (CD5<sup>−</sup>CD11b<sup>−</sup>IgM<sup>−</sup>B220<sup>−</sup>), B1b (CD5<sup>−</sup>CD11b<sup>−</sup>IgM<sup>−</sup>B220<sup>−</sup>), and B2 (CD5<sup>−</sup>IgM<sup>−</sup>B220<sup>−</sup>). Values (±SEM) indicate cell numbers (×10<sup>6</sup>) present in mice 7 days after mAb treatment (250 µg; n ≥ 4/value); blood shown as cells per milliter, and lymph node as pooled bilateral inguinal and axial lymph nodes. Significant differences between means are indicated; *, p < 0.05; **, p < 0.01.
joint sections using a scoring system from 0 to 5: 0, within normal limits; 1, minimal; 2, mild; 3, moderate; 4, marked; 5, severe as described (46).

Measurement of serum Ag-specific Abs

Serum collagen-specific Ab levels were assessed by ELISA as described (42). In brief, 96-well microtiter plates (Costar) were coated with collagen (100 µl/well, 10 µg/ml), with Ab binding detected using alkaline phosphatase-conjugated goat anti-mouse IgM, IgG1, IgG2a, IgG2b, and IgG3 secondary Abs (Southern Biotechnology Associates). ELISA color development was allowed to progress until the wells containing the highest Ab levels reached OD values of ~2.0. The relative Ig concentrations in individual samples were determined by comparing the mean OD values obtained for duplicate wells to a semilog standard curve of titrated

**FIGURE 3.** B cell depletion and delayed arthritis onset following CD20 mAb treatment. Mice were treated with MB20-16 (○) or isotype control (□) mAb 7 days before collagen (CII) immunizations on day 0 and were boosted with collagen on day 21. A, Numbers of mature BM B cells, blood B cells (per milliliter), T1, T2, mature, and marginal zone spleen B cells, peripheral lymph node B cells, and peritoneal B1a, B1b, and B2 B cells were determined by immunofluorescence staining with flow cytometry analysis. Because the mice were sacrificed to harvest their tissues, each data point represents a different group of littermates. Two factors contribute to changes in numbers of cells at each data point. First, each point represents different mice. Second, the mice are also aging which leads to associated increases and decreases in B cell numbers within the respective tissues. B, Arthritis severity in collagen-immunized mice. Mice were monitored for signs of clinical arthritis and given consensus clinical score as described (42, 43). Each mouse was also evaluated as described (44, 45) and given a total clinical score that was calculated by summing each individual score for inflammation, distortion, and ankylosis in each mouse examined. All values represent mean (±SEM) results for 4–12 individual mice at each time point. A and B, Significant differences between sample means are indicated. *, p < 0.05; **, p < 0.01.
standard Ab using linear regression analysis. These OD values were determined to be within the linear range of the ELISA using sera over multiple dilutions.

**Statistical analysis**

All data are shown as means ± SEM. Significant differences between sample means were determined using the Student t test.

**Results**

**B cell depletion in DBA mice following CD20 mAb treatment**

The use of mouse anti-mouse CD20 mAbs as a model for CD20 immunotherapy allows for detailed mechanistic studies and tissue analysis (36–38). The MB20-11 (IgG2c, Ighb allotype) mAb and its properties have been characterized extensively in vitro and in B6 mice (35–38). CIA is typically induced in DBA mice, which produce IgG2a (Igha allotype) but not IgG2c Abs. Therefore, the MB20-16 (IgG2a), another previously characterized mouse CD20 mAb, and the MB20-11 Ab were compared in vitro and in vivo for their abilities to bind and deplete B cells of DBA and B6 mice. When mAb reactivity with spleen B cells was assessed over a range of mAb concentrations, both mAbs stained B cells from DBA and B6 mice at saturating levels when used at 1 μg/ml as assessed by flow cytometry (data not shown). When used at 10 μg/ml, the two CD20 mAbs reacted similarly with splenic B220⁺ B cells from both strains of mice (Fig. 1). Thus, the MB20-16 and MB20-11 mAbs bound specifically to CD20⁺ B cells, with DBA and B6 mouse B cells expressing CD20 at similar densities.

