# Therapeutic B cell depletion impairs adaptive and autoreactive CD4$^+$ T cell activation in mice

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**CD20 antibody depletion of B lymphocytes effectively ameliorates multiple T cell-mediated autoimmune diseases through mechanisms that remain unclear. To address this, a mouse CD20 antibody that depletes >95% of mature B cells in mice with otherwise intact immune systems was used to assess the role of B cells in CD4$^+$ and CD8$^+$ T cell activation and expansion *in vivo*. B cell depletion had no direct effect on T cell subsets or the activation status of CD4$^+$ and CD8$^+$ T cells in naive mice. However, B cell depletion impaired CD4$^+$ T cell activation and clonal expansion in response to protein antigens and pathogen challenge, whereas CD8$^+$ T cell activation was not affected. *In vivo* dendritic cell ablation, along with CD20 immunotherapy, revealed that optimal antigen-specific CD4$^+$ T cell priming required both B cells and dendritic cells. Most importantly, B cell depletion inhibited antigen-specific CD4$^+$ T cell expansion in both collagen-induced arthritis and autoimmune diabetes mouse models. These results provide direct evidence that B cells contribute to T cell activation and expansion *in vivo* and offer insights into the mechanism of action for B cell depletion therapy in the treatment of autoimmunity.**

autoimmune disease | B lymphocyte | immunotherapy | antigen presentation

Central defects in T lymphocyte regulation contribute to most autoimmune diseases (1). Surprisingly however, mAb therapy directed against the CD20 cell surface molecule of human B cells (rituximab) has shown varying degrees of efficacy in rheumatoid arthritis, idiopathic thrombocytopenic purpura, hemolytic anemia, lupus erythematosus, and pemphigus vulgaris treatment (2, 3). Despite the benefits of this immunotherapy, the cellular and molecular basis for the protective effect mediated by B cell depletion is unknown (4). Understanding the mechanisms underlying the therapeutic benefit of B cell depletion is complicated by the fact that B cells not only produce autoantibodies (5) but also release inflammatory or immunomodulatory cytokines (6), regulate lymphoid tissue neogenesis and structure, provide costimulatory signals, alter dendritic cell (DC) function and homeostasis (7), can function as antigen-presenting cells, promote naïve CD4$^+$ T cell differentiation into Th1 or Th2 subsets, and may influence regulatory T cell numbers and function (8).

Examining the effects of B cell depletion in mice with intact immune systems is possible by using mouse anti-mouse CD20 mAbs in which B cells are depleted *in vivo* by monocyte-mediated antibody-dependent cellular cytotoxicity (9, 10). More than 95% of mature B cells in blood and primary lymphoid organs are depleted after 2 days by a single dose of CD20 mAb (MB20-11, 250 $\mu$g per mouse), with the effect lasting 8 weeks (11). B cell depletion prevents autoimmunity in mouse models of collagen-induced arthritis (CIA) (12), scleroderma (13), Sjogren's syndrome (14), and diabetes in nonobese diabetic (NOD) mice (Y. Xiu, C. P. Wong, J.-D.B., Y. Hamaguchi, Y.W., S. M. Pop, R.M.T., and T.F.T., unpublished work). In these models, B cell depletion after disease onset is less beneficial, indicating a possible role for B cells in the early stages of autoimmunity during autoreactive T cell activation and/or expansion. The effect of B cell depletion on T cell activation *in vivo* was therefore assessed to identify mechanisms by which B cell depletion ameliorates T cell-dependent autoimmune disease.

## Results

**CD20 Expression Is B Cell-Restricted in Mice.** CD20 mAb treatment depleted the majority of B220$^+$ B cells on days 7 and 28, whereas absolute numbers of other leukocyte subsets in all primary lymphoid organs were not affected (Table 1). Although a small subset of human T and natural killer cells has been reported to be depleted after CD20 mAb therapy in rheumatoid arthritis patients as a result of low-level CD20 expression (15, 16), immunofluorescence staining of all leukocytes subsets revealed that CD20 expression is B cell restricted in mice [supporting information (SI) Fig. 5], as in humans (17). Thereby, CD20 mAb treatment was unlikely to modify autoimmune responses by depleting functionally important non B cell leukocyte subsets.

**B Cell Depletion Does Not Affect Serum Cytokine Levels and T Cell Phenotypes or Functional Capacity.** Whether B cell depletion and/or monocyte activation could influence serum cytokines that in turn may modify T cell function was examined. CD20 mAb treatment did not significantly alter serum IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, TNF-$\alpha$, IFN-$\gamma$, GM-CSF, or TGF-$\beta$1 levels over the course of a week (SI Fig. 6*A* and data not shown). Therefore, B cell depletion was unlikely to induce a systemic cytokine release that altered T cell function.

To assess possible indirect effects of CD20 mAb treatment on T cells, expression of activation markers by CD4$^+$ and CD8$^+$ T cells was examined. Expression of CD25, CD28, CD18, CD69, CD40L, OX40, or CTLA-4 by spleen or lymph node CD4$^+$ or CD8$^+$ T cells was unaffected by B cell depletion over 7 and 28 days (SI Fig. 6*B* and data not shown). Likewise, B cell depletion did not impair the capacity of spleen or lymph node CD4$^+$ T cells to proliferate and produce cytokines *in vitro* after polyclonal stimulation (SI Fig. 6*C* and data not shown). To rule out more subtle effects on T cells, monocytes, and DCs after B cell depletion, gene expression profiles were assessed by quantitative real-time PCR analysis. Transcripts for 84 genes related to T cell activation and differentiation were analyzed by using B220$^-$ splenocytes from mice treated 4 days earlier with CD20 or control mAb. B cell depletion did not alter transcript levels for any of the 84 genes analyzed (SI Fig. 6*D* and

