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

Yasuhito Hamaguchi, Junji Uchida, Derek W. Cain, Guglielmo M. Venturi, Jonathan C. Poe, Karen M. Haas and Thomas F. Tedder

*J Immunol* 2005; 174:4389-4399; doi: 10.4049/jimmunol.174.7.4389

http://www.jimmunol.org/content/174/7/4389

**References**

This article cites 52 articles, 27 of which you can access for free at: http://www.jimmunol.org/content/174/7/4389.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
The Peritoneal Cavity Provides a Protective Niche for B1 and Conventional B Lymphocytes during Anti-CD20 Immunotherapy in Mice

Yasuhito Hamaguchi, Junji Uchida, Derek W. Cain, Guglielmo M. Venturi, Jonathan C. Poe, Karen M. Haas, and Thomas F. Tedder

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 the peritoneum. 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.

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. Therefore, 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.
**FIGURE 1.** CD20 mAb reactivity. A, MB20-11 mAb staining of B220<sup>+</sup> B cells in mice treated i.v. with MB20-11 or control mAb for 1 or 24 h. Washed cells were placed on ice and either stained directly with IgG2a-specific secondary Ab plus anti-B220 mAb, or the cells were incubated with saturating concentrations of MB20-11 mAb on ice for 30 min before staining. Negative controls (Neg C) represent secondary Ab staining of cells from control mAb-treated mice. Positive controls (Pos C) represent B cells from control mAb-treated mice stained using MB20-11 mAb in vitro. Each histogram represents the relative fluorescence staining of 10<sup>4</sup> cells. Results represent ≥3 experiments. B, In vivo MB20-11 mAb <i>t<sub>1/2</sub></i> in two wild-type or two CD20<sup>−/−</sup> littermates (○ and ●, represent individual mice). Each point represents serum MB20-11 mAb levels of individual mice after day 0 administration. Regression lines used values from both mice.

**Immunofluorescence analysis and immunohistochemistry**

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).

**Peritoneal B cell depletion assays**

Splenocytes from CD20<sup>−/−</sup> 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 × 10<sup>7</sup>) were mixed with equal numbers of CFSE-labeled CD20<sup>−/−</sup> 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 mAbs 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<sup>+</sup> 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+ B220med), 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 B220+ B cell subset, as in B. For the left panel, CD20+ B cell numbers 7 days after MB20-11 mAb treatment were significantly depleted from the bone marrow by as little as 1 μg of MB20-11 mAb (Fig. 2B). By contrast, significant depletion of IgM+ B220high pro/pre-B cells and IgM+ B220high 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, surface CD20 (22). After 7 days of MB20-11 mAb treatment, there was a small subpopulation of CD20high 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 cell depletion 7 days after MB20-11 or control mAb treatment of FcRγ−/− or C3−/− littermates. Bars graphs represent mean IgM+ B220high (mature) B cell numbers 7 days after MB20-11 (●) or isotype control (□) mAb treatment (≥5 mice per group), with significant differences between sample means 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⁺ B220high B cell depletion was therefore assessed using FcrγI common γ-chain-deficient (FcrγI−/−) mice that lack high affinity FcγR1 and low affinity FcγRIII (31). Mature B220high B cells were not significantly depleted in FcγRI−/− mice, while most IgM⁺ B220high B cells were depleted in C3−/− mice (Fig. 2E). Thus, CD20 mAb treatment depleted the majority of recirculating B cells in the bone marrow through FcR-dependent mechanisms, which predominantly included the IgM⁺ B220high B cell subset that expresses CD20 (22).