Mature CD20⁺ B cells are eliminated within 2 days after a single treatment with MB20-11 mAb (250 μg/mouse) in B6 mice (35, 37). Therefore, it was determined whether MB20-16 mAb-depleted B cells to the same extent in DBA mice when given at 250 μg/mouse. This single dose is ~10-fold lower than the 375 mg/m² dose of rituximab (chimeric anti-CD20 mAb) given four times weekly (or 1000 mg dose given twice 14 days apart) to humans with RA and other autoimmune diseases (47–50). The
MB20-16 mAb depleted the vast majority of blood, spleen, peripheral lymph node, and BM B cells in DBA mice after 7 days as determined by immunofluorescence staining with flow cytometry analysis (Fig. 2, A–F, Table I). Mature B cells were significantly depleted from BM (Fig. 2A). By contrast, significant depletion of IgM<sup>-</sup>B220<sup>low</sup> pro/pre-B cells and IgM<sup>-</sup>B220<sup>imm</sup> immature B cells was not observed after MB20-16 mAb treatment (Table I) since the majority of pro-/pre- and immature mouse B cells express little, if any, cell surface CD20 (39). Furthermore, MB20-16 mAb depleted 98% of blood B cells and 91–96% of splenic and peripheral lymph node B cells (Fig. 2, B, C, and F). Little difference was seen between T1, T2, mature, and marginal zone B cell depletion in spleen (Fig. 2, D and E). In contrast with other tissues, the MB20-16 mAb did not effectively deplete peritoneal B1a, B1b, or B2 cells (Fig. 2, G and H), as previously shown in B6 mice (35, 37). B cell depletion was also comparable in DBA and B6 mice when the MB20-16 and MB20-11 mAbs were used at doses between 10 and 250 μg/mouse (data not shown, Refs. 35 and 37). The MB20-16 mAb (250 μg/mouse) effectively depleted circulating and spleen B cells in DBA mice for ~1 mo, with BM, spleen, lymph node, and circulating B cell recovery starting between days 30–42 (data not shown). This contrasts with results from B6 mice where BM, blood and spleen B cells return on approximately day 57 (35, 37). Isotype-matched control mAb did not affect circulating or tissue B cell numbers. Thus, the MB20-16 mAb mediated effective B cell depletion in DBA mice, the strain most commonly used for CIA.

### B cell depletion delays CIA onset

DBA mice were used to assess the effects of CD20 mAb treatment on the depletion and recovery of various B cell subsets in vivo. Mice were treated with a single i.v. injection of MB20-16 mAb at day −7, which preceded collagen immunization on days 0 and 21. After CD20 mAb administration, mature CD20<sup>+</sup> B cells in the BM, blood, and spleen were significantly depleted (>95%) by day 0 (Fig. 3A). Spleen, T1, T2, and marginal zone B cells were also effectively depleted. Unexpectedly, B cell recovery in the BM (day 30–60) and spleen (day 30) occurred much earlier than for blood (day 60). Peripheral B cells returned to levels found in control mAb-treated mice by day 90. Peritoneal B cell numbers were reduced after 30 days of mAb treatment, as previously described, but were never eliminated (37). Thus, a single CD20 mAb treatment significantly reduced mature B cell subsets in the BM, blood, spleen, and lymph nodes.

Mice were monitored for arthritis development and severity and given a consensus clinical score as described (42, 43) or a total clinical score as described (44, 45). Total clinical scores were calculated by summing individual scores for inflammation, distortion, and ankylosis in each mouse limb (Fig. 3B). DBA mice treated with control mAb 7 days before collagen immunization developed a rapid and marked arthritis after the second collagen challenge. Joint inflammation was first observed in control mAb-treated mice at day 30, with inflammation peaking on day 68. By contrast, DBA mice treated with MB20-16 mAb on day −7 and immunized with collagen on days 0 and 21 showed a delay in arthritis onset until day 75. This finding suggests that sufficient collagen persisted in vivo to sensitize B cells after their regeneration from the BM. However, peak inflammation remained below that of control mAb-treated littermates. Joint distortion was apparent by day 40 and was maximal by day 82 in control mAb-treated mice, but was only first observed in B cell-depleted mice on day 93 and remained below the levels observed in control mAb-treated mice. Ankylosis was first apparent by day 84, becoming apparent in most joints of half the control mAb-depleted mice by day 114. Although ankylosis was a common manifestation of severe arthritis in control mAb-treated mice; CD20 mAb-treated mice did not develop ankylosis. Using this treatment protocol, B cell depletion was associated with significant reductions in total clinical scores, inflammation, distortion, and ankylosis compared with control mAb-treated mice (p < 0.05). Inflammation and arthritis were not observed in littermates that were not immunized with collagen and minimal inflammation was observed in mice immunized only once with collagen (data not shown). Thus, B cell depletion before collagen immunization had dramatic effects on CIA induction, but arthritis nevertheless developed after the reappearance of B cells in the periphery.

