The Peritoneal Cavity Provides a Protective Niche for B1 and Conventional B Lymphocytes during Anti-CD20 Immunotherapy in Mice

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The Peritoneal Cavity Provides a Protective Niche for B1 and Conventional B Lymphocytes during Anti-CD20 Immunotherapy in Mice

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Although anti-CD20 immunotherapy effectively treats human lymphoma and autoimmune disease, the in vivo effect of immunotherapy on tissue B cells and their subsets is generally unknown. To address this, anti-mouse CD20 mAbs were used in a mouse model in which the extent and kinetics of tissue B cell depletion could be assessed in vivo. CD20 mAb treatment depleted most mature B cells within 2 days, with 95–98% of B cells in the bone marrow, blood, spleen, lymph nodes, and gut-associated lymphoid tissues depleted by day 7, including marginal zone and follicular B cells. The few spleen B cells remaining after CD20 mAb treatment included pre-B, immature, transitional, and some B1 B cells that expressed CD20 at low levels. By contrast, peritoneal cavity B cells expressed normal CD20 densities and were coated with CD20 mAb, but only 30–43% of B1 cells and 43–78% of B2 cells were depleted by day 7. Spleen B cells adoptively transferred into the peritoneal cavity were similarly resistant to mAb-induced depletion, while transferred B cells that had migrated to the spleen were depleted. However, peritoneal B1 and B2 cells were effectively depleted in mAb-treated wild-type and C3-deficient mice by thioglycolate-induced monocyte migration into this otherwise privileged niche. Inflammation-elicited effector cells did not promote peritoneal cavity B cell depletion in FcR-deficient mice treated with CD20 mAb. Thus, the majority of CD20+ cells and B cell subsets within lymphoid tissues and the peritoneum could be depleted efficiently in vivo through Fc-dependent, but C-independent pathways during anti-CD20 immunotherapy. The Journal of Immunology, 2005, 174: 4389–4399.

Chimeric or radiolabeled mAb-based therapies directed against the CD20 cell surface molecule of mature B lymphocytes (1) represent an effective in vivo treatment for non-Hodgkin’s lymphoma (2–9). CD20 mAb therapy also ameliorates the manifestations of rheumatoid arthritis, systemic lupus erythematosus, idiopathic thrombocytopenic purpura, and hemolytic anemia, as well as other immune-mediated diseases (10–12). The molecular and cellular mechanisms for B cell depletion and tumor regression in humans following CD20 mAb treatment have remained uncertain (13), but are thought to include disrupted progression through cell cycle (1), B cell apoptosis (14, 15), the depletion of B cells through initiation of C-dependent cytotoxicity (16–20), and Ab-dependent cytotoxicity (21). However, mouse anti-mouse CD20 mAbs (22) provide a preclinical model of CD20 mAb immunotherapy amenable to mechanistic studies and genetic manipulation. This mouse model demonstrates that the innate mononuclear phagocytic network and IgG2a isotype CD20 mAbs, including the MB20-11 mAb, are primarily responsible for blood and spleen B cell depletion through FcR-dependent and C-independent mechanisms (23). Although monocytes were the primary, if not exclusive, effector cells for B cell removal from the blood and spleen of CD20 mAb-treated mice, their role in B cell depletion from other lymphoid and nonlymphoid tissues is unknown. Thus, these mouse anti-mouse CD20 mAbs provide an effective tool for assessing selective lymphocyte depletion in vivo through innate effector mechanisms.

It is difficult to carry out mechanistic studies in humans undergoing immunotherapy with CD20 mAbs. For example, human studies primarily measure changes in blood B cells, which represent <2% of B cells outside of the bone marrow. Thus, it is difficult to accurately ascertain the clinically relevant effects of CD20 therapies on the majority of human B cells, which are found in peripheral lymphoid tissues. Moreover, it has not been determined whether all B cells or only specific B cell subpopulations are affected in humans. Because mouse CD20 is B cell specific and is first expressed during the pre-B to immature B cell transition as in humans (22), studies in mice allow a detailed analysis of the effects and kinetics of CD20 mAb treatment on B cells in a variety of lymphoid tissues and the peritoneal cavity. Moreover, this model system allowed the identification of B cell subpopulations within different tissues that are resistant to mAb-mediated depletion and innate effector mechanisms.

Materials and Methods

Antibodies

Mouse anti-mouse CD20-specific mAbs were as described (22). Other mAbs included: CD19 mAb MB19-1 (24); B220 mAb RA3-6B2; Thy-1.2 mAb (Caltag Laboratories); and CD5 (53-7.3), CD11b (M1/70), CD21 (7G6), CD24 (M1/69), and CD1d (1B1) mAbs from BD Pharmingen. Goat anti-mouse Ig, IgM, or isotype-specific secondary Abs were from Southern Biotechnology Associates.
Immunofluorescence analysis and immunohistology

Single cell suspensions of bone marrow (bilateral femurs), spleen, peripheral lymph node (paired axillary and inguinal), mesenteric lymph node (superior mesenteric), and Peyer’s patch lymphocytes were generated by gentle dissection. Intraepithelial and lamina propria lymphocytes were isolated by modification of a previously described protocol (25). 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, with relative lymphocyte percentages determined by flow cytometry analysis. Blood erythrocytes were lysed after immunofluorescence staining using FACS lysing solution (BD Biosciences).