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**Table 1. Lymphocyte populations after B cell depletion**

| Tissue and lymphocyte subset | 7 days | | 28 days | |
|---|---|---|---|---|
| | Control mAb | CD20 mAb | Control mAb | CD20 mAb |
| Blood | | | | |
| $B220^+$ | 4.0 ± 0.9 | 0.013 ± 0.002* | 3.49 ± 0.75 | 0.010 ± 0.003* |
| $CD4^+$ | 1.7 ± 0.4 | 1.2 ± 0.2 | 1.2 ± 0.1 | 1.3 ± 0.2 |
| $CD4^+CD25^+FoxP3^+$ | 0.6 ± 0.1 | 0.6 ± 0.2 | 0.75 ± 0.08 | 0.9 ± 0.2 |
| $CD8^+$ | 0.8 ± 0.2 | 0.6 ± 0.1 | 0.7 ± 0.07 | 0.7 ± 0.1 |
| $NK1.1^+CD3^-$ | 0.5 ± 0.1 | 0.4 ± 0.1 | 0.4 ± 0.1 | 0.4 ± 0.05 |
| Spleen | | | | |
| $B220^+$ | 52.6 ± 13.3 | 0.7 ± 0.2* | 70.4 ± 10.6 | 0.27 ± 0.01* |
| $CD4^+$ | 14.9 ± 3.5 | 13.1 ± 2.3 | 20.6 ± 2.3 | 19.0 ± 1.5 |
| $CD4^+CD44^+CD62L^-$ | 3.4 ± 0.8 | 2.9 ± 1.1 | 3.1 ± 0.3 | 3.1 ± 0.4 |
| $CD4^+CD25^+FoxP3^+$ | 1.6 ± 0.2 | 1.4 ± 0.1 | 2.1 ± 0.3 | 1.9 ± 0.1 |
| $CD8^+$ | 7.8 ± 1.6 | 6.8 ± 1.6 | 9.7 ± 1.3 | 9.0 ± 1.1 |
| $CD8^+CD44^+CD62L^-$ | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.22 ± 0.04 | 0.18 ± 0.01 |
| $CD11c^{high}CD8\alpha^-$ | 3.4 ± 0.1 | 3.8 ± 0.1 | 3.5 ± 0.1 | 3.6 ± 0.1 |
| $CD11c^{high}CD8\alpha^+$ | 0.8 ± 0.1 | 1.0 ± 0.1 | 0.7 ± 0.1 | 0.9 ± 0.1 |
| $CD11c^{int}PDCA\text{-}1^+B220^+$ | 0.31 ± 0.04 | 0.27 ± 0.10 | 0.51 ± 0.12 | 0.65 ± 0.10 |
| Peripheral LN | | | | |
| $B220^+$ | 8.6 ± 1.2 | 0.09 ± 0.01* | 10.7 ± 1.8 | 0.03 ± 0.01* |
| $CD4^+$ | 12.3 ± 1.5 | 11.6 ± 2.3 | 15.4 ± 0.9 | 16.8 ± 2.2 |
| $CD4^+CD44^+CD62L^-$ | 0.71 ± 0.01 | 0.63 ± 0.02 | 0.85 ± 0.09 | 0.76 ± 0.08 |
| $CD4^+CD25^+FoxP3^+$ | 1.2 ± 0.1 | 1.1 ± 0.2 | 1.6 ± 0.1 | 1.5 ± 0.2 |
| $CD8^+$ | 8.4 ± 1.4 | 7.9 ± 0.9 | 10.5 ± 0.4 | 9.8 ± 1.6 |
| $CD8^+CD44^+CD62L^-$ | 0.04 ± 0.01 | 0.05 ± 0.01 | 0.05 ± 0.01 | 0.06 ± 0.01 |
| Thymus | | | | |
| $B220^+$ | 0.17 ± 0.01 | 0.05 ± 0.01* | 0.17 ± 0.02 | 0.07 ± 0.02* |
| $CD4^-CD8^-$ | 2.8 ± 0.5 | 2.5 ± 0.4 | 3.7 ± 0.4 | 4.1 ± 0.5 |
| $CD4^+CD8^+$ | 96 ± 24 | 76 ± 12 | 137 ± 20 | 175 ± 10 |
| $CD4^+CD8^-$ | 13 ± 4 | 13 ± 3 | 24 ± 4 | 36 ± 4 |
| $CD4^-CD8^+$ | 3.3 ± 0.5 | 2.4 ± 0.3 | 4.8 ± 0.6 | 5.6 ± 0.6 |

The values (±SEM) indicate cell numbers ($\times 10^{-6}$) present in tissues of at least four mice for each group either 7 or 28 days after mAb treatment (250 $\mu$g). The differences between mean lymphocyte numbers in CD20 and control mAb-treated littermates were significant: *, $P < 0.01$.

data not shown). Thus, *in vivo* B cell depletion did not significantly affect $CD4^+$ T cell phenotypes, functional capacity, or gene expression profiles.

**B Cell Depletion Inhibits Antigen-Specific $CD4^+$ T Cell Expansion and Activation *in Vivo*.** To evaluate the impact of B cell depletion on T cell responses to protein antigen challenge, B cell-depleted mice were immunized with keyhole limpet hemocyanin (KLH), with draining lymph node $CD4^+$ and $CD8^+$ T cells being purified 7 days later. Antigen-specific T cell proliferation was quantified *in vitro* by using purified mitomycin C-treated B cells from control mAb-treated littermates as antigen-presenting cells. $CD4^+$ T cell recall responses to KLH in B cell-depleted mice were reduced by 71 ± 13% ($P < 0.01$) at 1 $\mu$g/ml KLH and 63 ± 8% ($P < 0.01$) at 10 $\mu$g/ml KLH compared with control mAb-treated littermates (Fig. 1*A*), whereas antigen-specific $CD8^+$ T cell proliferation was unaffected.