**Blood, spleen, lymph node, and gut-associated B cell depletion**

The MB20-11 mAb had potent and long-lasting effects on lymphoid tissue B cells. A single 250 μg i.v. injection of MB20-11 mAb depleted most blood, spleen, peripheral lymph node, mesenteric lymph node, Peyer’s patch, and gut-associated intraepithelial and lamina propria B220⁺ cells (Fig. 3A and Table I), as previously shown for blood and spleen B cells (22). MB20-11 mAb depleted circulating B cells within 1 h, while maximal spleen and lymph node B cell depletion required ~2 days (Fig. 3B). MB20-11 mAb-induced clearance was durable for ~60 days before B220⁺ cells began to repopulate each tissue. Likewise, CD20 mAb treatment depleted mesenteric lymph node, Peyer’s patch, and gut-associated intraepithelial and lamina propria B220⁺ cells by 80–90% after 1 h and by 70–98% after 24 h of treatment (data not shown). An isotype control mAb did not affect circulating or tissue B cells, and Thy-1.2⁺ T cell numbers were unchanged by CD20 mAb treatment. When given as a single dose as low as 2.5 μg/g mouse, the MB20-11 mAb depleted the majority of circulating, spleen, and lymph node B cells by day 7 (Fig. 3C). In all cases, circulating B cells were the most rapidly cleared and were affected by the lowest doses of MB20-11 mAb, while CD20 mAb treatment affected spleen and lymph node B cells to similar extents. B cell depletion from lymphoid tissues 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 and spleen B cell depletion (22). Consistent with this, the MB20-11 mAb did not significantly deplete lymph node B cells in FcγRI−/− mice, although lymph node B cells were efficiently depleted in C3−/− mice (Fig. 3D), as previously shown for blood and spleen B cells (22).

**Spleen B cell subset depletion**

Following MB20-11 mAb treatment, it was not possible to identify histological structures resembling follicles within the spleens of mice (Fig. 4A). Rather, the remaining B220⁺ cells were found in rare and small lymphoid clusters that included Thy-1.2⁺ T cells. Rare B220⁺ cells were scattered through T cell-rich areas of the spleen, although these cells predominantly had a nonlymphoid morphology 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⁺ B220high cells (mature B cell-like), IgM⁺ B220low cells (immature B cell-like), IgM⁺ B220low cells (pro/pre-B cell-like), and an IgM⁻ subset 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⁺CD24high B220⁺ T1 cells with the virtual elimination of CD21⁺CD24high 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⁺ 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 CD220low 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