Single cell leukocyte suspensions were stained on ice using predetermined optimal concentrations of each Ab for 20–60 min, and fixed as described (24, 26). Cells with the light scatter properties of lymphocytes were analyzed by two- to four-color immunofluorescence staining, with FACSscan or FACSCalibur flow cytometer analysis (BD Biosciences). Background staining was determined using unreactive control mAbs (Caltag Laboratories) with gates positioned to exclude ≥98% of the cells. Serial frozen sections of spleen were fixed in acetone and stained with FITC-labeled anti-B220 mAb and PE-labeled anti-Thy-1.2 mAb at predetermined optimal dilutions in saline containing 2% FCS for 30 min at room temperature. For CD20 mAb titration determinations, serum (diluted 1/50 to 1/400) from mice given MB20-11 mAb (250 μg) i.v. on day 0 was used for indirect immunofluorescence staining of splenocytes. Known concentrations of MB20-11 mAb were used in parallel to generate a standard curve for determining MB20-11 mAb levels in each sample. Serum obtained from the mice before treatment served as negative controls.

Mice and immunotherapy

Wild-type C57BL/6 mice were used, unless indicated otherwise. CD20−/− mice bred onto a C57BL/6 background for 12 generations were as described (22). Human CD19 transgenic (hCD19TG) mice were as described (24). FeCr common γ-chain-deficient (FeCrγ−/−) mice (B6.129P2, Enemy [m]) were from Taconic Farms. C3−/− mice (provided by M. Carroll, Center for Blood Research, Boston, MA) were as described (27). Sterile anti-mouse CD20 and isotype-matched control mAbs in 200 μl of PBS were injected through lateral tail veins, as described (23). The transient decrease in circulating B cell numbers observed 1 h after control mAb treatment is a physiologic consequence of the mouse being handled, injected with mAb, and having blood harvested. Ab doses in humans and mice were compared using the Oncology Tool Dose Calculator (www.fda.gov/cder/cancer/animalframe.htm). For thioglycolate-induced peritonitis, 1 ml of thioglycolate solution (3% w/v, Sigma-Aldrich) was injected i.p. into wild-type mice. Mice were housed in a pathogen-free barrier facility and used at 6–8 wk of age. The Duke University Animal Care and Use Committee approved all studies.

Peritoneal B cell depletion assays

Splenocytes from CD20−/− and wild-type mice were labeled with 0.01 and 0.1 μM Vybrant carboxyfluorescein diacetate, succinimidyl ester, respectively (CFSE; Molecular Probes), according to the manufacturer’s instructions. CFSE-labeled wild-type splenocytes (2 × 107) were mixed with equal numbers of CFSE-labeled CD20−/− splenocytes and injected into the peritoneal cavity of wild-type mice before i.v. injection of either MB20-11 or control mAbs. By day 2, 8–15% of labeled cells were recovered from the peritoneum, while ~5% of the labeled cells were recovered on day 7.

Statistical analysis

All data are shown as means ± SEM. The significance of differences between sample means was determined using Student’s t test.

Results

CD20 mAb reactivity with B cells in vivo

The kinetics of mouse anti-mouse CD20 mAb binding to B cells in vivo was assessed using a panel of mouse anti-mouse CD20 mAbs that have been previously described (22). IgG2a isotype CD20 mAbs, including the MB20-11 mAb, are most effective in depleting blood and spleen B cells in vivo. The ability of anti-CD20 mAbs to bind CD20 on tissue B cells was therefore assessed in vivo using the MB20-11 mAb at 10 μg/mouse with i.v. administration. One hour after mAb treatment, mature B cells were depleted from the bone marrow and circulation, while B220+ cells from the spleen and peritoneal cavity were coated with similar levels of MB20-11 mAb (Fig. 1A). By contrast, lymph node B cells were coated with MB20-11 mAb at ~10-fold lower levels relative
FIGURE 2. Bone marrow B cell depletion after CD20 mAb treatment.

A. Representative B cell depletion 7 days following MB20-11 or isotype-matched control mAb (250 μg) treatment of mice, as determined by two-color immunofluorescence staining of cells with the light scatter properties of lymphocytes. Bar graphs indicate B220+ cells within the bilateral femurs of mAb-treated mice. B. Numbers of pre/pro (IgM+ B220low), immature (IgM+ B220medium), and mature (IgM+ B220high) B cells following MB20-11 (●) or control (○) mAb treatment. The value shown after time 0 represents data obtained at 1 h. A and B. Differences between means are indicated: *, p < 0.05; **, p < 0.01. C. Numbers of each B220+ B cell subset, as in B, 7 days after MB20-11 mAb treatment at the indicated doses (≥2 mice per data point). Values represent percentages of B cells in each subset, as determined by two-color immunofluorescence staining of cells with the light scatter properties of lymphocytes. Bar graphs indicate B220+ cells within the bilateral femurs of mAb-treated mice. D. Numbers of pre/pro (IgM+ B220low), immature (IgM+ B220medium), and mature (IgM+ B220high) B cells following MB20-11 (●) or control (○) mAb treatment. The value shown after time 0 represents data obtained at 1 h. E. Representative mature bone marrow B cell depletion 7 days after MB20-11 or control mAb treatment of FcRγ−/− or C3−/− littermates. Bar graphs represent mean IgM+ B220high (mature) B cells per 5 mice per group, with significant differences between sample means indicated: *, p < 0.05; **, p < 0.01.