The effect of B cell depletion on $CD4^+$ and $CD8^+$ T cell responses *in vivo* was directly investigated by using ovalbumin (OVA)-specific $Thy1.1^+$ $CD4^+$ and $CD8^+$ T cells prepared from OT-II and OT-I transgenic mice, respectively (18, 19). 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled OT-II $CD4^+$ and OT-I $CD8^+$ T cells were adoptively transferred into CD20 or control mAb-treated littermates before immunization with OVA. $CD4^+$ T cells proliferated similarly in B cell-depleted and control mAb-treated littermates after immunization with high-dose OVA (100 $\mu$g; Fig. 1*B*). By contrast, $CD4^+$ T cell expansion was significantly reduced in B cell-depleted mice compared with control mAb treated littermates immunized with low doses of OVA (at 10 $\mu$g, 40 ± 14% reduction, $P < 0.01$; at 1 $\mu$g, 75 ± 33% reduction, $P < 0.05$). Heterogeneity in the frequency and extent of T cell proliferation is likely to result from heterogeneity among T cells, heterogeneous frequencies of T cells interacting with appropriate antigen-presenting DCs or B cells in vivo, and/or the strength/duration of antigen-specific interactions. $CD8^+$ T cell proliferation was identical in B cell-depleted and control mAb-treated littermates at all OVA concentrations.

Whether B cells also influence T cell responses to pathogens was assessed by infecting mice with a strain of *Listeria monocytogenes* modified to secrete soluble OVA (20). *Listeria* replicate in macrophages and induce both $CD4^+$ and $CD8^+$ T cell responses (21). *Listeria* infection induced robust $CD4^+$ and $CD8^+$ T cell reactivity in control mAb-treated littermates, whereas B cell depletion significantly reduced the frequency of dividing $CD4^+$ T cell proliferation (14 ± 1% reduction; $P < 0.01$) (Fig. 1*C Left*), and the extent of $CD4^+$ T cell proliferation (evaluated by DFSE geometric mean fluorescence decrease; Fig. 1*C Right*) but not $CD8^+$ T cell proliferation. B cell depletion had no effect on mouse survival, consistent with the rapid expansion of $CD8^+$ T cells after *Listeria* infection. Moreover, characteristic of T effector cell generation, most $CD4^+$ T cells exhibited a $CD62^{low}CD44^{high}$ phenotype by day 7 after *Listeria* infection in control mAb-treated mice (Fig. 2*A*). By contrast, CD44 up-regulation and CD62L down-regulation were significantly reduced in B cell-depleted littermates (35 ± 5% reduction and 54 ± 4% reduction, $P < 0.01$, for CD44 and CD62L, respectively) (Fig. 2*A*). IL-2 and IFN-$\gamma$ production by $CD4^+$ T cells was also significantly reduced in B cell-depleted littermates after *Listeria* infection (for IL-2: 52 ± 15% reduction, $P < 0.05$; for IFN-$\gamma$: 82 ± 6% reduction, $P < 0.01$) (Fig. 2*B* and data not shown). Despite

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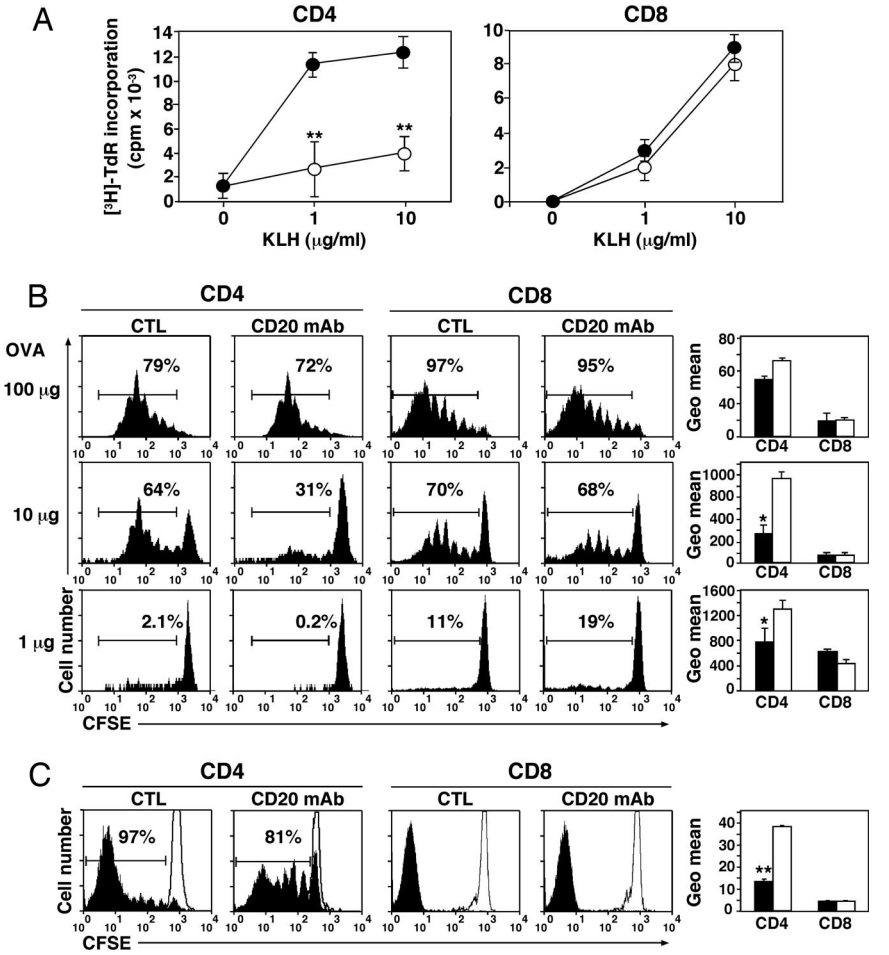