Table 1. *Tissue B cell depletion following CD20 mAb treatment*  

<table>
<thead>
<tr>
<th>Tissue</th>
<th>B Subset</th>
<th>Control mAb</th>
<th>MB20–11 mAb</th>
<th>% Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow:</td>
<td>Pro/pre</td>
<td>2.7 ± 0.3 (17)</td>
<td>3.2 ± 0.5 (20)</td>
<td>0</td>
</tr>
<tr>
<td>Immature</td>
<td>1.9 ± 0.2 (17)</td>
<td>1.6 ± 0.2 (20)</td>
<td>16</td>
<td></td>
</tr>
<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>B220⁺</td>
<td>4.6 ± 0.4 (9)</td>
<td>0.08 ± 0.02 (12)</td>
<td>98**</td>
</tr>
<tr>
<td>Spleen:</td>
<td>Mature</td>
<td>32.0 ± 3.4 (6)</td>
<td>0.7 ± 0.1 (6)</td>
<td>98**</td>
</tr>
<tr>
<td>T1</td>
<td>4.9 ± 1.0 (6)</td>
<td>0.62 ± 0.09 (6)</td>
<td>87**</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>2.5 ± 0.7 (6)</td>
<td>0.01 ± 0.01 (6)</td>
<td>&gt;99**</td>
<td></td>
</tr>
<tr>
<td>Marginal zone</td>
<td>1.6 ± 0.5 (6)</td>
<td>0.01 ± 0.01 (6)</td>
<td>&gt;99**</td>
<td></td>
</tr>
<tr>
<td>B1a</td>
<td>1.3 ± 0.4 (3)</td>
<td>0.53 ± 0.08 (3)</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>CD21⁺CD24⁺ B220⁺</td>
<td>2.0 ± 0.4 (6)</td>
<td>1.4 ± 0.2 (6)</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Peripheral LN:</td>
<td>B220⁺</td>
<td>1.1 ± 0.1 (16)</td>
<td>0.09 ± 0.02 (16)</td>
<td>95**</td>
</tr>
<tr>
<td>Mesenteric LN:</td>
<td>B220⁺</td>
<td>3.1 ± 0.8 (4)</td>
<td>0.10 ± 0.03 (4)</td>
<td>97**</td>
</tr>
<tr>
<td>Peyer’s patch:</td>
<td>B220⁺</td>
<td>0.7 ± 0.2 (4)</td>
<td>0.07 ± 0.02 (4)</td>
<td>91**</td>
</tr>
<tr>
<td>Peritoneum:</td>
<td>B220⁺</td>
<td>1.8 ± 0.3 (14)</td>
<td>0.75 ± 0.09 (15)</td>
<td>59**</td>
</tr>
<tr>
<td>B1a (day 7)</td>
<td>0.40 ± 0.07 (14)</td>
<td>0.27 ± 0.04 (15)</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>B1a (day 28)</td>
<td>0.73 ± 0.12 (5)</td>
<td>0.19 ± 0.09 (4)</td>
<td>74*</td>
<td></td>
</tr>
<tr>
<td>B1a (day 7)</td>
<td>0.41 ± 0.10 (10)</td>
<td>0.29 ± 0.05 (10)</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>B2 (day 28)</td>
<td>0.94 ± 0.23 (14)</td>
<td>0.21 ± 0.04 (15)</td>
<td>78**</td>
<td></td>
</tr>
</tbody>
</table>

* B cell subsets were: bone marrow pro/pre-B (IgM⁺ B220high), immature B (IgM⁺ B220low); spleen mature (CD24⁺CD21⁺ B220⁺), T1 (CD24highCD21⁺ B220⁺), T2 (CD24highCD21⁺ B220⁻), marginal zone (CD21hiCD1dhigh B220⁻), and peritoneal Bla (CD5⁺CD11b⁺ IgM⁺B220⁺), B1b (CD5⁺CD11b⁺ IgM⁺B220high), B1 (CD5⁺CD11b⁺ IgM⁺B220high). 

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.
CD20 mAb concentrations, indicating similar sensitivities to CD20 mAb treatment (Fig. 4F). By contrast, spleen CD5⁺CD20low 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⁺CD19highB220⁻ 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⁺ cells when given i.v. at 250 μg (Fig. 5A and Table I). Although the CD5⁻IgMlow-B220high subset of conventional (B2) cells was reduced the most by MB20-11 mAb treatment by day 7, CD5⁺CD11b⁺IgM⁺B220low B1a B cells and CD5⁻CD11b⁻IgMhigh-B220low B1b B 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

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⁺CD19highB220⁻ 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⁺ cells when given i.v. at 250 μg (Fig. 5A and Table I). Although the CD5⁻IgMlow-B220high subset of conventional (B2) cells was reduced the most by MB20-11 mAb treatment by day 7, CD5⁺CD11b⁺IgM⁺B220low B1a B cells and CD5⁻CD11b⁻IgMhigh-B220low B1b B 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 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
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 and Thy-1.2 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+ 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+ cells following MB20-11 mAb treatment. The negative control (Neg C) histogram shows CD19 staining by B220− splenocytes from MB20-11 mAb-treated mice. The positive control (Pos C) splenocytes were from control mAb-treated mice stained using IgG2a-specific 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 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+CD21+ B220+) and B1a (CD5+B220+) 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.