### Table II. Tissue B cell depletion following collagen immunizations and CD20 mAb treatment

<table>
<thead>
<tr>
<th>Tissue</th>
<th>B Subset&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Control mAb&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Day 35</th>
<th>Days −7 and 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM:</td>
<td>Pro/pre</td>
<td>1.4 ± 0.1</td>
<td>2.3 ± 0.1</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Immature</td>
<td>0.38 ± 0.07</td>
<td>0.38 ± 0.07 (34)</td>
<td>0.06 ± 0.01 (97**)</td>
</tr>
<tr>
<td></td>
<td>Mature</td>
<td>0.41 ± 0.06</td>
<td>0.02 ± 0.01 (95**)</td>
<td>&lt;0.01 (&lt;99**)</td>
</tr>
<tr>
<td>Blood:</td>
<td>B220&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.1 ± 0.2</td>
<td>0.01 ± 0.01 (&lt;99**)</td>
<td>0.01 ± 0.01 (&lt;99**)</td>
</tr>
<tr>
<td>Spleen:</td>
<td>B220&lt;sup&gt;+&lt;/sup&gt;</td>
<td>48.5 ± 3.9</td>
<td>1.4 ± 0.2 (97**)</td>
<td>1.8 ± 0.2 (96**)</td>
</tr>
<tr>
<td></td>
<td>Mature</td>
<td>43.7 ± 2.7</td>
<td>0.31 ± 0.10 (93**)</td>
<td>0.05 ± 0.03 (99**)</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>6.1 ± 0.6</td>
<td>0.59 ± 0.12 (&lt;99**)</td>
<td>0.68 ± 0.12 (99**)</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>5.7 ± 0.8</td>
<td>0.03 ± 0.02 (&lt;99**)</td>
<td>0.01 ± 0.01 (&lt;99**)</td>
</tr>
<tr>
<td></td>
<td>Marginal zone</td>
<td>7.7 ± 0.6</td>
<td>0.02 ± 0.01 (&lt;99**)</td>
<td>0.01 ± 0.03 (&lt;99**)</td>
</tr>
<tr>
<td>Peripheral lymph node:</td>
<td>1.21 ± 0.25</td>
<td>0.42 ± 0.04 (65**)</td>
<td>0.02 ± 0.01 (98**)</td>
<td></td>
</tr>
<tr>
<td>Peritoneum:</td>
<td>B220&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.61 ± 0.07</td>
<td>0.49 ± 0.08 (20)</td>
<td>0.07 ± 0.01 (89**)</td>
</tr>
<tr>
<td></td>
<td>B1a</td>
<td>0.08 ± 0.02</td>
<td>0.05 ± 0.01 (37)</td>
<td>0.01 ± 0.01 (87**)</td>
</tr>
<tr>
<td></td>
<td>B1b</td>
<td>0.16 ± 0.02</td>
<td>0.17 ± 0.02</td>
<td>0.01 ± 0.01 (94**)</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>0.37 ± 0.03</td>
<td>0.28 ± 0.04 (24)</td>
<td>0.05 ± 0.01 (86**)</td>
</tr>
</tbody>
</table>

<sup>a</sup>The experimental procedures for collagen immunizations and mAb treatments were as described for Fig. 4 except tissue B cell numbers were determined on day 42 (n = 4 mice/value).

