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B cell-depletion curtails CD4+ T cell memory and reduces protection against disseminating virus infection[1],[2]

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Abstract

Dynamic interactions between CD4+ T cells and B cells are needed for humoral immunity and CD4+ T cell memory. It is not known whether B cells are needed early on to induce the formation of memory precursor cells or are needed later to sustain memory cells. Herein, primary and memory CD4+ T cells responses were followed in wildtype mice that were depleted of mature B cells by anti-CD20 before or different times after acute lymphocytic choriomeningitis virus (LCMV) infection. The antibody treatment led to a 1000-fold reduction in B cell number that lasted 6 weeks. Primary virus-specific CD4+ Th1 cells were generated in B cell-depleted mice, however, there was a decrease in the $CD4^{+}Ly6C^{lo}Tbet^{+}$ memory precursor population and a corresponding 4-fold reduction in CD4+ memory cell number. Memory T cells showed impaired cytokine production when they formed without B cells. B cell-depletion had no effect on established memory populations. During disseminating virus infection, B cell depletion led to sustained weight loss, functional exhaustion of CD4+ and CD8+ T cells, and prevented mice from resolving the infection. Thus, B cells contribute to the establishment and survival of memory CD4+ T cells following acute infection and play an essential role in immune protection against disseminating virus infection.

Introduction

Millions of patients are treated with drugs to deplete autoreactive B cells. In rare instances, there is an association between the loss of B cells and reduced immunity against pathogens (1, 2). B cell depletion (such as by anti-CD20; eg Rituximab) is a successful therapy for treating rheumatoid arthritis and non-Hodgkin's lymphoma (3, 4), yet it compromises T cell immunity and increases susceptibility to opportunistic infections (1, 2). While some evidence indicates that B cell depletion therapies have minimal effects on patient disease course & infections (5, 6) other data indicate that B cell-depletion in increases the risk for progressive multifocal leukoencephalopathy, which is caused by re-activation of a common latent polyoma virus infection, the return of active hepatitis B virus infection, as well as other serious systemic infections, and potentially impaired vaccine-induced T cell responses (1, 2, 7, 8).

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[2]LCMV, lymphocytic choriomeningitis virus.

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Previously, we showed that congenitally B cell-deficient mice (μMT−/−) generate primary T cell responses to acute LCMV infection; however, those mice have a selective defect in CD4+ T cell memory (9). CD4+ T cells play a central role as the immune system confronts infection (10). Their frequency correlates with vaccine-induced protection in people: individuals with deficiencies in CD4+ T cell memory are not protected well by vaccines, are susceptible to opportunistic infections, and have recurring reactivation of latent virus infections. Antigen-specific CD4+ T cells promote vigorous humoral and cellular responses that protect against pathogens, including recall CTL responses that are protective against re-infection (11–14) and act during the memory phase to maintain and/or improve CD8 memory (15). Virus-specific CD4 T cells interactions actively sustain CD8 responses during persistent virus infection (16–21) in part by producing IL-21 (22–24). Memory CD4+ T cells can directly suppress infection due to their rapid production of IFNγ (25), directly kill MHCII+ target cells (26), and enhance innate responses (27). Our previous analyses showed that B cell-deficient μMT−/− mice are unable to resolve disseminating virus infections due to defects in cellular immunity (9).

B cells contribute to T cell responses in ways that are independent of antibody production (9, 28, 29). B cells express MHC-II, co-stimulatory molecules, lymphotoxin, TNF, and OX40L and other cytokines, to interact with and activate antigen-specific CD4+ T cells, influencing their differentiation into effector cells or memory (30–41). B cells stimulate memory CD4+ T cell differentiation and promote T_{FH} cell differentiation in infection and vaccination models (42–50). In other circumstances, unique regulatory signals may be communicated by B cells to T cells after infection or vaccination (51–53). B cells also contribute to lymphoid organogenesis, and mice that are congenitally deficient in B cells show profound defects in spleen organization and cellularity that may affect T cell responses. During development, B cells produce lymphotoxin and TNF to differentiate B cell and T cell zones that draw emigrants from the thymus. In this capacity, B cells are involved in normal T cell-B cell segregation and microstructure of the spleen and populating the spleen with other cell types (follicular dendritic cells, fibroblastic reticular endothelial cells, marginal zone populations, dendritic cells). Thus, congenital absence of B cells reduces the frequency other cell types, including dendritic cells and phagocytic macrophage populations (54) that produce sustained interferon responses (55), and the number of mature naïve T cells that are available in this organ to mount adaptive T cell responses. Finally, B cells can directly limit virus infection, for example, by expressing LTb to stimulate marginal zone macrophage type-1 interferon expression to limit the spread of VSV into neurons (56, 57).

It is not known whether B cells program early memory cell precursors, affect the establishment of memory levels, or act during the maintenance phase to regulate memory CD4 cell number. Moreover, it is unknown whether the effect of B cells on CD4+ T cell memory is mediated by direct B cell interaction, B cell cytokine production, or B cell-dependent lymphoid organ structure. Herein, we sought to better understand the role of B cells at different stages of the T cell response after acute virus infection by transiently depleting B cells early on or during established memory in immune mice. Using this approach, the effect of B cells on CD4+ T cell memory and immune protection could be disentangled from the effects of B cells on lymphoid architecture. We found that the depletion of B cells during the expansion and contraction phases reduced the number of memory cell precursors and the number and function of memory CD4+ T cells, but these effects were not as severe as observed in μMT−/− mice. B cell depletion in immune mice resulted in no apparent loss of CD4 memory cells, indicating that established memory CD4+ T cell populations are not linked to B cells. Finally, we show that B cell depletion had a profound detrimental effect on the immune response to persisting virus infection, highlighting the key role of B cells in sustaining T cell responses against disseminating virus infection.

Materials & Methods

Mice and virus

We used C57BL/6 mice, SMARTA TCR-transgenic mice specific for the I-A^b LCMV epitope GP_{61-80} (58) and μMT−/− (B cell-deficient) mice (59). Adult mice (8–10 weeks old) were infected by i.p. administration of 2×10^5 plaque forming units of LCMV (Armstrong strain). Some mice were given an intravenous injection of 2×10^6 PFU of LCMV-Armstrong, LCMV-t1b, or LCMV-A22 for re-challenge experiments. All mouse experiments were approved by the University of North Carolina Hill Institutional Animal Care and Use Committee. Viral stocks of plaque-purified LCMV were prepared from infected BHK-21 monolayers.

Anti-CD20 mAbs

We received anti-CD20 mAbs (aCD20: clone 5D2) from Genentech. Mice were given one or two doses of anti-CD20 (200 μg/mouse in 100μl volume, i.p.) or same amount of isotype control antibody (IgG2a, anti-KLH) diluted in phosphate buffered saline.

