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

Rheumatoid arthritis is a systemic autoimmune disease. B cells are likely to play a critical role in arthritis pathogenesis, although it is unclear whether they are necessary for disease induction, autoantibody production, or disease progression. To assess the role of B cells in inflammatory arthritis, B cells were depleted using mouse anti-mouse CD20 mAbs in a mouse model of collagen-induced arthritis. CD20 mAbs effectively depleted mature B cells from adult DBA-1 mice. When B cells were depleted using CD20 mAbs before collagen immunization, there was a delay in disease onset and autoantibody production, with significantly diminished severity of arthritis both clinically and histologically. B cell depletion further delayed disease onset if initiated before, as well as after, collagen immunization. However, in both cases, the eventual reappearance of peripheral B cells triggered autoantibody production and the subsequent development of arthritis in collagen-sensitized mice. By contrast, B cell depletion after collagen immunizations did not have a significant effect on arthritis progression or severity. Thus, disease symptoms were only induced when peripheral B cells and their autoantibody products were present in collagen-immunized mice, documenting a critical role for B cells during the elicitation phase of collagen-induced arthritis. These studies suggest that B cell depletion strategies will be most effective when initiated early in the development of inflammatory arthritis, with sustained B cell depletion required to inhibit the production of isotype-switched pathogenic Abs and the evolution of joint inflammation and destruction.

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**Abbreviations used in this paper:** RA, rheumatoid arthritis; RF, rheumatoid factor; CCP, cyclic citrullinated peptide; CIA, collagen-induced arthritis; BM, bone marrow.

Department of Immunology and Department of Medicine, Duke University Medical Center, Durham, NC 27710; and Department of Biological Sciences, University of Wisconsin, Milwaukee, WI 53201.

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2 E.W. St.Clair and T.F. Tedder contributed equally to this study and share first authorship.

3 Address correspondence and reprint requests to Dr. Thomas F. Tedder, Box 3010, Department of Immunology, Duke University Medical Center, Durham, NC 27710. E-mail address: thomas.tedder@duke.edu.

4 Abbreviations used in this paper: RA, rheumatoid arthritis; RF, rheumatoid factor; CCP, cyclic citrullinated peptide; CIA, collagen-induced arthritis; BM, bone marrow.
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 (24), implying that B cells play a pivotal role in the disease.

**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 (Caltag Laboratories); and CD1d (1B1), CD5 (53-7.3), CD11b (M1/70) CD21 (7G6), and CD24 (M1/69) mAbs from BD Pharmingen. Isotype-specific secondary Abs 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 (4°C) PBS was injected into the peritoneum of sacrificed mice followed by gentle massage of the abdomen. Viable cells were counted using a hemocytometer.

Table I. Tissue B cell depletion following CD20 mAb treatment

<table>
<thead>
<tr>
<th>Tissue</th>
<th>B Subset</th>
<th>Control mAb</th>
<th>CD20 mAb</th>
<th>% Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM:</td>
<td>Pro/pre</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Immature</td>
<td>0.42 ± 0.01</td>
<td>0.33 ± 0.03</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Mature</td>
<td>1.1 ± 0.1</td>
<td>0.13 ± 0.02</td>
<td>88**</td>
</tr>
<tr>
<td>Blood:</td>
<td>B220b</td>
<td>1.4 ± 0.3</td>
<td>0.03 ± 0.01</td>
<td>98**</td>
</tr>
<tr>
<td>Spleen:</td>
<td>B220b</td>
<td>51.2 ± 1.6</td>
<td>1.9 ± 0.3</td>
<td>96**</td>
</tr>
<tr>
<td></td>
<td>Mature</td>
<td>32.4 ± 1.7</td>
<td>1.2 ± 0.2</td>
<td>96**</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>7.8 ± 0.1</td>
<td>0.38 ± 0.04</td>
<td>87**</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>8.8 ± 1.1</td>
<td>0.03 ± 0.01</td>
<td>&gt;99**</td>
</tr>
<tr>
<td>Peritoneum:</td>
<td>B220b</td>
<td>0.56 ± 0.13</td>
<td>0.05 ± 0.02</td>
<td>91*</td>
</tr>
<tr>
<td></td>
<td>B220b</td>
<td>1.11 ± 0.05</td>
<td>1.09 ± 0.11</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>B1a</td>
<td>0.29 ± 0.02</td>
<td>0.19 ± 0.03</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>B1b</td>
<td>0.48 ± 0.02</td>
<td>0.61 ± 0.07</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>0.38 ± 0.06</td>
<td>0.33 ± 0.06</td>
<td>13</td>
</tr>
</tbody>
</table>

* B cell subsets were: BM: pro-pre-B (IgM+ B220low), immature B (IgM+ B220mid), mature B (IgM+ B220high); spleen: mature (CD24+ CD21+ B220+), T1 (CD24highCD21 B220+), T2 (CD24highCD21+ B220+), and marginal zone (CD24high CD1d+ B220+); peritoneal B1a (CD5+ CD11b+ IgMhighB220mid), B1b (CD5+ CD11b+ IgMhighB220mid), and B2 (CD5+ IgMhigh B220low).