**Fig. 1.** B cell depletion inhibits antigen-specific CD4$^+$ but not CD8$^+$ T cell expansion. (*A*) Impaired CD4$^+$ T cell proliferation in B cell-depleted mice after KLH restimulation *in vitro*. CD20 (open circles) or control (CTL) (filled circles) mAb-treated mice were immunized with KLH. Seven days later, CD4$^+$ (*Left*) or CD8$^+$ (*Right*) T cells were purified from draining lymph nodes and cultured *in vitro* with KLH and mitomycin C-treated B cells from control mice. The values represent mean (±SEM) [$^3$H]thymidine (TdR) uptake from triplicate cultures. (*B*) Impaired CD4$^+$ T cell proliferation *in vivo* in B cell-depleted mice after low-dose OVA immunization. CD4$^+$ cells from OT-II Thy1.1$^+$ mice or CD8$^+$ cells from OT-I Thy1.1$^+$ mice were CFSE-labeled and transferred into Thy1.2$^+$ recipients that had been treated with CD20 or control mAb 7 days earlier. After adoptive transfers, the mice were immunized with graded OVA doses (1, 10, and 100 $\mu$g). Three days later, splenocytes were harvested and stained to analyze divisions of donor cells. The bar graphs show CFSE geometric mean fluorescence of the whole histogram, which is inversely proportional to cell divisions (CD20 mAb, open bars; control mAb, closed bars). (*C*) Impaired CD4$^+$ T cell proliferation in B cell-depleted mice after *Listeria* infection. The same experimental protocols were used as in *B* except that mice were infected 1 day after T cell adoptive transfers with *Listeria* that secretes OVA peptide and that splenocytes were harvested 7 days after infection. All data are representative of two independent experiments with at least three mice in each group. Significant differences between sample means are indicated: *, $P < 0.05$; **, $P < 0.01$.

this, B cell depletion did not significantly alter CD8$^+$ T cell phenotypes or cytokine production after *Listeria* infection. Thus, short-term B cell depletion significantly affects antigen-specific CD4$^+$ T cell expansion, activation, and effector cell differentiation *in vivo*.

**B Cell and DC Interactions Regulate CD4$^+$ T Cell Expansion *in Vivo*.** The relative contributions of B cells and DCs to CD4$^+$ T cell priming *in vivo* were compared by using B6.CD11c–DTR transgenic mice, a mouse model for inducible DC ablation *in vivo* (22). A single injection of diphtheria toxin (DT) (100 ng) into CD11c–DTR mice induced a selective >10-fold depletion of conventional splenic DCs after 10 h that persisted for 3 days (Fig. 3*A*). CD20 mAb-mediated B cell depletion was similar in WT and B6.CD11c–DTR littermates treated with DT (data not shown). B cell depletion significantly reduced the frequency of dividing OT-II CD4$^+$ T cells after