CD20 mAb t1/2 in vivo

The extent that B cell expression of CD20 affected circulating MB20-11 mAb levels was assessed using CD20−/− and wild-type littermates. One day after mAb administration (250 μg, i.v.), the serum concentration of free MB20-11 mAb was ~50 μg/ml in both wild-type and CD20−/− littermates, with a calculated t1/2 of 4.6 days in wild-type mice (Fig. 1B). The MB20-11 mAb had a t1/2 of 6.8 days in CD20−/− mice. This is consistent with the reported 5- to 8-day t1/2 of IgG2a mAbs in mice (28, 29). Similarly, the serum t1/2 for Rituximab is 4.4 days in humans (30). Thus, B cell expression of CD20 hastens MB20-11 mAb clearance, although free mAb persists in the serum of both wild-type and CD20−/− littermates for extended periods of time.

Bone marrow B cell depletion

Consistent with its long t1/2 in vivo, the MB20-11 mAb had potent and long-lasting effects on bone marrow B cells. B220+ cells were reduced by ~30% when the MB20-11 mAb was given to wild-type mice at 250 μg/mouse, with elimination of the majority of mature IgM+ B220high B cells (Fig. 2A and Table I). Mature B cell depletion was observed by 1 h after MB20-11 mAb treatment, with the majority of recirculating B cells depleted by day 2 (Fig. 2B). Remarkably, mature B cells were eliminated from the bone marrow for up to 60 days following mAb treatment. Mature B cells were significantly depleted from the bone marrow by as little as 1 μg of MB20-11 mAb (Fig. 2C). By contrast, significant depletion of IgM+ B220low pro/pre-B cells and IgM+ B220medium immature B cells was not observed at any dose or time point after MB20-11 mAb treatment. However, this was expected as the majority of pro/pre- and immature mouse B cells express little, if any, cell surface CD20 (22). After 7 days of MB20-11 mAb treatment, there was a small subpopulation of CD20−/−IgM+ B220+ cells with surface-bound MB20-11 mAb with an overall decrease in the frequency of IgM+ B cells (Fig. 2D). Mature B cell depletion from
Table 1. Tissue B cell depletion following CD20 mAb treatment

<table>
<thead>
<tr>
<th>Tissue</th>
<th>B Subseta</th>
<th>Control mAb</th>
<th>MB20–11 mAb</th>
<th>% Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro/pre</td>
<td>2.7 ± 0.3 (17)</td>
<td>3.2 ± 0.5 (20)</td>
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<tr>
<td>Immature</td>
<td>1.9 ± 0.2 (17)</td>
<td>1.6 ± 0.2 (20)</td>
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<tr>
<td>Mature</td>
<td>2.6 ± 0.3 (17)</td>
<td>0.06 ± 0.01 (20)</td>
<td>98**</td>
<td></td>
</tr>
<tr>
<td>Blood:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B220</td>
<td>4.6 ± 0.4 (9)</td>
<td>0.08 ± 0.02 (12)</td>
<td>98**</td>
<td></td>
</tr>
<tr>
<td>Splenic</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>T1</td>
<td>4.9 ± 1.0 (6)</td>
<td>0.62 ± 0.09 (6)</td>
<td>87**</td>
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<td>T2</td>
<td>2.5 ± 0.7 (6)</td>
<td>0.01 ± 0.01 (6)</td>
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<tr>
<td>Marginal zone</td>
<td>1.6 ± 0.5 (6)</td>
<td>0.01 ± 0.01 (6)</td>
<td>&gt;99**</td>
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<tr>
<td>B1a</td>
<td>1.3 ± 0.4 (3)</td>
<td>0.53 ± 0.08 (3)</td>
<td>60</td>
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<tr>
<td>CD21⁺CD24⁻B220⁺</td>
<td>2.0 ± 0.4 (6)</td>
<td>1.4 ± 0.2 (6)</td>
<td>32</td>
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<td>Peripheral LN:</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B220</td>
<td>1.1 ± 0.1 (6)</td>
<td>0.09 ± 0.02 (6)</td>
<td>95**</td>
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<td>Mesenteric LN:</td>
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<tr>
<td>B220</td>
<td>3.1 ± 0.8 (4)</td>
<td>0.10 ± 0.03 (4)</td>
<td>97**</td>
<td></td>
</tr>
<tr>
<td>Peyer’s patch:</td>
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<td></td>
</tr>
<tr>
<td>B220⁺</td>
<td>0.7 ± 0.2 (4)</td>
<td>0.07 ± 0.02 (4)</td>
<td>91**</td>
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<td>Peritoneum:</td>
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<tr>
<td>B220⁺</td>
<td>1.8 ± 0.3 (4)</td>
<td>0.75 ± 0.09 (15)</td>
<td>59**</td>
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<td>B1a (day 7)</td>
<td>0.40 ± 0.07 (14)</td>
<td>0.27 ± 0.04 (15)</td>
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<td>B1a (day 28)</td>
<td>0.73 ± 0.12 (5)</td>
<td>0.19 ± 0.09 (4)</td>
<td>74*</td>
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<td>B1a (day 7)</td>
<td>0.41 ± 0.10 (10)</td>
<td>0.29 ± 0.05 (10)</td>
<td>30</td>
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<tr>
<td>B2 (day 7)</td>
<td>0.94 ± 0.23 (14)</td>
<td>0.21 ± 0.04 (15)</td>
<td>78**</td>
<td></td>
</tr>
<tr>
<td>B2 (day 28)</td>
<td>2.3 ± 0.3 (5)</td>
<td>0.26 ± 0.06 (4)</td>
<td>89**</td>
<td></td>
</tr>
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a B cell subsets were: bone marrow pro/pre-B (IgM⁺ B220⁺), immature B (IgM⁺ B220⁻), spleen mature (CD24⁺CD21⁺ B220⁺), T1 (CD24⁺CD21⁺ B220⁺), T2 (CD24⁺CD21⁻ B220⁺), marginal zone (CD21⁻CD19⁺ B220⁺), and peritoneal B (CD5⁻CD11b⁻ IgM⁺ B220⁺), B1b (CD5⁺CD11b⁺ IgM⁺ B220⁺), and B2 (CD5⁺ IgM⁺ B220⁺); LN, lymph node.