Flow cytometry

Single-cell suspensions of splenocytes were surface stained with combinations of fluorescently labeled monoclonal antibodies that were specific for CD4 (clone RM4-5), CD8 (53–6.7), CD19 (6D5), B220 (RA3-6B2), CD11a (M17/4), KLRG1 (2F1/KLRG1), CD127 (A7R34), Thy1.1 (HIS51), CCR7 (4B12), ICOS (7E.17G9), CD20 (AISB12), MHC II (M5/114/15/2), F4/80 (BM8), and Ly6c (HK1.4). CXCR5 was detected through a 3-step staining protocol (45) using purified rat anti-CXCR5 (clone2G8), biotin-anti-rat IgG (polyclonal sera), then streptavidin-allophycocyanin (Invitrogen). Monomers or Streptavidin-APC-conjugated tetramers ($DbGP_{33-41}$ and I-$AbGP_{67}$) were provided by the NIH Tetramer core facility at Emory University. The intracellular staining (ICCS) assay was performed by culturing splenocytes with or without LCMV peptide in the presence of brefeldin A. After 5 h of incubation, cells were stained for surface markers, washed, fixed with formaldehyde, then permeabilized and exposed to mAbs specific for IFN-γ (XMG1.2), IL-2 (JES6-5H4), T-bet (4B10), and TNF (MP6-XT22). Antibody stained cells were detected by a FACSCalibur cytometer (BD Biosciences) and the data were analyzed with FlowJo software (Tree Star). All listed mAbs above were purchased from Biolegend, except for CXCR5 (BD Biosciences) and CD11a (eBioscience).

ELISAs

Serum IFN-α was quantified by VeriKine Mouse Interferon-Alpha ELISA Kit (PBL, Piscataway, NJ) according to manufacturer's instructions. LCMV-specific serum antibody was quantified by using ELISA plates (Greiner bio-one, Monroe, NC) that were coated with lysates from LCMV-infected BHK cells as described previously (60).

Statistics

Statistical analyses and graphing were done with Prism software (www.graphpad.com). An unpaired two-tailed Student's t-test was employed to evaluate the significance of differences between groups.

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Results

SMARTA CD4+ T cells fail to establish a memory population in μMT−/− mice

Our earlier analyses showed that endogenous CD4+ T cells in μMT−/− mice respond to LCMV infection but fail to establish memory cells that make cytokine (9). The μMT−/− mice have smaller spleens than WT mice before infection, so it is plausible that the repertoire of virus-reactive CD4+ T cells in these mice does not include subpopulations of effector cells that are capable of further differentiation into memory. A limitation with the earlier analyses was that the detection of virus-specific T cells was based on a functional readout (eg, ICCS), thus it is plausible that virus-specific memory CD4+ T cells were present in immune μMT−/− mice but were unable to produce cytokine. To better understand the underlying CD4+ T cell defects in these mice and circumvent these concerns, we utilized an adoptive transfer model where a small number of SMARTA CD4+ T cells, which express a TCR-transgene specific for LCMV-$GP_{61\text{-}80}$ in I-Ab, were adoptively transferred into either WT or μMT−/− mice followed by LCMV-Armstrong infection. In this experimental setup, the differentiation process of the same population of T cells is examined in mice with B cells or with no B cells. Additionally, the differentiating cells can be identified due to their surface expression of congenic markers (Ly5a or Thy1.1) and quantified by direct surface staining, independent of their production of cytokine.

At day 8, the primary CD4+ T cell response was vigorous in both groups of mice (Fig. 1A), consistent with our earlier analyses of the endogenous response (9), and the mice resolved the infection in the liver, lung, and kidney as measured by plaque assay (data not shown). We observed that the majority of SMARTA CD4+ T cells made IFNγ, TNF, and IL-2 in both hosts, but there was no significant difference in the percentage of SMARTA cells co-expressing IFNγ with IL-2 (Fig. 1B and Fig. 4D). Interestingly, the amount of IFNγ and IL-2 made per cell, as revealed by gMFI of cytokine staining, was greater for SMARTA CD4+ T cells in the μMT−/− mice than in the WT mice (Supplemental Figure 1B). Thus, the expansion of SMARTA CD4+ T cells is largely normal, but the cytokine output of SMARTA CD4+ T cells is increased when the cells differentiate in the absence of B cells.

The T cell response was examined at day 44, which is during the memory phase. The memory SMARTA CD4+ T cells represented ~0.3% of splenocytes in the WT mice; however, SMARTA CD4+ T cells were near the limits of detection in the μMT−/− mice (Fig. 1C). These frequencies corresponded to approximately 6×10^4 memory SMARTA CD4+ T cells in WT mice and ~300 memory SMARTA CD4+ T cells in the μMT−/− mice (Fig. 1D). Thus, there was a 220-fold reduction in memory in the μMT−/− mice. The defect was specific for CD4+ T cells, since the memory CD8+ T cell response in the μMT−/− mice appeared normal (**data not shown** and (9, 61)). These data indicate that the loss of CD4 memory in immune μMT−/− mice is due to the physical absence of memory cells rather than the persistence of functionally impaired CD4 memory cells.

The above data show that the same cells undergo different fates depending on the recipient mouse. Memory cell differentiation begins early on with a subset of effector cells (memory precursor cells) transiting into the memory pool (62). The loss of CD4 memory in the B cell-deficient mice could represent a defect in the formation of memory precursor cells among the effector cells, or it could be that the mice generate memory precursor cells but fail to sustain them during the contraction or memory phases. Thus, we wished to determine whether the defect in memory was intrinsic to the T cells by day 8 or if it was related to the B-less host environment. To address these two possibilities, we engrafted SMARTA cells into WT or μMT−/− mice followed by LCMV-Armstrong infection to induce effector T cells, including memory precursors. At day 8, the reactive SMARTA CD4+ cells were isolated from the 2 groups of mice, mixed in equal proportion, and then transferred to the same infection-matched WT hosts (Fig. 2A). In this experimental setup, the early differentiation steps of CD4+ T cells occurred in the presence or absence of B cells, but the subsequent survival of the CD4+ T cells could be compared in the same host. After another 32 days, which is sufficient for the donor cells to transit into memory, the mice were either left unchallenged to evaluate the establishment/maintenance of memory cells under homeostatic conditions or challenged with high-dose LCMV-Armstrong to evaluate the recall response of the donor memory cells. The effector CD4+ T cells from the μMT−/− mice and WT mice established comparable levels of memory when transferred into WT mice (Fig. 2B, **left, white bars**). Thus, the effector CD4+ T cells generated in μMT−/− mice are capable of forming memory cells. These quiescent memory cells were also functional, as they were responsive to the re-challenge infection and underwent expansion (Fig. 2B, **left, shaded bars**).