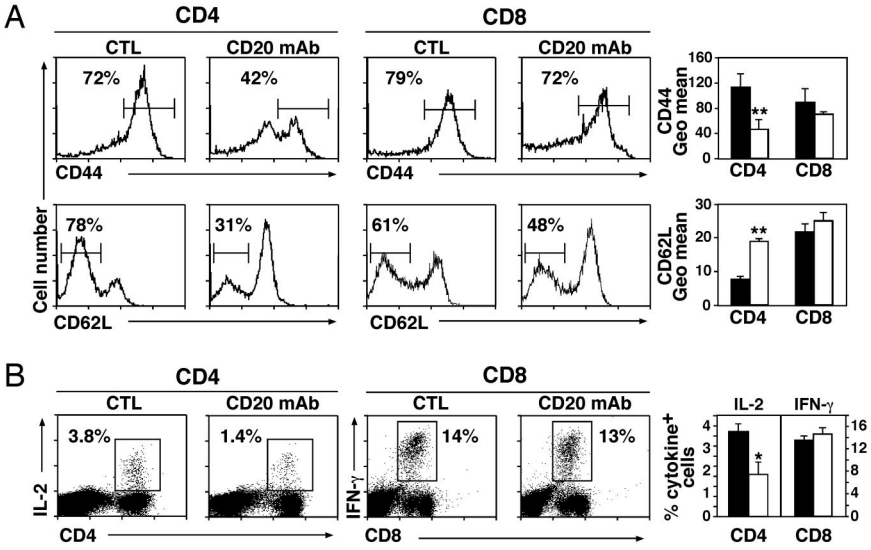


**Fig. 2.** Impaired CD4$^+$ T cell activation after B cell depletion. WT mice treated with CD20 or control (CTL) mAb were infected with *Listeria* 7 days later. Splenocytes were harvested 7 days after infection. (*A*) CD44 and CD62L expression by CD4$^+$ and CD8$^+$ T cells. Flow cytometric histograms from a representative mouse in each group are shown. (*Left* and *Center*) Numbers denote the percentage of CD4$^+$ (*Left*) and CD8$^+$ (*Center*) T cells that were CD44$^{high}$ or CD62L$^{low}$. (*Right*) Bar graphs show CD44 and CD62L geometric mean fluorescence intensities for CD4$^+$ and CD8$^+$ T cells (CD20 mAb, open bars; control mAb, closed bars). (*B*) (*Left* and *Center*) Intracellular IL-2 production by CD4$^+$ T cells (*Left*) and IFN-$\gamma$ production by CD8$^+$ T cells (*Center*) after *Listeria* infection. Flow cytometric histograms from a representative mouse in each group are shown. (*Right*) The bar graph denotes the percentage of CD4$^+$ and CD8$^+$ T cells that produce IL-2 and IFN-$\gamma$, respectively (CD20 mAb, open bars; CTL mAb, closed bars). All data are representative of two independent experiments with three mice in each group. Significant differences between sample means are indicated: *, $P < 0.05$; **, $P < 0.01$.

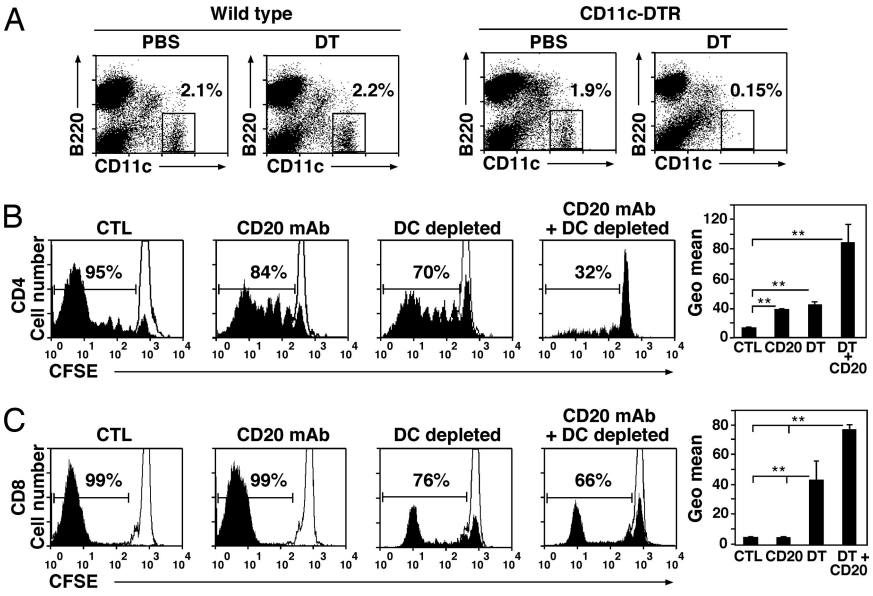


**Fig. 3.** Both B cells and DCs contribute to CD4$^+$ T cell expansion *in vivo*. (*A*) DT depletion of CD11c$^+$ DCs in CD11c–DTR mice. WT or CD11c–DTR mice were given DT or PBS. Splenocytes were harvested 10 h later and stained for CD11c and B220 expression. Frequencies of DCs are indicated by the gated subpopulations. (*B* and *C*) CD4$^+$ and CD8$^+$ T cell proliferation *in vivo* after B cell and/or DC depletion and *Listeria* infection. OT-II Thy1.1$^+$ CD4$^+$ cells or OT-I Thy1.1$^+$ CD8$^+$ cells were CFSE-labeled and transferred into Thy1.2$^+$ recipients. WT recipients were treated with control or CD20 mAb, whereas CD11c–DTR mice were treated with DT (DC-depleted) or DT and CD20 mAb (DC- and B cell-depleted). One day after T cell adoptive transfer, mice were infected with *Listeria*. At day 7 after infection, splenocytes were harvested to analyze divisions of donor cells. The thin lines represent adoptively transferred T cells without *Listeria* infection. The bar graphs show CFSE geometric (Geo) mean fluorescence of the whole histogram, which is inversely proportional to cell divisions. The data are representative of two experiments with three mice in each group. Significant differences between means are indicated: *, $P < 0.05$; **, $P < 0.01$.

adoptive transfer (14 ± 1% reduction; $P < 0.01$) and the extent of divisions of OT-II CD4$^+$ T cells in response to *Listeria* infection (Fig. 3*B*). The absence of DCs in B6.CD11c–DTR mice also reduced the frequency of proliferating OT-II CD4$^+$ T cells in response to *Listeria* infection (33 ± 4% reduction; $P < 0.01$), and also dramatically reduced the extent of CD4$^+$ T cell proliferation. Combined B cell and DC depletion further inhibited the frequency of proliferating OT-II CD4$^+$ T cells (76 ± 9%; $P < 0.01$) and almost eliminated their proliferation extent after *Listeria* infection. By contrast, only DC depletion significantly affected the proliferation of adoptively transferred OT-I CD8$^+$ T cells in response to *Listeria* infection (34 ± 11% reduction; $P < 0.01$) (Fig. 3*C*). Thus, DCs alone were not capable of inducing optimal CD4$^+$ T cell responses to a pathogen challenge.