b Values (± SEM) indicate cell numbers present in mice 7 days after mAb treatment (250 μg). The number of mice examined is indicated in parentheses. Significant differences between means are indicated: *, p < 0.05; **, p < 0.01.

the bone marrow was also obtained using the MB20-1, -2, and -14 (IgG1); MB20-6 and -16 (IgG2a); and MB20-7, -8, and -18 (IgG2b) mAbs (data not shown), although the degree of B cell depletion was greatest with IgG2a > IgG1 > IgG2b mAbs, as previously described for blood B cell depletion (22). The role of FcR in IgM⁺ B220⁺ B cell depletion was therefore assessed using FcR common γ-chain-deficient (FcRγ⁻/⁻) mice that lack high affinity FcγRI and low affinity FcγRII (31). Mature B220⁺ B cells were not significantly depleted in FcRγ⁻/⁻ mice, while most IgM⁺ B220⁺ B cells were depleted in C3⁻/⁻ mice (data not shown). CD20 mAb-induced clearance was durable for 60 days before B220⁺ B cells were found in rare and small lymphoid clusters that included Thy-1.2⁻ T cells. Rare B220⁺ B cells were scattered throughout T cell-rich regions of the spleen, although these cells predominantly had a nonlymphoid phenotype suggesting that they were either plasmablasts or dendritic cells (data not shown). The remaining B220⁺ cells were also phenotypically distinct from normal spleen B cells (Fig. 4B), and were reminiscent of those found in bone marrow, IgM⁺ B220⁺ cells (mature B cell-like), IgM⁺ B220⁻ cells (immature B cell-like), IgM⁺ B220⁻ cells (pro/pre-B cell-like), and an IgM⁻ subpopulation that expressed B220 at very low levels. The majority of mature CD21⁺ heat-stable Ag (CD24⁺) B220⁺ B cells and marginal zone B cells were also eliminated by MB20-11 mAb treatment (Fig. 4, C and D, and Table I). There was also a significant reduction in transitional CD21⁺CD24⁻/⁻ B220⁺ T1 cells with the virtual elimination of CD21⁺CD24⁻/⁻ B220⁻ T2 cells. As a result, there was a dramatic increase in the frequency of CD21⁺CD24⁻ B220⁺ B cells (Fig. 4D), although this is normally only a very minor spleen B cell subpopulation (Table I). Consistent with an immature phenotype, most B220⁺ B cells expressed CD19 at levels lower than mature B cells found in control mAb-treated mice (Fig. 4D) and did not bear cell surface CD20 or MB20-11 mAb (Fig. 4E). The small B cell subpopulation (<20%) that was CD20⁻/⁻ and bore cell surface MB20-11 mAb primarily had a T1 phenotype (data not shown). There was little difference between mature, T1, T2, and marginal zone B cell depletion over a range of
CD20 mAb concentrations, indicating similar sensitivities to CD20 mAb treatment (Fig. 4F). By contrast, spleen CD5⁺B220low B1a cells were far less sensitive to CD20 mAb treatment and were only reduced 60% following MB20-11 mAb treatment at 250 μg (Table I). Human CD19TG mice were also examined after MB20-11 mAb treatment because B1a cells can represent ~30% of their spleen B cells (24). Spleen B1a cells were depleted by only 70% following MB20-11 mAb treatment of CD19TG mice on day 7, while mature conventional B cells were depleted by 95% (Fig. 4G). Thus, the few B cells remaining in the spleens of MB20-11 mAb-treated mice were predominantly CD24⁺CD21⁻CD20⁻IgM⁺CD19+cells and B1a cells.