Values (±SEM) indicate cell numbers (×10⁶) present in mice 7 days after mAb treatment (250 μg; n ≥ 4/value): blood shown as cells per milliliter, and lymph node pool as pooled bilateral inguinal and axillary lymph nodes. Significant differences between means are indicated; *, p < 0.05; **, p < 0.01.

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 tuberculosi s (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 appearance; 1, slight inflammation and redness; 2, severe erythema and swelling affecting the entire paw; 3, deformed paw or joint with ankylosis, joint rigidity, and loss of function as described (42, 43). Overall scores were calculated for each mouse by summing the parameter scores in each mouse limb with a maximum score of 12 per mouse. In addition, each mouse was also evaluated as described (44, 45) and given a “total clinical score” as follows. Redness and swelling (inflammation) were quantified according to a scoring system: 0, normal; 1, mild; 2, moderate; 3, severe. Swollen digits were noted but paws were only considered arthritic when the entire paw was inflamed for two consecutive evaluations, with arthritis onset recorded as the first date 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 in each mouse limb (maximum total clinical score of 36/mouse), although 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
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](http://www.jimmunol.org)
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 1/9262 g/ml as assessed by flow cytometry (data not shown). When used at 10/9262 g/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 (250/9262 g/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⁺ B220low pro/pre-B cells and IgM⁺ B220⁺ 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, T1, T2, mature, and marginal zone B cell depletion in spleen (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⁺ 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 gave 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 of the control mAb-treated 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</th>
<th>Control mAb</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⁺</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⁺</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>7.6 ± 0.7</td>
<td>0.02 ± 0.01 (99**)</td>
<td>0.01 ± 0.03 (&lt;99**)</td>
</tr>
<tr>
<td>Peripheral lymph node:</td>
<td>B220⁺</td>
<td>1.21 ± 0.25</td>
<td>0.42 ± 0.04 (65*)</td>
<td>0.02 ± 0.01 (98**)</td>
</tr>
<tr>
<td>Peritoneum:</td>
<td>B220⁺</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>

* 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).

* B cell subsets were: BM pro/pre-B (IgM⁺ B220⁻), immature B (IgM⁺ B220⁻), mature B (IgM⁺ B220⁺), spleen mature (CD24⁺ CD21⁺ B220⁺), T1 (CD24⁺ CD21⁻ B220⁺), T2 (CD24⁺ CD21⁻ B220⁻), and marginal zone (CD21⁺ CD14⁻ B220⁻); peritoneal B1a (CD5⁺ CD11b⁺ IgM⁺ B220⁺), B1b (CD5⁺ CD11b⁻ IgM⁺ B220⁺), and B2 (CD5⁻ IgM⁺ B220⁻).

* Values (±SEM) indicate cell numbers (×10⁶) present in each tissue: blood shown as cells per milliliter, and lymph nodes as pooled bilateral inguinal and axial 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. The other group was treated with CD20 mAb at days -7, 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%).
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, and MB20-16 mAb alone as shown in Fig. 4. Paw joint tissue sections were obtained 400 days after the initial collagen immunization and stained with H&E. Joints representing average histological scores are shown for (A) untreated mice, (B) mice treated with isotype control mAb alone, and (C) mice treated with MB20-16 mAb alone (original magnification, ×50). D, Histological sections were blindly scored on a scale of 0–5 for the presence of synovial hyperplasia, cartilage damage, bone erosion, and ankylosis. Values represent means (±SEM) from three to six mice of each group. Significant differences between groups of mice are indicated (*, p < 0.05; **, p < 0.01).

**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 Ab levels 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 littermates by day 150. However, collagen-specific IgG2a Ab levels remained significantly lower than those found in control mAb-treated littermates. 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 Freund’s adjuvant, with IgG2a, IgG2b, and IgG3 levels remaining similar to those found in uninimmunized wild-type littermates (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 observed 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 littermates 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 littermates, 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 Abs 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 FcγRs (54). Although autoantibodies are important for initiating joint inflammation by binding to cartilage and initiating inflammation through FcγR-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 lymphotoxin, 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 anti-bacterial 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).
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References