**B Cell Depletion Inhibits Autoantigen-Specific CD4$^+$ T Cell Proliferation.** The effect of CD20 mAb treatment on autoreactive CD4$^+$ T cell expansion was evaluated in murine models of autoimmune diabetes (NOD mice) and arthritis, two T cell-dependent autoimmune diseases in which B cell-depletion significantly reduces disease severity (ref. 12 and Y. Xiu, C. P. Wong, J.-D.B., Y. Hamaguchi, Y.W., S. M. Pop, R.M.T., and T.F.T., unpublished work). Five-week-old female NOD mice were treated with CD20 or control mAb and then received an injection of CFSE-labeled CD4$^+$ T cells from transgenic BDC2.5 NOD mice. BDC2.5 T cells proliferate in response to $\beta$ cell autoantigens including glutamic acid decarboxylase within the pancreatic lymph nodes of NOD mice without any immunization (23). Four days after adoptive transfer, CD20 mAb treatment had no effect on the numbers of BDC2.5 CD4$^+$ T cells migrating to pancreatic and superficial lymph nodes (data not shown). However, B cell depletion significantly reduced the frequency of proliferating BDC2.5 CD4$^+$ T cells (42 ± 6%; $P < 0.05$; Fig. 4*A Left*) and the total relative extent of labeled CD4$^+$ T cell proliferation within pancreatic lymph nodes (Fig. 4*B*), whereas BDC2.5 CD4$^+$ T cell proliferation was not detected in superficial lymph nodes or spleen of either group of recipients (Fig. 4*A Right* and data not shown). Thus, B cell depletion reduced autoreactive T cell proliferation in response to endogenous autoantigens.

The role of B cells in CIA was assessed in DBA/1J mice treated with CD20 or control mAb before collagen immunization as described in ref. 24. DBA/1J mice have a high penetrance of arthritis after heterologous collagen immunizations (25). CD4$^+$ T cells were purified from draining lymph nodes 14 days after immunization, with collagen-specific T cell proliferation being quantified *in vitro*. Responses of CD4$^+$ T cells to recall collagen challenge at 10 and 100 $\mu$g/ml were reduced by 61 ± 8% and 60 ± 2%, respectively, in B cell-depleted mice when compared with control mAb-treated littermates (Fig. 4*C Left*), whereas polyclonal mitogen-induced proliferation remained intact (Fig. 4*C Right*). Thus, B cell depletion inhibited collagen-specific CD4$^+$ T cell proliferation in response to autoantigen challenge.

## Discussion

This study demonstrates that B cell depletion in mice with otherwise intact immune systems significantly reduced CD4$^+$ T cell responses to foreign and self antigens, whereas CD8$^+$ T cell reactivity was unaffected (Figs. 1 and 4). B cell depletion also reduced the conversion of CD4$^+$ T cells from a naive CD44$^{low}$CD62L$^{high}$ phenotype to a memory CD44$^{high}$CD62L$^{low}$ phenotype in response to *Listeria* challenge, whereas CD8$^+$ T cell phenotypes were only modestly affected (Fig. 2). These effects likely result from the absence of B cell and antigen-specific CD4$^+$ T cell interactions because CD20 expression and mAb-mediated depletion were restricted to mature B cells (SI Fig. 5 and Table 1). Moreover, circulating and tissue T cell numbers, subsets, and phenotypes were not affected by short-term B cell depletion (SI Fig. 6 *B–D* and Table 1), and *ex vivo* T cell proliferation in response to polyclonal stimuli remained intact in the absence of B cells (SI Fig. 6*C*). Consistent with the established role of DCs in T cell activation, both B cells and DCs were required for optimal CD4$^+$ T cell activation (Fig. 3). This suggests that CD20 mAb-induced changes in lymphoid tissue architecture (11) do not eliminate DC and CD4$^+$ T cell interactions, their respective functions, or their migration. Moreover, CD20 mAb-induced B cell depletion and monocyte activation did not result in the release of inflammatory cytokines into the serum that could affect T cell responses (SI Fig. 6*A*). Therefore, B cell depletion had specific and selective effects on CD4$^+$ T cell responses *in vivo*.

Inhibiting autoantigen-specific CD4$^+$ T cell activation may elucidate the therapeutic benefit of B cell depletion in both the CIA and NOD mouse models. Arthritogenic collagen-specific CD4$^+$ T cells are essential for CIA induction, perpetuation, and exacerbation (26). CD20 mAb-mediated B cell depletion significantly delays CIA development whether B cells are depleted before collagen immunizations, with modest effects once arthritis first appears (12). Therefore, therapeutic B cell depletion may prevent disease by