<sup>b</sup>B cell subsets: BM pro/pre-B (IgM<sup>-</sup>B220<sup>+</sup>), immature B (IgM<sup>-</sup>B220<sup>imm</sup>), mature B (IgM<sup>-</sup>B220<sup>imm</sup>), splenic mature (CD20<sup>-</sup>CD11b<sup>-</sup>CD22<sup>+</sup>), T1 (CD24<sup>low</sup>CD21<sup>+</sup>CD22<sup>+</sup>), T2 (CD24<sup>high</sup>CD21<sup>+</sup>CD22<sup>+</sup>), and marginal zone (CD21<sup>high</sup>CD14<sup>+</sup>CD22<sup>+</sup>); peritoneal B1a (CD5<sup>+</sup>CD11b<sup>+</sup>IgM<sup>high</sup>B220<sup>+</sup>), B1b (CD5<sup>+</sup>CD11b<sup>+</sup>IgM<sup>high</sup>B220<sup>+</sup>), and B2 (CD5<sup>+</sup>IgM<sup>low</sup>B220<sup>+</sup>).

<sup>c</sup>Values (±SEM) indicate cell numbers (×10<sup>6</sup>) present in each tissue: blood shown as cells per milliliter, and lymph nodes as pooled bilateral inguinal and axillary lymph nodes. Significant differences between sample means of mice treated with isotype control mAb and MB20-16 mAbs (*, p < 0.05; **, p < 0.01) are shown.
Prolonged B cell depletion delays CIA onset, but does not ameliorate ongoing disease

Additional experiments were conducted to determine whether prolonged B cell depletion more effectively inhibited CIA induction and if B cell depletion following arthritis onset was effective in ameliorating disease. Three groups of mice were sensitized with collagen on days 0 and 21, as in previous experiments. A control group of mice was first treated with control mAb 7 days before collagen sensitization and on days 35 and 70 after the first collagen immunization. Another group was treated with CD20 mAb on days 35 and 70 to produce a long-lasting B cell-depleted state before and after normal disease induction. In the latter two groups of mice, CD20 mAb treatment depleted >95% of circulating B cells immediately after administration, while Thy-1.2+ T cell numbers were not affected (Fig. 4). Circulating B cells reappeared in both groups of CD20 mAb-treated mice on day 100, with circulating B cell numbers returning to normal by days 133–150 and thereafter for up to 400 days. Control mAb treatments had no effect on circulating lymphocyte numbers. Similar results were obtained for independent groups of mice where blood and tissue B cell numbers were quantified on day 42 (Table II). Control mAb treatment had no effect on B cell numbers in collagen-immunized mice. CD20 mAb treatment on day 35 significantly reduced mature B cell numbers in the BM, blood, spleen, and lymph nodes by 65–99%, but there was not a significant reduction in peritoneal B cell numbers at this time. In mice treated with CD20 mAb 7 days before the first collagen immunization and on day 35, B cell numbers were significantly reduced in all tissues (86–99%).

FIGURE 5. Effect of B cell depletion on collagen-specific Ab and serum Ig levels. Mice were immunized with collagen and treated with isotype control mAb alone (○), isotype control mAb in combination with MB20-16 mAb (□), and MB20-16 mAb alone (■) as shown in Fig. 4. Serum was obtained on the indicated days, with (A) relative collagen-specific Ab levels and (B) total serum IgM and IgG levels determined by ELISA. Values represent mean (±SEM) results for 4–12 mice in each group. Significant differences between sample means of mice treated with isotype control mAb and MB20-16 mAbs only (*, p < 0.05), or isotype control and isotype control plus MB20-16 mAb (†, p < 0.05) are shown.
Inflammation was first observed in control mAb-treated mice on day 33, with inflammation peaking on day 75 (Fig. 4). Joint distortion was apparent by day 35, and was maximal by day 100. Ankylosis was apparent by day 88, and was present in most joints of control mAb-treated mice (60%) by day 133. Mice treated with MB20-16 mAb alone (Fig. 4A) had minimal effects on arthritis development. Depletion of B cells on days 35 and 70 followed collagen immunization and coincided with disease onset, albeit delayed in comparison with control mAb treatments. Depletion of B cells on days 35 and 70 followed collagen immunization and coincided with disease onset. This therapeutic approach attenuated to some extent but did not significantly ameliorate clinical arthritis, as compared with control mAb-treated littermates (Fig. 4). B cell recovery was equivalent to that observed in mice treated with CD20 mAb on days −7, 35, and 70. The time to onset of inflammation, joint distortion, and ankylosis was similar in CD20 mAb-treated (day 35 and day 70) and control mAb-treated littermates. Overall severity scores were lower for this group of CD20 mAb-treated mice during the course of disease and the incidence of ankylosis after day 100 was reduced compared with control mAb-treated mice. Thus, initiating B cell depletion before CIA induction significantly delayed arthritis onset and reduced its severity, while B cell depletion after collagen exposure had minimal effects on arthritis development.