The peritoneal cavity provides a protective environment for mAb-coated B1 and B2 cells

In contrast with other tissues, the MB20-11 mAb did not effectively deplete peritoneal B220⁺B1a cells when given i.v. at 250 μg (Fig. 5A and Table I). Although the CD5⁻IgMlowB220high subset of conventional (B2) cells was reduced the most by MB20-11 mAb treatment by day 7, CD5⁺CD11b⁺IgM⁺B220low B1a cells and CD5⁻CD11b⁻IgM⁺B220low B1b cells were not significantly affected (Fig. 5B). Despite their resistance to CD20 mAb treatment, peritoneal B1 and B2 cells expressed CD20 at nearly wild-type levels, and their cell surface CD20 was saturated with bound MB20-11 mAb at nearly wild-type levels, and their cell surface CD20 was saturated with bound MB20-11 mAb in vivo (Fig. 5C). Furthermore, the increased proportion of peritoneal B1a and B1b cells relative to B2 cells following MB20-11 mAb treatment resulted in an increase in overall IgM expression by peritoneal B cells (Fig. 5C). Similar results were also obtained using the MB20-1, -2, and -14 (IgG1); MB20-6 and -16 (IgG2a); and MB20-7, -8, and -18 (IgG2b) mAbs (data not shown), although the degree of B cell depletion was greatest with IgG2a > IgG1 > IgG2b mAbs, as previously described for spleen B cell depletion (22). Consistent with this, peritoneal B1 and B2 cell numbers were not altered in MB20-11 mAb-treated FcR⁺/⁻ mice, although peritoneal B cell depletion in C3⁻/⁻ mice was similar to what was observed in wild-type mice (Fig. 5D). However, both B1a
and conventional B cells within the peritoneum were significantly reduced by prolonged MB20-11 mAb treatment on days 28–58 (Fig. 5, A–G, and Table I). Although the relative ability of MB20-11 mAb to deplete peritoneal B cells appeared to vary in the different sets of mice used in different experiments (Fig. 5, A, B, D, and E), this predominately reflects the normal variability in numbers of peritoneal B cells harvested from individual mice in each group. Because the values shown in Table I represent large numbers of mice, these results should be considered the most representative consensus measurements. Nonetheless, peritoneal B1 and B2 cell depletion after CD20 mAb treatment was much less efficient than B cell depletion from lymphoid tissues.

**Peritoneal B1 and B2 cell depletion**

The route of mAb delivery did not affect peritoneal B cell clearance because i.p. (Fig. 6A) or i.p. plus i.v. (data not shown) MB20-11 mAb administration did not accelerate peritoneal B1 or B2 cell depletion by day 7. However, i.p. MB20-11 mAb treatment effectively depleted spleen B cells. Whether peritoneal B cell resistance to CD20 mAb treatment was environmental or due to intrinsic properties was assessed by introducing spleen B2 cells into the peritoneal cavity of MB20-11 mAb-treated mice. Spleen B cells served as internal controls because they are not affected by CD20 mAb treatment (23). Similarly, the relative frequencies of CFSE<sup>+</sup> B220<sup>−</sup> splenocytes served as internal controls for relative numbers of wild-type or CD20<sup>−/−</sup> splenocytes injected into each mouse. By day 2, the relative frequencies of peritoneal CFSE<sup>+</sup> wild-type and CD20<sup>−/−</sup> B

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**FIGURE 4.** Spleen B cell subsets present following CD20 or control mAb (250 μg, 7-day) treatment. A, Spleen sections from MB20-11 (middle panel; right panel is higher magnification) or control (left panel) mAb-treated mice stained with FITC-labeled B220 and PE-labeled Thy-1.2 mAbs. Yellow regions indicate the overlapping localization of B220<sup>+</sup> and Thy-1.2<sup>+</sup> cells (bar length = 100 μm). B, Spleen B cells remaining after MB20-11 mAb treatment. To allow rare B cell visualization, 200,000 cells are shown for MB20-11 mAb-treated mice, while 10,000 cells are shown for control mAb-treated mice. C, Marginal zone (MZ) B cells defined by high CD1d and CD21 expression. D, T1, T2, and mature (M) B220<sup>−</sup> B cells in MB20-11 or control mAb-treated mice defined by relative heat-stable Ag (CD24) and CD21 densities. Right panel, Demonstrates CD19 expression by B220<sup>−</sup> cells following MB20-11 mAb treatment. The negative control (Neg C) histogram shows CD19 staining by B220<sup>−</sup> splenocytes from MB20-11 mAb-treated mice. The positive control (Pos C) histogram shows CD19 staining by B220<sup>−</sup> splenocytes from control mAb-treated mice. E, CD20 and IgM expression by B220<sup>−</sup> splenocytes. Left panels, Splenocytes stained for MB20-11 mAb binding in vivo using IgG2a-specific secondary Ab in vitro. Middle panel, The cells were also stained with saturating concentrations of MB20-11 mAb and secondary Ab in vitro. Negative control (Neg C) splenocytes were from control mAb-treated mice stained using IgG2a-specific secondary Ab. Positive control (Pos C) splenocytes were from control mAb-treated mice stained using MB20-11 mAb and secondary Ab in vitro. F, B cell subset sensitivity to MB20-11 mAb treatment over a range of doses with numbers of each B cell subset determined as in C and D. G, B cell depletion in hCD19TG mice. Bar graph values represent the mature (CD24<sup>−</sup>CD21<sup>−</sup>B220<sup>−</sup>) and B1a (CD5<sup>−</sup>CD24<sup>−</sup>B220<sup>−</sup>) cells remaining in MB20-11 (■) or isotype control (□) mAb-treated mice. A–G, All results represent those obtained with ≥3 pairs of mice. Significant differences between means: *, p < 0.05; **, p < 0.01.
FIGURE 5. Peritoneal cavity B cells are resistant to CD20 mAb treatment. A and B, Representative peritoneal lymphocytes 7 days after MB20-11 or control mAb (250 μg) treatment. Bar graph values represent total numbers of B220<sup>-</sup>CD5<sup>-</sup>B220<sup>+</sup>B1a, CD5<sup>+</sup>CD11b<sup>-</sup>B220<sup>+</sup>B1b, and CD5<sup>-</sup>CD11b<sup>+</sup>B220<sup>+</sup>B2 (conventional) B cells, as identified in the histogram gates shown. B1b and B2 cells were distinguished based on their differential expression of CD11b (data not shown). C, CD20 and IgM expression following MB20-11 or control mAb treatment, as in A. Left panel, MB20-11 mAb binding in vivo was assessed by IgG2a-specific secondary Ab staining in vitro. Peritoneal cells stained with saturating concentrations of MB20-11 mAb and anti-mouse IgG2a-specific secondary Ab in vitro (middle panel) or with anti-mouse IgM-specific Ab (right panel). For the left and center panels, negative controls (Neg C) were peritoneal cells from control mAb-treated mice stained using IgG2a-specific secondary Ab, while positive controls (Pos C) were stained using MB20-11 mAb plus secondary Ab in vitro. Right panel, Controls are B220<sup>-</sup> mononuclear cells from MB20-11 mAb-treated mice stained using IgG2a-specific secondary Ab and positive control IgM staining of cells from control mAb-treated mice. D, Representative B cell depletion 7 days after MB20-11 or isotype control mAb treatment of Fcγ<sup>-/-</sup> or C3<sup>-/-</sup> mice. Bar graphs represent mean B cell numbers 7 days after MB20-11 (□) or isotype control (○) mAb treatment (n = 5 mice per group). A–D, Results represent those obtained with ≥3 mouse pairs, with significant differences between sample means indicated: *, p < 0.05; **, p < 0.01. E, Numbers of B1a and B2 cells within the peritoneal cavity following MB20-11 (□) or control (○) mAb treatment. The value shown after time 0 represents data obtained 1 h after mAb treatment. Each value point represents mean ± SEM results for three individual mice at each time point. Thus, peritoneal B cells from B1 mice were used for this time course analysis, with the results reflecting, in part, the normal variation in numbers of peritoneal B cells harvested from individual sets of three mice. Otherwise, significant differences between sample means are indicated: *, p < 0.05; **, p < 0.01.