The 2 populations of effector SMARTA CD4 T cells were also transferred to μMT−/− mice. However, the abundance of the memory SMARTA CD4+ T cells was greatly reduced in the μMT−/− mice compared to the WT recipients (Fig. 2B, **right, white bars**), indicating that both donor cells poorly transitioned to memory in these hosts. Neither donor cell population responded vigorously upon re-challenge (Fig. 2B, **right, shaded bars**). These data show that CD4 memory precursors can form without B cells, however μMT−/− mice fail to sustain these cells into memory.

Sustained depletion of B cells by anti-CD20 monoclonal antibody

The above data show that despite the early induction of CD4+ T cells that are capable of becoming memory cells, the long-term survival of CD4 memory was defective in μMT−/− mice due to the physical loss of memory cells. There are several possible explanations for the defect in memory in the μMT−/− mice. The μMT−/− mice have a disorganized spleen structure (63), so there may be a deficiency in stromal cell numbers resulting in low basal homeostatic cytokines, such as IL-7, that are needed to sustain memory CD4+ T cells. Alternatively, the μMT−/− mice have defects in critical B cell-derived cytokines that affect CD4+ T cell survival. To better understand the role of B cells at different stages of CD4 memory formation, we analyzed T cell responses in WT mice that have normal stromal cell organization but were depleted of B cells by monoclonal anti-CD20 antibody at different times after infection.

CD20 is expressed on immature B cells, mature B cells, and a subset of memory B cells but not pro-B cells, plasmablasts, or plasma cells (3, 64). The expression of CD20 has been reported for unchallenged mice (40, 64), but the expression pattern of molecules can change dramatically in the context of infection due to the inflammatory environment. We found that CD20 in the spleen was confined to B cell populations and not induced on T cells, macrophages, or DC subsets after infection (**data not shown)**. We next evaluated the effect of anti-CD20 treatment on B cell frequencies in uninfected mice or in mice that were given LCMV-Armstrong. The depleting monoclonal antibody against CD20 acts by inducing either FcγR-dependent (ADCC) or complement-mediated lysis of target cells (4, 65). In uninfected mice, B cells were effectively depleted by anti-CD20, as CD19+B220+ B cell populations were reduced from 45% to < 0.5% in the spleen and the depletion was maintained for 45 days (Fig. 3A). A similar loss of CD20+ B cells was also observed (**data not shown**). A kinetic analysis of B cell depletion in uninfected mice revealed that the vast majority of B cells (>98%) were eliminated within 1 week after anti-CD20 was given, and the depletion lasted 40 days (Fig. 3B). After day 45, the frequency of B cells began to increase until normal frequencies were observed by day 60. A similar analysis of anti-CD20-treated mice that were subsequently given LCMV-Armstrong showed that there was a 2-log decrease in B cell numbers within one week and the B cells remained low in number for 40 days (Fig. 3C). The anti-CD20 resulted in serum levels of anti-LCMV antibody were at or below detection at day 40 (Fig. 3D). Thus, anti-CD20 treatment is specific, long-lasting with a half-life of nearly 1 month, and is unaffected by the inflammation associated with live infection.

Previously, it was shown that μMT−/− mice and other mice with disrupted splenic organization have deficient type-1 interferon responses (54). B cells maintain a subcapsular sinus population of macrophages that are responsive to VSV infection and make protective amounts of type-1 interferon (56, 57). The early interferon production during LCMV infection is from pDC and then macrophage populations (54, 55). Therefore, we examined the B cell-depleted mice for serum levels of IFN and found that they were partially reduced in the anti-CD20-depleted mice, but not to the extent observed in μMT−/− mice (Fig. 3E). These results show that even when lymphoid organogenesis unfolds normally, B cells contribute indirectly to the interferon response.

Vigorous primary T cell response but reduced CD4 memory to acute LCMV infection in the absence of B cells

The data in Figure 2 indicate that B cells are required after day 7, during the contraction or memory phases, to sustain CD4 memory. To better understand when B cells are needed, cohorts of WT mice were treated with anti-CD20 or isotype control antibody at days -14 and day -7 to remove B cells (Fig. 4A). LCMV-specific Thy1.1+ SMARTA CD4+ T cells were engrafted into these mice so that we could follow the same population of cells in the different hosts without the concern of B cell effects on stromal architecture. The engraftment of the naïve SMARTA CD4+ T cells was not affected by the depletion of B cells when there was no infection (data not shown). The mice were then infected with LCMV-Armstrong and the viral load and virus-specific T cell and B cell responses were quantified at 8 days after infection. This treatment regimen led to a significant loss of B cells at day 8 (Fig. 4B), and the mice controlled the infection in the liver, lung, and kidney at this time (data not shown). Consistent with our earlier analyses of endogenous CD4+ T cells, control-treated WT mice, B cell-depleted mice, and μMT−/− mice generated elevated frequencies of SMARTA CD4+ T cells (Fig. 4C), representing ≥1000-fold expansion of cells in all groups. However, there was a significant ~2-fold lower number of SMARTA CD4+ T cells in the B cell-depleted mice compared to B cell-sufficient mice, as observed previously for μMT−/− mice (9). The proportion of SMARTA CD4+ T cells expressing IFNγ, IL-2, and TNF was unaffected by B cell-depletion or marginally increased (Fig. 4D, Supplemental Fig. 1A–B & data not shown), indicating that effector differentiation was not inhibited by the absence of B cells. Primary virus-specific CD8+ T cell numbers were unaffected by the loss of B cells (Fig. 4E), as observed previously for μMT−/− mice.

Following the peak response, T cells undergo contraction in number and establish a pool of memory cells. We assessed whether the early depletion of B cells would impact T cell memory. At day 40 post-infection, the B cell-depleted mice continued to show a 1000-fold reduction in B cell number compared to isotype-treated mice (Fig. 4F). The immune control-treated mice contained ~15×10^4 memory SMARTA CD4+ T cells. However, there was a reduction in this number to ~5×10^4 memory CD4+ T cells in the B cell-depleted mice (Fig. 4G), indicating that approximately 2/3rds of CD4 memory requires B cells, even when lymphoid organogenesis is normal. The number of memory CD4+ T cells in μMT−/− mice was reduced ~1000-fold compared to the immune control mice, consistent with our earlier findings concerning endogenous CD4+ T cell responses and the data in Figure 1 (9). The memory SMARTA CD4+ T cells in B cell-depleted mice showed significant reductions in the percentage of cells that expressed IFNγ, IFNγ with IL-2, or IFNγ with TNF compared to cells in control immune mice (Fig. 4H **& data not shown**). In contrast to CD4 memory cells, there was no loss in the number of cytokine+ve CD8+ memory T cells (Fig 4I), consistent with earlier analyses (9). Thus, B cells are required for most of the memory CD4+ T cell pool and improve the capacity of memory CD4+ T cells to make cytokine. The greater loss of CD4+ T cell memory in μMT−/− mice suggests that lymphoid architecture plays a major role in the maintenance of CD4+ T cell memory but not CD8+ T cell memory.