**B cell depletion delays collagen-specific Ab production**

Because collagen-specific autoantibodies play a pivotal role in CIA pathogenesis (22, 25, 28), the effect of CD20 mAb treatment on serum Ab responses was assessed. Serum Ab levels from control and CD20 mAb-treated mice (shown in Fig. 4) were examined using isotype-specific ELISAs (Fig. 5). Collagen-specific autoantibodies were not detected in any of the mice 7 days before collagen immunization (data not shown). Control mAb-treated mice generated significant collagen-specific Ab responses by day 14 after a primary immunization on day 0 (p < 0.01, Fig. 5A). Collagen-specific IgM and IgG Ab levels were generally maximal by day 50 and slowly declined over the subsequent months. Littermates treated twice with CD20 mAb after collagen immunization generated Ab responses that reached similar, if not identical levels to those of control mAb-treated mice. The exception was decreased IgG2b collagen-specific Ab levels after day 56 compared with control mAb-treated mice. By contrast, mice treated with CD20 mAb before and after collagen immunizations generated relatively modest Ab responses until the return of B cells. Mice treated with CD20 mAb at days −7, 35, and 70 generated low, but significant (p < 0.01), levels of collagen-specific IgM, IgG1, IgG2a, and IgG2b Abs which peaked on days 42–56, but were decreased by day 85. Low-level collagen-specific Ab production is likely to have been generated by residual B cells within the peritoneal cavity (Figs. 2 and 3). By day 100, collagen-specific IgM, IgG1, IgG2a, and IgG2b Abs had increased (Fig. 5A), consistent with the predicted regeneration of mature B cells in the BM, spleen, lymph nodes, and peritoneal cavity of DBA mice, along
with the reappearance of mature B cells in the circulation on day 100 after CD20 mAb treatment (Fig. 3A). In this group of mice, serum levels of collagen-specific IgM, IgG1, and IgG2b Abs reached levels equivalent to those found in control mAb-treated littersmates by day 150. However, collagen-specific IgG2a Ab levels remained significantly lower than those found in control mAb-treated littersmates. Collagen-specific IgG3 Ab levels remained low throughout the follow-up period, but were similar to those found in control mAb-treated mice by day 200. Thus, collagen-specific Abs were observed in all mice that developed arthritis.

B cell depletion at days 35 and 70 after collagen immunization did not have a significant effect on total serum IgM, IgG1, IgG2a, IgG2b, or IgG3 levels compared with control mAb-treated mice (Fig. 5B). B cell depletion in mice treated with CD20 mAb at days −7, 35, and 70 had modest reductions in serum IgM and IgG1 levels, but more significant decreases in serum IgG2a, IgG2b, and IgG3 levels. Specifically, B cell depletion prevented the induction of high IgG2a, IgG2b, and IgG3 levels after immunizations with in Freund’s adjuvant, with IgG2a, IgG2b, and IgG3 levels remaining similar to those found in uninmunized wild-type littersmates (data not shown). Thereby, B cell depletion did not affect existing serum Ab levels, but inhibited new Ab production.