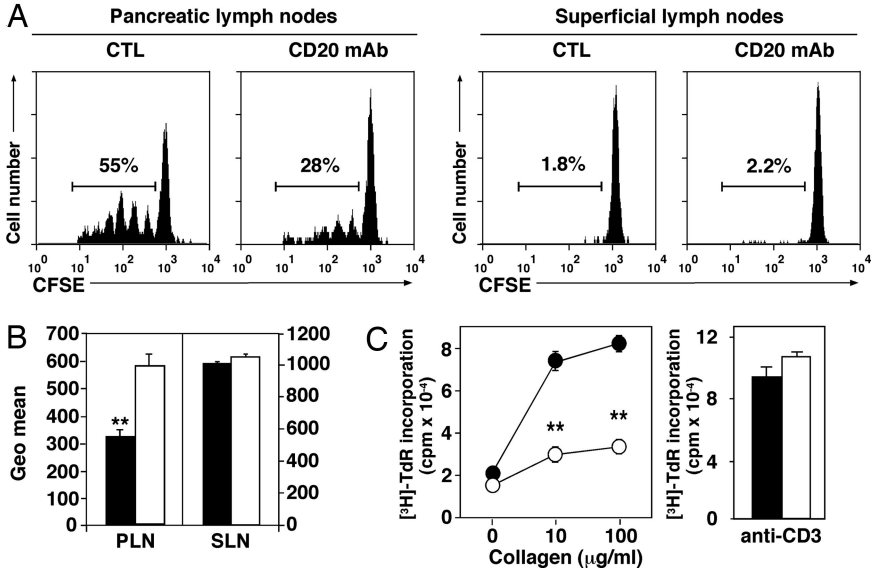


**Fig. 4.** B cell depletion inhibits autoantigen-driven CD4$^+$ T cell expansion *in vivo*. (*A*) B cell depletion impairs β cell-specific CD4$^+$ T cell expansion in pancreatic lymph nodes of NOD mice. CFSE-labeled BDC2.5 CD4$^+$ cells were transferred into NOD recipients that had been treated with CD20 or control (CTL) mAb 7 days earlier. Four days later, pancreatic and superficial lymph node cells were stained for Vβ4/CD4 expression and analyzed for CFSE flourescence intensity by flow cytometric analysis. Representative CFSE profiles are shown. (*B*) Mean CFSE geometric mean fluorescence intensities (± SEM) for CD20 (open bars) and control (closed bars) mAb-treated mice shown in *A*. (*C*) B cell depletion impairs CD4$^+$ T cell proliferation in response to collagen immunization. CD20 (open circles) or control (filled circles) mAb-treated DBA/1J mice were immunized with collagen to induce arthritis. Fourteen days later, CD4$^+$ T cells were purified from draining lymph nodes and incubated with collagen plus mitomycin C-treated B cells from control mice (*Left*) or stimulated with CD3 mAb (*Right*). Values represent mean (± SEM) [$^3$H]thymidine uptake from triplicate cultures. (*A–C*) All data represent results obtained in at least four mice for each group. Significant differences between samplemeans are indicated: **, $P < 0.01$.

reducing collagen-specific CD4$^+$ T cell activation (Fig. 4*C*) in addition to inhibiting autoantibody production. Likewise, B cell depletion delayed autoreactive CD4$^+$ T cell expansion in response to islet β cell autoantigens in NOD mice (Fig. 4*B*). Inhibiting autoantigen-specific T cell activation by B cell depletion may explain diabetes prevention in mature NOD mice with otherwise intact immune systems (Y. Xiu, C. P. Wong, J.-D.B., Y. Hamaguchi, Y.W., S. M. Pop, R.M.T., and T.F.T., unpublished work).

In both the CIA and NOD mouse models, B cell depletion from birth ameliorates disease (27–29). Studies demonstrating a role for B cells in CD4$^+$ T cell priming have also predominantly used mice given anti-IgM antibody since birth (30–33) or mice with genetic defects in B cell development (34). In some studies, the absence of B cells can impair CD4$^+$ T cell priming (28, 31, 32, 35, 36), whereas in other studies, CD4$^+$ T cell priming was not affected (7, 37–40). However, the absence of B cells during mouse development results in significant quantitative and qualitative abnormalities within the immune system, including a remarkable decrease in thymocyte numbers and diversity (41), significant defects within spleen DC and T cell compartments (7, 42, 43), an absence of Peyer's patch organogenesis and follicular DC networks (44, 45), and an absence of marginal zone and metallophilic macrophages, with decreased chemokine expression (43, 45). That T cell numbers and function appear intact in mature mice after CD20 mAb-mediated B cell depletion demonstrates that B cells are critical for normal immune system development but not its maintenance. Thus, induced B cell depletion in mice with intact immune systems may dampen some cell-mediated immune responses without the pleiotropic effects induced by congenital B cell depletion.

The current B cell depletion studies demonstrate that both B cells and DCs are essential for optimal CD4$^+$ T cell responses (Fig. 3). Although an intact B cell compartment was unable to support optimal CD4$^+$ T cell activation when DCs were depleted, B cells were not required for CD4$^+$ T cell proliferation *in vivo* after high-dose OVA challenge (100 μg; Fig. 1*B*). However, low-dose OVA challenge (1–10 μg; Fig. 1*B*) and *Listeria* infection (Fig. 1*C*) required B cells for optimal CD4$^+$ T cell activation *in vivo*. B cells were also required for optimal CD4$^+$ T cell activation in response to high-dose KLH challenge *in vivo* (100 μg) when assessed *in vitro* by using restimulation assays with exogenous B cells as antigen-presenting cells (Fig. 1*A*). That B cells may be required for effective low-dose antigen recognition *in vivo* may reflect a functional balance between DCs, B cells, and other antigen-presenting cell populations. This balance is most likely influenced by the molecular nature of the antigen being presented, its concentration and route of exposure, and the adjuvants present. Additionally, DCs may prime naïve CD4$^+$ T cells *in vivo*, whereas B cells may have specific roles in stimulating antigen-specific CD4$^+$ T cell proliferation after activation by DCs (40).

B cell depletion therapy most likely affects autoimmune disease onset by regulating CD4$^+$ T cell expansion, thereby delaying the inflammatory phase of disease that leads to tissue destruction and pathology. That B cells provided extra and essential functions over and above those provided by DCs for CD4$^+$ T cell activation in response to low-level antigen may be important during early autoimmune disease development, when continuous waves of low-level self-antigen stimulation occur. The effects of B cell depletion on CD4$^+$ T cell activation may explain rare infections in lymphoma patients receiving rituximab with microorganisms generally associated with T cell immunosuppression such as Jamestown Canyon (JC) papovavirus, CMV, or parvovirus B19 (46). B cell depletion may also have other unanticipated effects on T cell effector functions including the growth regulation of non-B cell malignancies. An optimal therapy for disease quiescence may not only require the effective elimination of mature B cells but may also require continuous B cell depletion because environmental triggers are likely to persist indefinitely. Nonetheless, it must be taken into account that autoimmune disease patients are normally given CD20 mAb in combination with immunosuppressive drugs (47). In rheumatoid arthritis, rituximab is often administered in combination

with methotrexate. Therefore, the synergistic combination of B cell depletion and immunosuppression of T cell activation may lead to more significant clinical effects than B cell depletion alone. For example, B cell depletion may block nascent autoantigen-specific T cell activation and autoantibody development, whereas immunosuppression may arrest the clonal expansion of existing autoreactive lymphocytes, thereby ameliorating disease progression. Although multiple factors contribute to autoimmune disease pathogenesis, this insight into the potential mechanism of action for CD20 immunotherapy may lead to the development of additional therapeutic strategies for autoimmune disease management.