In this same cohort of mice, B cells returned to normal abundance by day 70 (Fig. 4J). At this time, memory SMARTA CD4+ T cells returned to normal number in the mice that had been depleted of B cells but not the μMT−/− mice (Fig. 4K). Despite the recovery in B cell number and the increase in SMARTA cell abundance, memory SMARTA CD4+ T cells continued to show deficits in cytokine output. For example, the percentage of SMARTA CD4+ cells that expressed IFNγ with IL-2 remained lower than that observed in isotype-treated immune mice (Fig. 4L). The per cell amount of IFNγ was reduced; although there was no significant difference in IL-2 or TNF output between the two groups of mice (Supplemental Fig. 1C & data not shown). By comparison, the few detectable SMARTA CD4+ T cells present in the μMT−/− mice showed highly significant reductions in their ability to produce multiple cytokines and there were major reductions in cytokine output per cell (Fig. 4L, Supplemental Fig 1C). These data suggest that memory CD4+ T cells accumulate deficiencies in cytokine production across time when there are no B cells. The recovery in memory CD4+ T cell frequency that is associated with increases in B cell number does not rescue memory CD4+ T cell defects in cytokine production.

Throughout these times after infection, virus-specific CD8+ T cells remained at elevated frequencies without B cells (Fig. 4E, 4I, 4M), and the recovery of B cells did not result in more memory CD8+ T cells, underscoring the independence of B cells and CD8+ T cell number. However, there were functional and phenotypic changes in that compartment that emerged during the memory phase. There were no apparent alterations in cytokine output early on (Supplemental Fig. 2A–C), but by day 40 there were reductions in the percentage IFNγ+IL-2+ double positive CD8+ T cells in B cell-depleted mice and in μMT−/− mice and lower amounts of cytokine made by the memory CD8+ T cells in these mice (Supplemental Fig. 2D–E). These reductions in cytokine production continued to day 70 when B cell numbers recovered in the B cell-depleted mice (Supplemental Fig. 2F–G). During the memory phase, the CD8+CD11a^{hi} population that contains all of the virus-specific memory CD8+ T cells (66, 67) were evenly distributed between CD8+ T cells with the short-lived effector cell (SLEC) phenotype and those with the characteristic markers of long-lived memory cells (MPEC) (68) that express IL-7R (Supplemental Fig. 2H). In contrast, the majority of memory phenotype CD8+ T cells in the B cell-deficient mice were SLEC. Thus, stable pools of memory CD8+ T cells form in B cell-depleted mice as observed for μMT−/− mice (9, 61, 69–71), but the T cells appear activated and show reductions in cytokine production.

B cell depletion does not diminish established T cell memory

The analyses in above involve mice that were depleted of B cells before infection, but the duration of B cell-loss continues through the contraction and memory phases of CD4+ T cells (Fig. 3). To better understand whether B cells function early on or during the contraction phase or memory maintenance phases, we explored the effect of staggering the B cell depletion to later times after infection. A cohort of mice containing SMARTA cells were given acute LCMV. At day 7, the mice were depleted of B cells or not, and the number and function of memory cells was examined on day 21 (Fig. 5A). At day 21, the control-treated mice had 2×10^7 B cells (Fig. 5B) and 6×10^5 memory SMARTA cells (Fig. 5C). In contrast, B cell-depleted mice had ~10^5 B cells and a 2.4-fold reduction of memory CD4+ T cells (Fig. 5B–C) and these cells were less able to make IFNγ (Fig. 5D). By day 70 the

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number of B cells and memory CD4+ T cells was equivalent to the un-depleted mice (**data not shown)**. Thus, B cell-depletion initiated during the contraction phase exaggerates the loss of early memory cells, but the effect is reversed when B cells recover in number.

Another cohort of mice were immunized and allowed to establish T cell memory. During the memory phase (day 40 post-infection), some of the immune mice were given isotype-control antibody or anti-CD20 to deplete B cells (Fig. 5E). Two weeks later (day 54), the number of B cells and memory T cells was quantified. There was a 200-fold reduction in B cells in the spleen (Fig. 5F); although there remained approximately 10^5 B cells, some of which are likely memory B cells, plasmablasts, or germinal center B cells (64, 72, 73). The numbers of memory SMARTA CD4+ T cells and CD8+ T cells were unaffected by this reduction in B cells (Fig. 5G & **data not shown**), although there was a modest reduction in the percentage of SMARTA CD4+ T cells that could make IFNγ (Fig. 5H). Another set of immune mice that were allowed to recover B cells and where analyzed at day 210 post-infection also showed no apparent change in the number or cytokine production of memory T cells compared those in mice that had B cells the entire time. Thus, B cell-depletion does not cause major changes in T cell memory once it is established.

Early B cell depletion reduces memory precursor populations

Thus far, the data show that early B cell-depletion before infection or during the contraction phase reduces the formation of CD4 memory (Fig. 4 & 5). We next examined whether this is due to a defect in the formation of memory precursor cells. Th1 and T_{FH}-like CD4+ T cells can form early after infection and represent distinct lineages that develop memory (43–47). Among the virus-specific CD4+ T cells at day 8, memory precursors are found that re-express IL-7R and are Ly6C^{lo} and T-bet^{+} (62). In the infected isotype-control treated mice, approximately 37% of SMARTA CD4+ T cells were Ly6C^{lo}Tbet^{+} (Fig. 6A & B). By comparison, there was a statistically significant decrease in the proportion of T cells that were Ly6C^{lo}Tbet^{+} in B cell-depleted mice and a greater decrease in the μMT−/− mice (Fig. 6A & B). Thus, the lower level of memory in the B cell-depleted mice is associated with a reduction in early effector cells with memory potential.

Among the memory precursors within the effector cell population, a subset expresses the “central memory” phenotypic marker, CCR7, which maintains CD4+ T cells in T cell zones and is associated with improved homeostatic survival across time (48, 62). About a quarter of effector SMARTA CD4+ T cells expressed CCR7 in WT mice and in B cell-depleted mice (Fig. 6C), but this population was greatly reduced in the μMT−/− mice. These data suggest that memory precursors form at reduced levels without B cells but these cells are capable of undergoing homeostatic cell division to recover their number when placed in mice with B cells (Fig. 2) or when B cells return in B cell-depleted mice (Fig. 4).