**B cell depletion inhibits the development of histological CIA**

Articular destruction in CD20 mAb-treated mice was assessed histologically. Whole ankle and ankle joints were collected 400 days after primary collagen immunizations (shown in Fig. 4) for histological evaluation (Fig. 6). No articular changes were observed in the joints of age-matched control mice that had not been immunized with collagen (Fig. 6, A and D), while the joints of control mAb-treated littersmates showed extensive synovial hyperplasia, cartilage damage, bone erosion, and ankylosis (Fig. 6, B and D). B cell depletion at days 35 and 70 tended to reduce mean histological scores compared with control mAb-treated littersmates, but the differences were not statistically significant (Fig. 6D). By contrast, histological changes observed in mice treated with CD20 mAb at days −7, 35, and 70 were reduced significantly, but not completely abrogated (Fig. 6, C and D). Thus, B cell depletion using a prevention strategy significantly inhibited articular damage, while B cell depletion following disease onset did not appear to have a significant impact on joint pathology.

**Discussion**

These studies confirm that B cells play an important role in the induction of CIA, with B cell depletion significantly delaying disease onset and severity. Unexpected, however, was the finding that B cell depletion 2 wk after a second collagen immunization and at the first indications of disease did not have a significant effect on the emergence of collagen-specific autoantibodies, or the severity of arthritis progression, as demonstrated by joint inflammation, distortion, and cartilage and bone destruction (Figs. 4–6). Equally important was the finding that B cell recovery after mAb treatment was sufficient to trigger subsequent autoantibody production in collagen-sensitized mice, with the development of joint inflammation, distortion, and cartilage and bone destruction similar to that induced in control mice with intact B cell compartments (Figs. 4–6). B cell depletion before collagen immunizations induced resistance to CIA, consistent with previous findings in mice that lack B cells constitutively or have compromised B cell development and humoral immunity (22, 23). Because CD20 mAb treatment allows the effective depletion of most B cells from adult mice with fully developed immune systems, the current experiments eliminate prior concerns that constitutive B cell absence or dysfunction affects T cell development or shaping of the immune repertoire (51). Thus, B cells are critical for the initiation of CIA, but may play a less significant role in disease progression once robust autoantibody production is initiated.

B cell depletion before collagen immunizations eliminated Ag-specific mature B cells before plasma cell differentiation, thereby reducing the generation of IgM and IgG autoantibody-secreting plasma cells (Fig. 5). Effective B cell depletion before collagen immunizations also delayed disease onset, and significantly diminished arthritis severity both clinically and histologically (Figs. 3–6). A single MB20-16 mAb treatment 7 days before collagen immunizations rapidly depleted B cells, with reappearance of peripheral B cells by days 30–60 (Fig. 3). Similarly, CD20 mAb treatments on days 35 and 70 in addition to 7 days before collagen immunizations prolonged B cell depletion, with the reappearance of tissue and circulating B cells 30–45 days after the last mAb treatment (Fig. 4, data not shown). In parallel with the return of tissue and circulating B cells, CD20 mAb-treated mice developed high anti-collagen Ab levels (Fig. 5) and developed arthritis within 35–50 days after the return of B cells, with joint inflammation and distortion, but not ankylosis (Figs. 3 and 4). It is unlikely that pathogenic B cell clones induced arthritis by escaping CD20 mAb-mediated depletion since most blood and tissue B cells were depleted by CD20 mAb treatments following collagen immunizations (Table II) and anti-collagen Ab levels in CD20 mAb-treated mice did not increase significantly until B cell reconstitution (Fig. 5). Furthermore, activated and germinal center B cells express CD20 at higher densities than follicular B cells, and are thus better targets for depletion (D. J. DiLillo, Y. Hamaguchi, Y. Ueda, J. Uchida, K. M. Haas, G. H. Kelsoe, T. F. Tedder, manuscript submitted for publication). In fact, the extent of CD20 mAb-mediated B cell depletion is not diminished by immunizations, Ag challenge, or B cell activation, so it is likely that the majority of Ag-specific B cells were depleted before and after collagen immunizations (D. J. DiLillo, Y. Hamaguchi, Y. Ueda, K. Yang, J. Uchida, K. M. Haas, G. Kelsoe, and T. F. Tedder, manuscript submitted for publication). However, peritoneal B cells were not eliminated by CD20 mAb treatment (Fig. 2, Table II) as described (37). Peritoneal B cells are thereby the likely source of the low-level anti-collagen Ab responses observed in mice treated with CD20 mAb before collagen immunizations, but did not appear to be sufficient for generating robust arthritogenic Ab responses (Fig. 5). Because a threshold level of collagen-specific IgG is required for CIA development (16, 52, 53) as observed in the current studies (Fig. 5), B cells are critical for CIA initiation.