cells were not altered by MB20-11 mAb treatment (Fig. 6B). By contrast, the relative frequency of spleen CFSE<sup>+</sup> wild-type B cells that had migrated to the spleen was 95% lower than the frequency of CFSE<sup>+</sup> CD20<sup>-/-</sup> B cells in MB20-11 mAb-treated mice. Similar results were obtained after 7 days of CD20 mAb treatment (Fig. 6B), with spleen CFSE<sup>+</sup> B2 cells preserved to a similar extent as endogenous peritoneal B2 cells (Fig. 5B). Thus, the peritoneal cavity is a protected environment that fosters the survival of mAb-coated B cells.

That peritoneal B1 and B2 cells were readily coated with MB20-11 mAb in vivo from 1 h to 7 days after mAb treatment (Figs. 1A and 5C), but were not effectively depleted, suggested an absence of effector cells necessary for B cell depletion. To address this, mice were treated i.p. with thioglycolate to induce macrophage migration into the peritoneal cavity over a 24- to 48-h period, as described (32). One day after thioglycolate treatment, mice were given MB20-11 mAb with peritoneal cells harvested 2 or 7 days later. On day 2, there was no significant difference in numbers of B1a, B1b, or B2 cells in the peritoneum of MB20-11 or control mAb-treated mice (Fig. 6C). In mice treated with thioglycolate, there was a 61% reduction in numbers of B2 cells in the peritoneum of MB20-11 mAb-treated mice relative to control mAb-treated mice. However, thioglycolate treatment did not alter the numbers of B1a or B1b cells in the peritoneum of MB20-11 or control mAb-treated mice by day 2 (Fig. 6C). By contrast, 89–98% of peritoneal B1a, B1b, and B2 cells were deleted in thioglycolate-treated mice after MB20-11 mAb treatment for 7 days (Fig. 6D). Peritoneal cavity B cells were not decreased in MB20-11 mAb-treated Fcγ<sup>-/-</sup> mice, although B cells were efficiently depleted in C3<sup>-/-</sup> mice. Thus, thioglycolate elicited effector cell recruitment facilitated the depletion of resident B cells in the peritoneal cavity through FcR-dependent and C3-independent pathways, with B2 cells being more acutely sensitive than B1a or B1b cells.
The vast majority of circulating and tissue B220⁺ B cells were rapidly depleted following CD20 mAb treatment (Figs. 2–4). However, peritoneal cavity B1a and B1b cells were remarkably resistant to depletion (Figs. 5–6 and Table I). The peritoneal cavity also provided a protective environment for Ab-coated conventional B cells because intrinsic B2 cells (Fig. 6A) and ectopic spleen B2 cells persisted in the peritoneal cavity after CD20 mAb treatment, while labeled spleen B cells that migrated to the spleen in adoptive transfer experiments were depleted normally (Fig. 6B). Conventional B2 cells in the peritoneum were reduced by 7 days, while significant depletion of the B1a cell subset was only achieved by 28 days of CD20 mAb treatment. By contrast, blood and recirculating bone marrow B cells were effectively depleted within just 1 h, with the majority of mature B cells in the spleen, lymph nodes, Peyer’s patches, and gut-associated lymphoid tissues depleted by 2 days of MB20-11 mAb treatment (Figs. 2B and 3B, data not shown). Within the spleen, ~98% of mature B220⁺ B cells, T2, and marginal zone B cells were depleted (Fig. 4, C and D, and Table I) by similar low mAb doses (Fig. 4F). Even though marginal zone B cells are juxtaposed with metallophilic and marginal sinus-associated macrophages in the spleen, they were equally sensitive to CD20 mAb-mediated deletion. The small number of B cells that remained within the spleen after CD20 mAb treatment predominantly had an immature/transitional phenotype and most likely represents recent emigrants destined for elimination as they expressed CD20 at higher densities (Fig. 4B). Consistent with this, immunohistochemical staining of spleen after CD20 mAb treatment revealed the focal localization of rare B220⁺ cells within T cell areas (Fig. 4A) that were similar to foci formed by transitional B cells migrating from the bone marrow (33). The majority of lymph node B cells required only 4 days for depletion (Fig. 3B). Therefore, subset- and tissue-specific differences in B cell depletion predict that CD20 mAb therapy is most effective in
removing circulating cells from blood and bone marrow, is effective in depleting B cells from the spleen and other lymphoid tissues, but may be less effective in removing B cells from nonlymphoid tissues such as the peritoneal cavity. This may have important ramifications for treatment of extranodal B cell tumors or B1 cells contributing to autoimmune disease.