A number of studies have shown a direct relationship between B cells and the formation of T_{FH} CD4+ T cells (43–48). Consistent with those studies, far fewer T_{FH} developed in μMT −/− mice than B cell-sufficient mice (Fig. 6D–E). By comparison, B cell-depleted mice showed a significant reduction in the number of CXCR5+ICOS+ CD4+ T cells compared to B cell-sufficient mice, yet there were significantly more T_{FH} in the B cell-depleted mice than in the μMT−/− mice (Fig. 6D–E). Thus, approximately 1/3 of the T_{FH} response can occur without B cells when stromal architecture is preserved.

B cell depletion impairs T cell responses to disseminating virus infection and results in exaggerated weight loss

μMT−/− mice are highly susceptible to disseminating virus infections (9, 74–76). When confronted with LCMV-Clone13, LCMV-t1b, or LCMV-A22 strains, μMT−/− mice lose virus-specific CD4+ T cell responses as well as CD8+ T cell responses, partly through exaggerated T cell loss and through functional T cell exhaustion. The data above (Fig. 4) for acute infection show that early B cell-depletion results in an intermediate phenotype where CD8+ T cells are largely unaffected numerically and CD4 memory is transiently reduced until B cells recover in number. We next asked whether these effects would impair immunity to a more aggressive infection. It is well established that CD4+ T cells are required to sustain CD8+ T cell responses during infections with disseminating strains of LCMV (17, 19, 22, 23, 77–80). Without T-help, virus-specific CD8+ T cells undergo deletion or functional exhaustion. We sought to learn whether B cell-depletion affects the ability of mice to eliminate disseminating LCMV infection. Groups of mice were depleted of B cells or given isotype-control antibody and then exposed to LCMV-t1b. All mice showed similar amounts of weight loss during the first week after infection (Fig. 7A). However, the B cell-sufficient mice recovered weight over the next week. In contrast, μMT −/− mice showed greater weight loss that was sustained until day 21. The B cell-depleted mice demonstrated even more weight loss than the infected μMT−/− mice with no evidence of recovery (Fig. 7A).

The viral load and antiviral T cell responses were evaluated at day 21 post-infection. The B cell-sufficient mice controlled replicating LCMV-t1b in the serum, liver, lung, and had reduced levels of virus in the kidneys at day 21 (Fig. 7B). In contrast, B cell-depleted mice and μMT−/− mice showed several logs increase in viral titers in each of these compartments (Fig. 7B). The inability of B cell-deficient mice to resolve this disseminating infection correlated with poor T cell responses. Tetramer+ve (DbGP$_{33\text{-}41}$) CD8+ T cells were present in all three groups, but there was a greater than 10-fold reduction in tetramer+ CD8+ T cells in B cell-depleted mice compared to B cell-sufficient mice (Fig. 7C–D, **top**). Compared to the B cell-sufficient mice, there was a ~50-fold reduction in the number of CD8+ T cells capable of making IFNγ in the B cell-deficient mice (Fig. 7C–D, **bottom**). Tetramer+ve (I-AbGP$_{67}$) CD4+ T cells and IFNγ-competent CD4+ T cells were reduced 10–20-fold in the B cell-depleted mice (Fig. 7E–F). Thus, B cell-depletion severely decreases CD4+ and CD8+ T cell responses through a combination of reduced T cell number and induction of functional exhaustion, preventing virus control and increasing pathogenesis.

Discussion

Several drugs that deplete B cells are given to treat people with autoimmune disorders. These therapies are highly tolerated and adverse problems are relatively rare. However, there is evidence that some individuals develop recurrent infections that depend on cellular immunity (1, 2). To better understand the role of B cells in the formation of T cell responses, we compared antiviral T cell responses in B cell-depleted mice, μMT−/− mice, and B cell-sufficient mice. We show that early T cell differentiation unfolds normally in μMT−/− mice, but these mice fail to support CD4+ T cell memory. However, effector T cells that form in the absence of B cells are capable of surviving on into memory when transferred to B cell-sufficient mice. We evaluated the role of B cells in mice that are not congenitally deficient in B cells but were depleted early on or during the memory phase of the T cell response after virus infection. We confirmed that B cells are not needed to generate primary T cell responses or eliminate acute virus infection. However, eliminating B cells before infection or during the contraction phase reduced memory CD4 T cell number and cytokine production, although not to the extent observed in mice that are congenitally deficient in B cells. Once T cell memory was established, B cell depletion had minimal affect on CD4 or CD8 memory. There appears to be a direct linkage between the frequency of B cells and CD4+ T cell memory: when B cells recovered from antibody-mediated depletion, memory SMARTA cells also increased numerically. We also found that B cells play a key role in sustaining cellular immunity to a disseminating virus infection. Taken together, the data indicate that B cells improve the early establishment & survival of memory T cells following acute infection and are involved in the vigorous T cell responses that are needed to eliminate aggressive infections. A prediction based on our study is that B cell depletion therapies in people who are otherwise immunologically intact will leave their pre-existing T cell memory intact, but they will form weaker CD4 T cell memory to new antigens and will be at risk for systemic infections.

A major limitation with using μMT−/− mice is that their spleens do not form properly, which can potentially impact T cell responses (81). Herein, we circumvented this problem by following T cell responses in mice that generated spleens with proper microarchitecture. One caveat to our analyses is that long-term B cell depletion mice may eventually disrupt some of the existing microarchitecture. For example, there was a gradual loss of marginal zone cells when B cells were conditionally eliminated by an interferon inducible Cre/Igα-floxed mouse model (82). Likewise, CD70-transgenic mice gradually loose B cells and marginal zone structure (82). These long-term changes in lymphoid organization may impact subsequent cellular immune responses to infection. For example, phagocytic populations in the marginal zone contribute to sustained interferon production during active LCMV infection (54, 55) and direct IFN signaling drives T cell differentiation into memory (83–85). We observed a reduction in type-1 interferon in μMT−/− mice, as reported previously (54), and a partial reduction in interferon in the B cell-depleted mice (Fig 3E). The reduction in interferon may be caused by the loss of a macrophage population or pDC in the spleen following B cell-depletion. Nevertheless, the reduction in type-1 interferon was not so severe to prevent significant induction of T cell responses and memory as observed in type-1 interferon-deficient mice. Moreover, delaying the B cell depletion until after the early inflammatory response (Fig. 5C) showed that B cells increase early CD4 memory.