B cell depletion 2 wk after primary plus secondary collagen immunizations did not significantly reduce collagen-specific Ab levels or arthritis (Fig. 5). Likewise, B cell depletion does not affect autoantibody generation (38) or serum Ab levels (D. J. DiLillo, Y. Hamaguchi, Y. Ueda, K. Yang, J. Uchida, K. M. Haas, G. Kelsoe, and T. F. Tedder, manuscript submitted for publication). That B cell reappearance was sufficient to trigger subsequent autoantibody production and arthritis in collagen-sensitized mice correlates with a known requirement for high levels of collagen-specific IgG autoantibodies (16, 19, 22, 25, 26, 28, 52, 53). Collagen-specific IgG1 and IgG2b levels rebounded quickly after B cell repopulation of the periphery, while collagen-specific IgG2a and IgG3 Ab levels were slower to recover. Thereby, the effect of long-term B cell depletion on arthritis severity (Fig. 4) may result in part from reduced IgG2a Ab levels (Fig. 5A) because IgG3 Abs do not interact significantly with FcYRs (54). Although autoantibodies are important for initiating joint inflammation by binding to cartilage and initiating inflammation through FcYR-dependent pathways (55), there is little agreement as to whether individual
collagen-specific autoantibody isotypes or their titers cause or correlate with disease severity in CIA (24). In fact, collagen-specific IgG1, IgG2a, and IgG2b Abs alone can induce transient arthritis in recipient mice (19, 26, 27), with collagen-reactive T cells required for normal CIA induction and progression (19). Although CIA and anti-collagen Ab-induced arthritis in the absence of B cell function exacerbates disease (19, 56), B cell depletion before or following collagen immunization did not exacerbate arthritis development, joint inflammation, distortion, or ankylosis (Figs. 3 and 4). Thereby, the role for B cells in autoantibody production and the very early stages of disease initiation is likely to be as complex as the roles that T cells play during arthritis induction and perpetuation.

A role for B cells in CIA initiation is likely to involve T cell activation in addition to autoantibody production. In support of this concept, arthritogenic collagen-specific T cells are essential for CIA induction, perpetuation, and exacerbation (19, 26). In addition, human T cell activation in human rheumatoid synovial xenotransplants is B cell dependent (11). Consistent with this, our preliminary studies indicate that CD20 mAb treatment alters the activation, cytokine production, and function of T cells and other immune cells (our unpublished observations). However, the mechanisms leading to these effects appear complex. In addition to autoantibody production, B cells have a determinative role as APCs in the elicitation of T cell-mediated immune responses (57). B cells also provide costimulatory molecules and cytokines necessary for T cell activation. For example, B cells produce multiple cytokines including IL-6, IL-10, IL-12, TNF-α, and lymphotixin, which can act as autocrine growth and differentiation factors that amplify immune responses (58, 59). IL-1, IL-6, and TNF-α are crucial in the development of CIA (60, 61), whereas loss of IL-12 or IFN-γ can increase CIA severity (62, 63). IL-1 and TNF-α are also important cytokines for arthritis development in mice given anti-collagen autoantibodies (64). Thereby, CD20 mAb treatment not only affects B cell cytokine production, but may also alter cytokine production by T cells and other immune cells. Although B cells are required for collagen-induced T cell proliferation/function in vitro and in vivo (65–67), some studies have found that primary CD4+ T cell responses can also develop in mice that genetically lack B cells (68, 69), perhaps due to constitutive B cell deficiency affecting T cell development and repertoire (51). Thus, B cell depletion before collagen immunization may disrupt both the elicitation of isotype-switched IgG autoantibodies and also influence the proliferation and activation of collagen-specific T cells. Our current studies are focused on understanding the complex cellular changes and changes in cytokine levels that induce these effects in vivo.