## Materials and Methods

**Mice and Immunotherapy.** C57BL/6, B6.PL THy1$^a$/Cy (B6.Thy1.1$^+$), DBA/1J, NOD/LtJ, C57BL/6-Tg(TcraTcrb)425Cbn/JB6 (OT-II), and C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) were from The Jackson Laboratory. OT-II and OT-I transgenic mice generate CD4$^+$ and CD8$^+$ T cells that respectively respond to peptide 323–339 and 257–264 of OVA (18, 19). OT-II and OT-I mice (Thy1.2$^+$) were crossed to B6.Thy1.1$^+$ mice to generate Thy1.1-expressing T cells for adoptive transfer experiments. CD20$^{-/-}$ mice were as described in ref. 48. NOD.Cg-Tg(TcraBDC2.5)1Doi Tg(TcrbBDC2.5)2Doi/DoiJ mice (BDC2.5) (49) were housed and bred at the University of North Carolina at Chapel Hill. CDB6.FVB-Tg(Itgax-DTR/EGFP)57Lan/J (B6.CD11c DT receptor transgenic mice, CD11c–DTR) as originally described were provided by M. D. Gunn (Duke University). To deplete CD11c$^+$ DCs *in vivo*, CD11c–DTR transgenic mice were treated i.p. with 100 ng DT (Sigma) in 200 $\mu$l of PBS as described in ref. 22. To induce *in vivo* B cell depletion, sterile CD20 (MB20-11, IgG2c) or isotype-matched control mAbs (250 $\mu$g) were injected in 200 $\mu$l of PBS through lateral tail veins (9). All mice were bred in a specific pathogen-free barrier facility and used at 8–12 weeks of age unless otherwise noted. Duke University and University of North Carolina, Chapel Hill, Animal Care and Use Committees approved all studies.

**Immunizations and *in Vitro* T Cell Proliferation Assays.** For KLH assays, mice were injected s.c. with KLH (100 $\mu$g; endotoxin free; Sigma) in complete Freund's adjuvant (Sigma). After 7 days, $3 \times 10^5$ purified CD4$^+$ or CD8$^+$ T cells harvested from draining lymph nodes were cultured in 96-well plates in 200 $\mu$l of complete RPMI 1640 culture medium with $1.5 \times 10^5$ mitomycin C-treated (Sigma) B cells and KLH (0, 1, and 10 $\mu$g/ml). Collagen immunizations in male DBA1/J mice used type II chicken collagen (Chondrex) dissolved in 10 mM acetic acid solution (5 mg/ml) overnight at 4°C. Dissolved collagen (100 $\mu$g) was emulsified with an equal volume of complete Freund's adjuvant, with 100 $\mu$l being injected s.c. into the base of the tail. For collagen-specific CD4$^+$ T cell proliferation, draining lymph nodes were harvested 14 days after collagen immunization, with $5 \times 10^5$ T cells being cultured with $1 \times 10^6$ mitomycin C-treated (Sigma) B cells and T cell proliferation grade type II collagen (0, 10, and 100 $\mu$g/ml; Chondrex), as recommended by the manufacturer. Proliferation was measured by [$^3$H]thymidine incorporation during the final 12-h of 4-day cultures, followed by scintillation counting. In some experiments, thymidine incorporation was measured after 48 h of CD4$^+$ T cell stimulation with tissue culture plate-bound CD3 mAb (5 $\mu$g/ml; BD PharMingen).

**Adoptive Transfer Experiments.** Donor Thy1.1$^+$ OT-II CD4$^+$ or OT-I CD8$^+$ T cells from pooled spleens and lymph nodes were labeled with CFSE fluorescent dye (1 $\mu$M; Vybrant CFDA SE; Invitrogen–Molecular Probes) according to the instructions of the manufacturer. Labeled Thy1.1$^+$ cells ($5 \times 10^6$) were given i.v. to Thy1.2$^+$ congenic recipients. Recipient mice were immunized i.p. with alum precipitated OVA (Sigma) or were given *Listeria* monocytogenes producing OVA peptide (amino acids 134–387) capable of activating both OT-II CD4$^+$ and OT-I CD8$^+$ T cells (20). Bacteria were grown in brain–heart infusion media supplemented with 5 $\mu$g/ml erythromycin (Sigma), with $5 \times 10^5$ colony-forming units (0.1 $LD_{50}$)/mouse in 200 $\mu$l of PBS injected i.v. (lateral tail vein). The proliferation of transferred cells was visualized by flow cytometric analysis of $1 \times 10^4$ CFSE-labeled Thy1.1$^+$ cells. Transferred OT-II CD4$^+$ or OT-I CD8$^+$ T cells were identified by Thy1.1 and CD4 or CD8 mAb staining, respectively. For adoptive transfer experiments involving BDC2.5 CD4$^+$ T cells, $2 \times 10^6$ CFSE-labeled cells were injected i.v. into female NOD recipients, and T cell proliferation was analyzed after staining with CD4 and V$\beta$4 mAbs.

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