CD4 memory is reduced when B cells are depleted. We observed that SMARTA CD4+ T cells were CCR7+ in the B cell-depleted mice (Fig. 6C), and memory CD4+ T cells that are CCR7+ are thought to have greater proliferative and self-renewing capacity compared to CCR7− CD4+ T cells (43). In contrast, μMT−/− mice induced CD4+ T cells that were mostly CCR7− and likely had reduced memory cell potential (43), and the μMT−/− mice fully loose memory cells with time (Fig. 1). The μMT−/− mice and, to a lesser extent, B cell-depleted mice generated CD4+ T cells that were shifted to the effector cell phenotype with enhanced IFNγ, IL-2, and TNF output (Fig 4D and Supplemental Fig. 1B), consistent with the formation of cells that are intrinsically shorter-lived. Additionally, the B cell-sufficient environment appears to stimulate intrinsic changes in SMARTA CD4+ T cells that enable them to undergo a better recall response compared to SMARTA CD4 T cells in μMT −/− mice (Fig. 2B). Among other potential changes, greater levels of CCR7 expression are associated with improved recall responses (43, 62). Nevertheless, virus-specific CD4+ T cells could be affected by regulatory cells. We do not see reductions in the abundance of Treg cells (CD4+CD25+FoxP3+) in the B cell-deficient mice after LCMV infection (data not shown), however, it is plausible that regulatory B cells restrain cytokine output by effector CD4+ T cells, as observed in other models (31, 49, 51, 81).

In addition to influencing the formation of memory cell precursors, B cells contribute to memory during the contraction phase by sustaining virus-specific CD4+ T cells. CD4+ T cell memory could be rescued by isolating the T cells from μMT−/− mice and placing them into B cell-sufficient mice, where they recovered numerically (Fig. 2). This notion is further supported by the finding that B cell depletion initiated during the contraction phase reduced the number of memory CD4+ T cells compared to B cell-sufficient mice (Fig. 5C). Thus, B cells enhance the formation of CD4+ memory precursors and improve their survival during the contraction phase; however, we do not know which cytokine(s) produced by B cells is required for these effects. B cells and sustained antigenic stimulation contribute to the formation of T_{FH} cells (45, 72, 86, 87). T_{FH} differentiation begins with the expression of Bcl6 by day 3 after acute LCMV infection (88). Both, T_{FH} and Th1 cells enter the memory pool and respond to reinfection (44, 62, 89–92). However, memory T_{FH} can respond to infection in the absence of B cells and remain CXCR5+ up to day 10 post-infection, whereas naïve CD4+ T cells fail to efficiently form CXCR5+ cells (44). Thus, established T_{FH} memory cells appear to be less dependent on B cells than primary effector cells. Our findings are consistent with this, since B cell-depletion during the expansion or contraction phases reduced CD4 memory, whereas depletion initiated at day 40 (Fig 5G) had minimal effect on memory. Further studies are needed to determine the temporal requirements of B cells for the establishment and maintenance of T_{FH} and Th1 memory cells.

B cells eventually recover in mice that had been given anti-CD20 antibody (Fig. 4K) and, at this time, memory CD4+ T cells repopulate the spleen. It is plausible that memory CD4+ T cells reside in another niche when B cells are absent but return to the spleen when B cells recover. Alternatively, we speculate that the recovery of memory T cells could be caused by enhanced homeostatic cell division due to increased lymphopenia associated cytokines. Marginal zone cells express, or trans-present, IL-15 that can act on memory T cells and stimulate their survival (93). Thus, B cell-depletion may reduce monocyte populations or stromal cell number to deprive T cells of essential survival/maintenance cytokines, such as IL-7 or IL-15. The return of these sources of homeostatic cytokines may induce memory T cells to proliferate and accumulate to normal levels.

Chronic virus infections cause significant amounts of morbidity and mortality throughout the world, and many of the immunological and pathological consequences of chronic infection can be modeled in mice that are given variants of LCMV that persist over time. Our data show that B cells improve T cell responses following acute infection and these positive effects are somewhat more apparent during chronic virus infection, where robust CD4+ T cell and CD8+ T cell responses need to be sustained to eliminate the infection. The data in Figure 7 show that B cell-depletion impairs immunity to disseminating virus infection, implying that B cells directly or indirectly contribute factors that prevent T cell exhaustion. B cell production of cytokines or antibody may contribute to the resolution of infection (76, 94, 95). Overall, our findings suggest that B cell-depletion therapies in people will increase their susceptibility to opportunistic infections. Further studies directed at understanding how B cells improve cellular immunity may reveal therapeutic approaches to sustain T cell responses during persisting infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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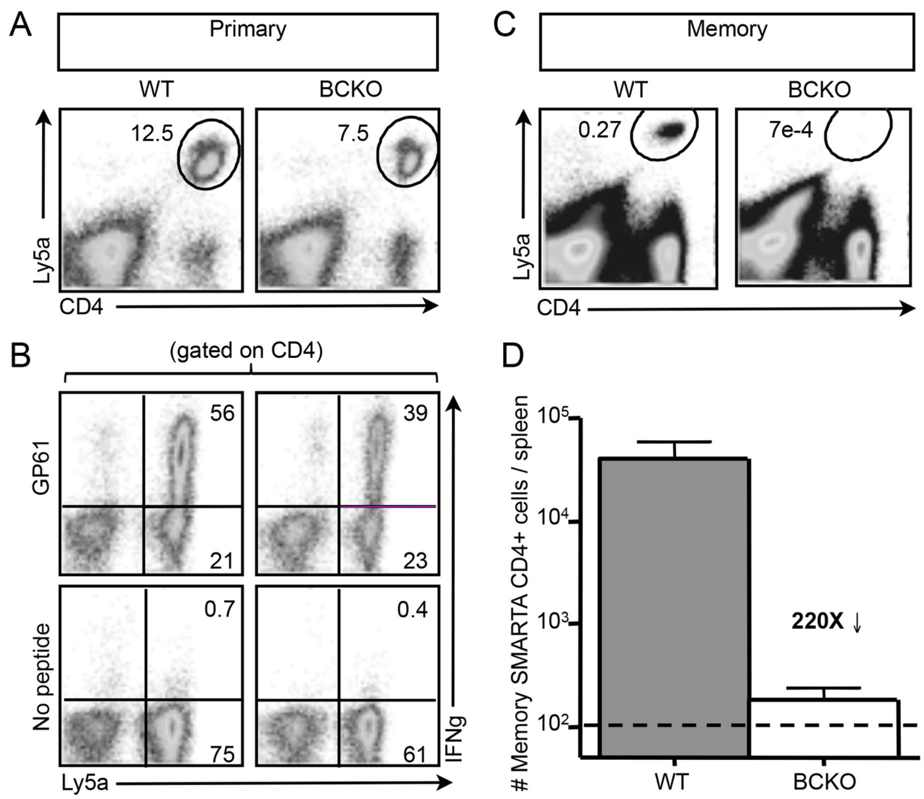