The results of the current study are consistent with recent findings in tight skin (Tsk) mice, a genetic model for human scleroderma (38), and Id3-deficient mice, a genetic model for human Sjögren’s disease (70). In Tsk mice, continuous B cell depletion after birth prevents autoantibody production and significantly reduces skin hyperplasia. Incomplete B cell depletion has a less dramatic therapeutic benefit in Tsk mice, something that is likely to also occur with the development of CIA. We were unable to detect B cell transcripts in the lesional skin of Tsk mice, suggesting that B cell depletion affects central immune responses rather than removing B cells from the skin where disease is observed. Similarly, B cell presence or deletion from joints may not influence CIA because only ~30% of patients with RA develop extralymphoid follicles containing B cells within their joints. By contrast, B cell depletion in Tsk mice reduces IL-4, IL-6, IL-10, and TGF-β transcription levels, while significantly increasing TNF-α and IFN-γ production when started early in the course of disease (38). However, prolonged B cell depletion in adult Tsk mice with disease does not affect disease severity, RF levels, autoantibody levels, or serum Ig levels. Consistent with these findings, serum Ig levels in RA and lymphoma patients mostly remain within normal ranges following CD20 mAb treatment (47, 71–73). Although antibacterial Ab levels are also preserved in RA patients following CD20 mAb treatment, IgM RF and anti-CCP Ab levels are reported to decrease significantly (72). This suggests that collagen-specific autoantibodies, anti-bacterial Abs, and the autoantibodies generated in Tsk mice may be derived from different plasma cell subsets when compared with RF and anti-CCP autoantibodies in humans. Alternatively, the immunosuppressive drugs normally administered with CD20 mAbs in patients may indirectly affect autoantibody production. Regardless, B cell depletion has the most significant therapeutic benefits in multiple models of autoimmunity when conducted during early disease development.

Although human RA is mainly an indolent, relapsing and remitting disease and CIA develops quickly, CIA models have value for elucidating the mechanisms by which arthritis develops in humans and how B cell depletion may be an effective therapy. Some of the findings from CD20 mAb therapy in CIA parallel observations made in patients with RA being treated with rituximab. For example, the return of circulating B cells after depletion was sufficient to trigger subsequent autoantibody production and arthritis development in collagen-sensitized mice (Figs. 3–5). In patients with RA, rituximab therapy significantly diminishes ongoing joint inflammation (30, 72), with B cell recovery often accompanied by a recrudescence of disease activity (31, 72). Some rituximab-treated patients with a beneficial therapeutic response also show a decrease in serum IgG and IgA RF levels and to a lesser extent, IgG anti-CCP Ab levels, with subsequent rise in titer after B cell repopulation heralding a disease relapse (72). Ag-specific induction of CIA may mimic the environmental triggers of RA, which may include citrullinated protein Ags. The current results indicating that B cells play critical roles in the initiation of CIA after autoantibody production suggests that B cell depletion may reduce the activity and progression of disease in a subset of patients with RA whose disease may be driven by continuous waves of self-Ag stimulation. Because it is often difficult to begin treatment before Ag exposure and disease onset, B cell depletion alone may be insufficient to produce long-lasting robust clinical responses in the setting of autoimmune disease. Nonetheless, it must be taken into account that patients with autoimmune disease are normally given CD20 mAb in combination with other immunosuppressive drugs (30). In RA, rituximab is often administered in combination with methotrexate, which may produce synergistic clinical effects. Thereby, the combination of B cell depletion and immunosuppression of T cell activation may lead to a more significant outcome than B cell depletion alone. For example, B cell depletion may block nascent autoantibody development and autoantigen-specific T cell activation, while immunosuppression may arrest the clonal expansion of existing autoreactive lymphocytes, thereby ameliorating disease progression. Unfortunately, pharmacologically relevant doses of methotrexate do not suppress collagen-induced arthritis in DBA mice (74–77). Thus, B cell-directed therapies may not only require the effective elimination of all mature B cells, but an optimal therapy for disease quiescence may require continuous B cell depletion because environmental triggers are likely to persist indefinitely. Although multiple factors in addition to B cells contribute to arthritis pathogenesis, understanding the contributions of B cells to disease may allow the development of complementary therapeutic strategies for both short-term benefit and long-term management (78).