The characteristic compartmentalization of B1 cells in the peritoneal cavity reflects Ag receptor-driven selection and their specialized functions in mucosal and innate immunity against the bacterial Ags within this microenvironment (34, 35). However, the preferential localization of B1 cells in the peritoneal cavity may also sequester them from effector mechanisms because peritoneal B1 and B2 cells were not readily depleted after CD20 mAb treatment (Fig. 5C) despite high levels of cell surface CD20 and CD20 mAb binding (Fig. 1A). Because B1 cells produce natural and polyreactive Abs that are self-reactive in some cases, their preferential residence in a protective microenvironment may reduce the depletion of B1 cells coated with self-generated autoreactive Abs or cell surface immune complexes. This may be particularly important given the limited capacity of B1 cells for self-renewal in a competitive environment (36, 37). That the peritoneal cavity provides a protective environment for Ab-coated B1 and B2 cells may also have important implications for the clearance of tumor cells within the peritoneal cavity that may repopulate lymphoid tissues once mAb therapy is discontinued. Furthermore, protective niches in addition to the peritoneal cavity may exist.

In addition to their localization within a protective microenvironment, B1a cells appear to also possess intrinsic properties that promote survival in the presence of CD20 mAb treatment because Ab-bathed B1a cells were more difficult to deplete from the spleens of wild-type and hCD19TG mice than conventional B cells (Fig. 4F and Table I). This characteristic may provide new insights into the resistance of chronic lymphocytic leukemia (CLL) and other malignant B cells to CD20 therapy. In some cases, CLL cells are postulated to be the malignant counterparts of B1a cells (38) and are intrinsically more resistant to Rituximab therapy (16, 39, 40). Consistent with this, FcγRIIa polymorphisms may not predict patient responses in CLL (41). Thereby, cell intrinsic resistance to CD20 mAb-mediated depletion in combination with the finding that FcγRI, FcγRIII, and potentially other FcγR can contribute to CD20 mAb-mediated depletion (22) may make CLL more resistant to therapy. That intrinsic factors can make B1a cells more resistant to CD20 mAb treatment may also explain in part why disease can progress during CD20 mAb therapy in some malignancies, despite CD20 expression by the malignant cells (9). Resistance of CLL cells to anti-CD20 therapy may also be partly explained by their characteristically low levels of CD20 expression (13). Consistent with this, the density of CD20 mAb binding to B cells significantly influenced the effectiveness of B cell depletion. CD20 expression is quite heterogeneous as B cells mature and exit the bone marrow (22) and residual CD20low B cells were observed in tissues following CD20 mAb treatment (Figs. 2–5). Thus, therapeutic strategies that can either increase CD20 density on target cells or the density of FcγR expression on effector cells are likely to be beneficial.

Although CD20 expression density is important, other factors also influence optimal B cell depletion. When 10 µg of MB20-11 mAb was given to mice, a single i.v. dose ~250-fold lower than the 375 mg/m2 dose primarily given four times for CD20 therapy in humans (2–6), there were differences in mAb distribution within the first hour. Although bone marrow and circulating B cells were primarily eliminated by 1 h, spleen and peritoneal B cells were already coated with MB20-11 mAb at saturating or near saturating levels (Fig. 1A). By contrast, lymph node B cells were not optimally coated with mAb by 1 h, but were saturated for MB20-11 mAb binding by 24 h. Nonetheless, mouse spleen and lymph nodes were cleared of B cells with similar kinetics (Fig. 3B). However, studies in three cynomolgus monkeys have suggested that lymph nodes may be more difficult to clear of B cells than the spleen after Rituximab therapy (42). Therefore, tissue-specific differences in the distribution of MB20-11 mAb after administration may influence B cell depletion following CD20 mAb treatment when free mAb concentrations are limiting. This was not the primary explanation for delayed depletion of peritoneal B1a and conventional B cells because i.p. (Fig. 6A) or combined i.p. and i.v. therapy (data not shown) did not significantly affect the kinetics of peritoneal B cell depletion. The inability to deplete peritoneal B cells by CD20 mAb treatment did not extend to gut-associated lymphoid tissues because Peyer’s patch, intraepithelial, and lamina propria B cells were depleted rapidly (1–24 h) and to the same extent as spleen B cells (Fig. 3A). Thus, B cells in different anatomical locations have different sensitivities to CD20 mAb-mediated depletion, which could have significant ramifications for lymphoma therapy.