Figure 1. Absence of virus-specific CD4+ T cell memory in μMT−/− mice

WT (B6) and B cell-deficient (μMT−/−) mice were given 2×10^4 SMARTA/Ly5a CD4+ T cells and then infected with LCMV-Armstrong a few days after engraftment. The T cell response was analyzed at day 8 (**A–B**) or day 44 (**C–D**) after infection by flow cytometry. (**A**) At day 8 post-infection, spleen cells were harvested and costained for CD4, Ly5a to identify the SMARTA CD4+ T cells. The dot plots show examples of surface stained splenocytes with the SMARTA cells identified within the ovals. The numbers represent their percentage among all spleen cells. (**B**) ICCS was used to quantify T cell reactivity to LCMV-$GP_{61\text{-}80}$ peptide. The dot plots are gated on CD4+ T cells and show donor cell (Ly5a +) expression of IFNγ. The numbers represent the percentage of CD4+ T cells in each quadrant. (**C**) The dot plots show surface staining for CD4 and Ly5a at day 45, with the SMARTA CD4+ T cells identified within the ovals. (**D**) The bar graph shows the total number of splenic SMARTA CD4+ T cells in the two groups of mice. The number above the white bar indicates the ratio of memory SMARTA cells in WT mice to the number in BCKO mice. The data represent 2 mice per group from a representative experiment.

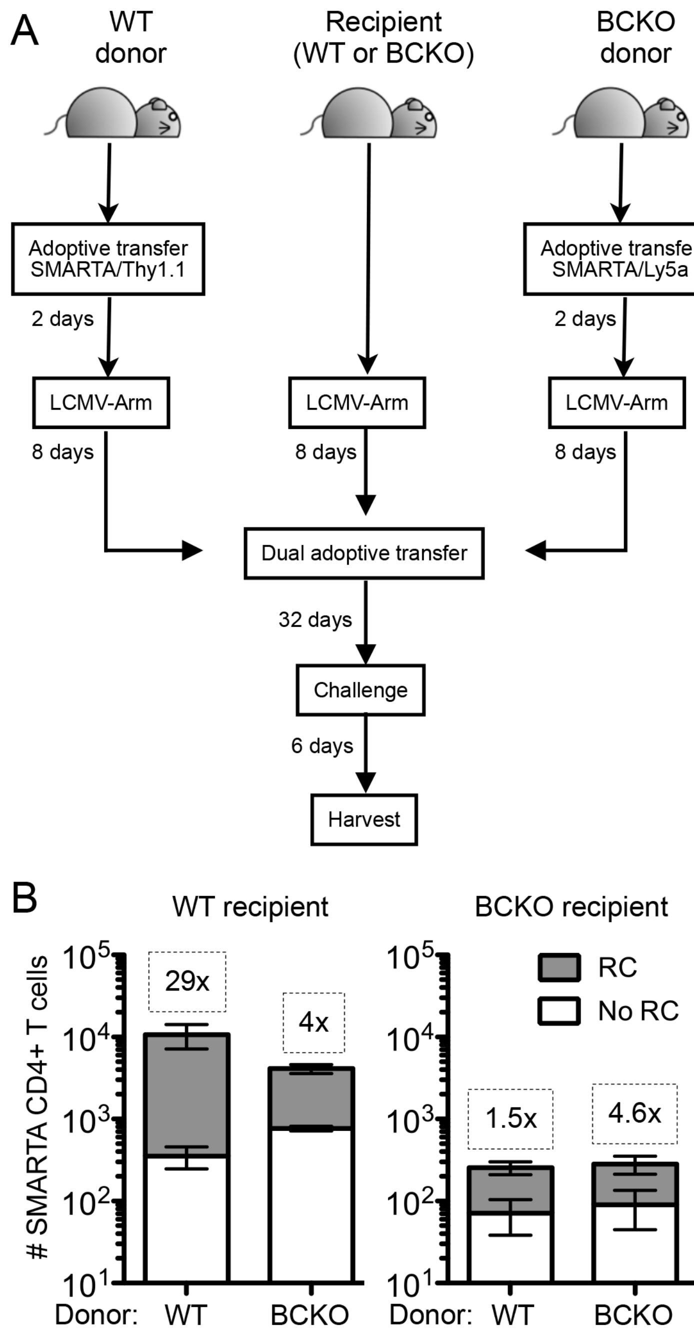

Figure 2. B cells are required during the contraction phase to produce functional memory CD4+ T cells

CD4+ T cells were allowed to differentiate into effector cells and memory precursor cells in either B cell-sufficient or B cell-deficient (BCKO) mice. The cells were harvested and their relative survival into the memory phase was compared in the same hosts. (**A**) An illustration of the experimental set up. A set of B6 and μMT−/− mice were given $2{\times}10^4$ SMARTA (Thy1.1+) CD4 T-cells 2 days before the primary infection with LCMV-Armstrong. In parallel, sets of other infection-matched B6 and μMT−/− mice were generated. At 8 days post-infection $5{\times}10^5$ SMARTA CD4 T cells from each donor mouse were co-transferred into the infection-matched B6 or BCKO recipients. Thirty-two days after the co-transfer (40 days after the primary infection), the recipients were re-challenged with LCMV-Armstrong (2×10^6 PFU, i.v.) or left unchallenged. Six days days later, splenocytes were isolated and surface stained for CD4, Thy1.1, and Ly5a to identify the 2 donor cell populations. (**B**) The left graphs show the number of memory Thy1.1+ SMARTA CD4+ T cells (generated in WT mice) or Ly5a+ SMARTA CD4+ T cells (generated in μMT−/− mice) in the unchallenged B6 recipients (open bars) or in the challenged B6 recipients (shaded). The right graph shows the number of memory SMARTA CD4+ T cells in the unchallenged (open bars) or challenged (shaded) B cell-deficient recipients. The numbers above the bars indicate the fold expansion of the memory cells upon challenge. The data are pooled from 2 independent experiments with 6–7 recipient mice/group.