and conventional B cells within the peritoneum were significantly reduced by prolonged MB20-11 mAb treatment on days 28–58 (Fig. 5E 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 predominantly 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 from wild-type and CD20−/− mice were labeled with CFSE at different intensities and injected i.p. into mice before MB20-11 mAb treatment. Two or 7 days later, peritoneal and spleen B cells were harvested and assessed for numbers of peritoneal or spleen CFSE− B cells. CD20−/− B cells served as internal controls because they are not affected by CD20 mAb treatment (23). Similarly, the relative frequencies of CFSE− B220− splenocytes served as internal controls for relative numbers of wild-type or CD20−/− splenocytes injected into each mouse. By day 2, the relative frequencies of peritoneal CFSE− wild-type and CFSE− CD20−/− B
that had migrated to the spleen was 95% lower than the frequency of CFSE+ CD20+ B cells in MB20-11 mAb-treated mice. Similar results were obtained after 7 days of CD20 mAb treatment (Fig. 6B), with spleen CFSE+ B 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 CD20 mAb was also indicated by the absence of 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 means ± 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.

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+ CD5+CD20+ B1a, CD5+CD11b+B220+ B1b, and CD5+CD11b+B220+B (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 Ab and positive control IgM staining of cells from control mAb-treated mice. Bar graphs represent mean B cell numbers 7 days after MB20-11 (■) or isotype control (○) mAb treatment (±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 means ± 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.

By contrast, the relative frequency of spleen CFSE+ wild-type B cells that had migrated to the spleen was 95% lower than the frequency of CFSE+ CD20+ B cells in MB20-11 mAb-treated mice. Similar results were obtained after 7 days of CD20 mAb treatment (Fig. 6B), with spleen CFSE+ 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.

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 depleted 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 FcγR−/− mice, although B cells were efficiently depleted in C3−/− mice. Thus, thioglycolate-elicted effector cell recruitment facilitated the depletion of resident B cells in the peritoneal cavity through FcγR-dependent and C3-independent pathways, with B2 cells being more acutely sensitive than B1a or B1b cells.
Discussion

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 CD20 mAb-bound B cells in the absence of inflammation. A, CD20 mAb administration i.p. does not augment peritoneal B cell clearance. Wild-type mice were given MB20-11 (■) or isotype control (□) mAb (250 \(\mu\)g) 7 days before assessing peritoneal and spleen B cell numbers. B, Ab-coated spleen B cells are not depleted from the peritoneal cavity of CD20 mAb-treated mice. Splenocytes from wild-type and CD20\(^{-/-}\) mice were labeled with CFSE and mixed equally, and injected i.p. into wild-type littermates before treatment with MB20-11 or control mAb (250 \(\mu\)g). After 2 or 7 days, peritoneal cells and splenocytes were isolated and stained for B220 expression before flow cytometry analysis. The gates indicate CFSE-labeled B220\(^+\) and B220\(^-\) lymphocytes from wild-type and CD20\(^{-/-}\) mice on day 2. Bar graphs indicate relative ratios of cells from wild-type and CD20\(^{-/-}\) donors within the CFSE-labeled B220\(^+\) and B220\(^-\) lymphocyte populations. Without depletion of wild-type B220\(^+\) cells, the relative ratios of CFSE-labeled B220\(^+\) cells would equal the ratios for CFSE-labeled B220\(^-\) splenocytes. C and D, Thioglycollate-induced inflammation results in B cell depletion from the peritoneum following CD20 mAb treatment. Littermates were either untreated or injected i.p. with thioglycollate 1 day before MB20-11 (■) or control (□) mAb (250 \(\mu\)g) treatment. On days 2 (C) and 7 (D) after mAb treatment, peritoneal lymphocytes were isolated for immunofluorescence staining and B cell quantification, as in Fig. 5B. A–D, Results represent those obtained with ≥5 mouse pairs, with significant differences between sample means (±SEM) indicated: *, \(p < 0.05\); **, \(p < 0.01\).
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, FcγRIIIa 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, FcγRII, 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 FcRy, 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 FcRy, 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 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.

Thus, the number and localization of mononuclear phagocytes within tissues may significantly affect the rate and degree of 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.

References