That B cells in different anatomical locations have different sensitivities to CD20 mAb-mediated depletion is difficult to correlate with current clinical results using Rituximab due to the heterogeneity in patient cohorts, in lymphoma origin and dissemination, in pretreatment therapy, and in the combinations of therapies normally given to patients. However, Rituximab clinical trials have demonstrated that the presence of extranodal disease significantly reduces the period of progression-free survival (43) and overall response rates (44) in lymphoma patients. In one patient with extranodal MALT lymphoma, CD20 therapy was therapeutically active, but CD20+ lymphoma cells remained within lymphoepithelial lesions (45). These results suggest impaired penetration of the Ab into these and perhaps other extranodal sites, or a paucity of effector monocytes within these sites. Likewise, Rituximab can deplete body cavity-based lymphoma or primary effusion lymphoma tumor cells (46, 47), but determining the relative sensitivity of pleural effusion and ascites cells to CD20 mAb therapy vs other lymphoma types or tumor cells within lymphoid tissues remains difficult. Thus, the current finding that B cells in different anatomical locations have different sensitivities to CD20 mAb-mediated depletion complements results obtained in human clinical trials, but illustrates the continuing need for further preclinical and clinical evaluation of this issue.

The extent that the MB20-11 mAb induced B cell depletion in mice was noteworthy when compared with results obtained using Rituximab in primates. Moreover, there was little intermouse variability in tissue B cell depletion (Figs. 2–5). In lymphoma patients, lymph node biopsies performed 2 wk after a single dose of Rituximab (>100 mg/m2) showed only 19–40% decreases in the percentage of B cells in six of seven patients (30), while ~95% depletion was achieved by day 7 in the current studies (Fig. 3A). This Rituximab dose would be equivalent to a >667 µg mAb dose in mice. Human blood B cells are depleted within 24–48 h of Rituximab treatment (7–9, 48), while mouse blood B cell depletion was nearly complete by 1 h (Fig. 3B). However, interpatient variability in Rituximab pharmacokinetic parameters might be explained in part by the differences in tumor volume and numbers of CD20 molecules expressed by B cells and lymphoma cells. Regardless, lymphoid organ B cells are only partially depleted in primates with considerable interanimal and intersite variability despite the use of relatively high Rituximab doses (7, 42, 49, 50). By contrast, the MB20-11 mAb effectively eliminated the majority of peripheral B cells when used as a single 5–10 µg treatment, a 0.5 mg/kg dose that is 400-fold lower than the 20 mg/kg or higher dosages of Rituximab used commonly in primates. Doses as low as 0.4–1.6 mg/kg have been shown to effectively reduce B cell levels.
in the peripheral blood of primates (7), but we have found that blood B cell clearance involves multiple factors in addition to FcγR-mediated depletion and does not necessarily predict tissue B cell depletion (22). Whether the greater potency of MB20-11 mAb relative to Rituximab reflects differences between mice and primates or has a different molecular explanation is currently under investigation. Regardless, the remarkable efficiency of tissue B cell depletion with anti-mouse CD20 mAbs in the current study does not necessarily imply that Rituximab will be comparable or have the same efficiency for tissue B cell depletion in humans.

These studies are consistent with the phagocytic network of the innate immune system eliminating B cells through FcR-dependent mechanisms following CD20 mAb therapy. First, B cell clearance from the circulation and all tissues examined in this study required FcRγ, but not C3 expression (Figs. 2, 3, 5, and 6). Consistent with this, efficient B cell depletion from tissues was not restricted to the MB20-11 mAb. B cells were depleted from each tissue by the MB20-6, -11, and -16 (IgG2a); MB20-1, -2, and -14 (IgG1); and MB20-7, -8, and -18 (IgG2b) mAbs (data not shown), although the degree of B cell depletion was greatest with IgG2a > IgG1 > IgG2b mAbs, as previously described for blood and spleen B cell depletion (22). Second, inflammation within the peritoneum, which elicits macrophage immigration, resulted in effective B cell depletion in the normally protective environment of the peritoneal cavity (Fig. 6D). Again, inflammation-induced B cell depletion from the peritoneal cavity required FcRγ, but not C3 expression. Thus, the number and localization of mononuclear phagocytes within tissues may significantly affect the rate and degree of B cell depletion, and tumor burden will also influence response rates. Thus, the current results have important clinical implications for CD20 mAb immunotherapies. The successful elimination of resistant B1 cells may further enhance the benefit of CD20 therapy for autoimmune disease (51). B cell depletion was also found to be influenced by multiple other factors, including B cell tissue localization, B cell subset differences, CD20 cell surface density, and the differential access of mAb to different tissues. Hastened mAb clearance due to global CD20 target density cell surface density, and the differential access of mAb to different tissues. Hastened mAb clearance due to global CD20 target density.

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