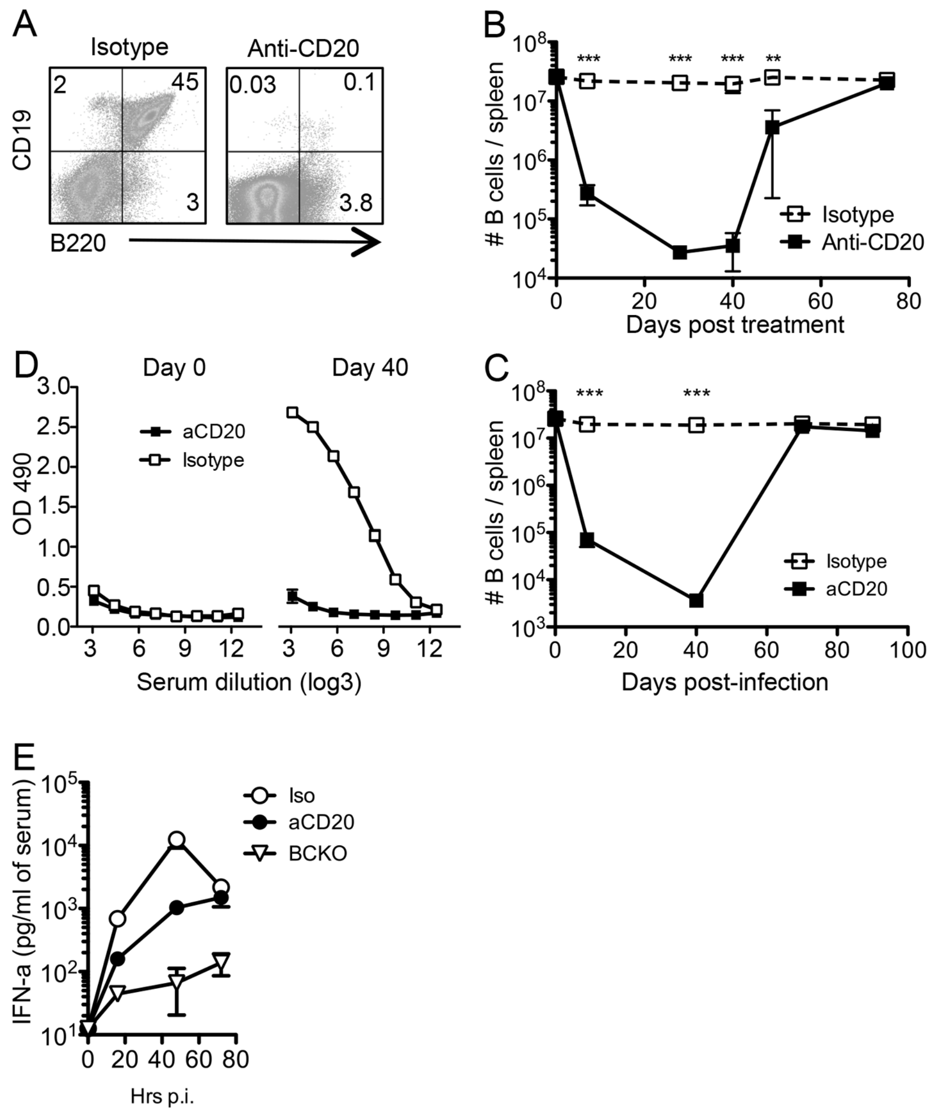

Figure 3. Anti-CD20 treatment leads to durable loss of B cells followed by cell recovery

Uninfected or infected mice were given 200ug anti-CD20 or isotype control (anti-KLH IgG2a mAb) into the peritoneal cavity to evaluate the effectiveness and longevity of the B cell-depletion. (**A**) The dot plot shows an example of splenocytes surface stained for B cell markers (B220 and CD19) 28 days after anti-CD20 was given. (**B**) The line graph shows the number of CD19+B220+ cells per spleen as measured by flow cytometry at day 0, 7, 28, 40, 49, and 75 post treatment in uninfected mice. The data represent 2 experiments with 3–6 mice/group at each time point. (**C**) B6 mice were treated with mouse anti-CD20 or isotype control at fourteen and seven days before infection. The line graph shows the number of B220+CD19+ cells in the spleen at the indicated days after infection. (**D**) Mice were depleted of B cells by two injections of anti-CD20 at day-14 and day-7 before infection. At the indicated times after infection, sera were collected and the amount of LCMV-specific IgG was quantified by ELISA. (**E**) Mice were depleted of B cells at day-7 and then infected with LCMV. At the indicated times after infection, serum was collected and analyzed for IFNα levels by ELISA. (**B–C**) The data represent 2–3 experiments with 3 (day 0 p.i.), 11–13 (day 40 p.i.), and 11–13 (day 70–90 p.i.) mice/group. (**D**) The data represent 2 experiments with 2–6 mice/group at each time point. A two-tailed Student's t-test was used to evaluate significance with **P <0.01; ***P <0.001.

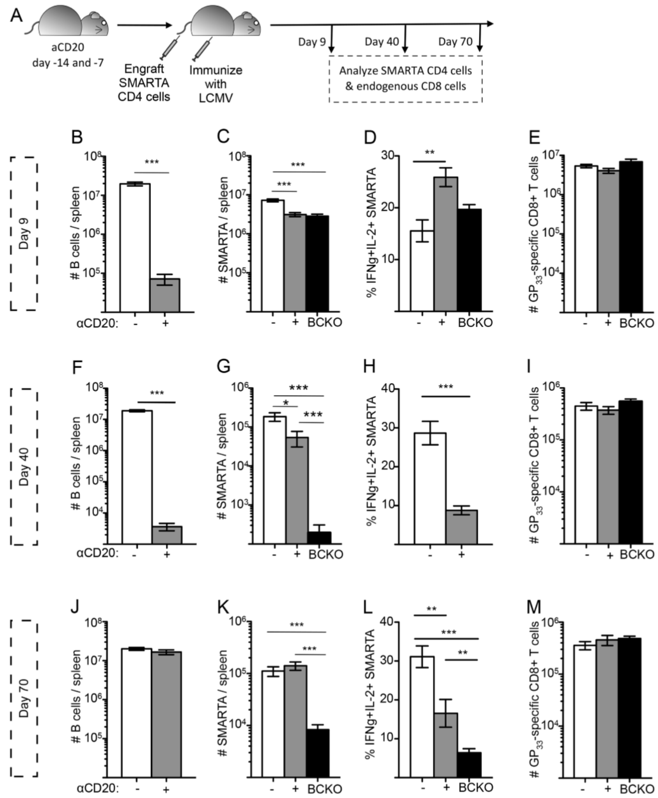

Figure 4. Early B cell depletion transiently reduces CD4 memory but not CD8 memory

(**A**) An illustration of the experimental approach to assess the effect of early B cell depletion on a defined population of virus-specific CD4+ T cells. B6 mice were treated with mouse anti-CD20 mAbs to deplete B cells or isotype control antibody at day-14 & day-7 before infection. The antibody-treated mice or μMT−/− mice were engrafted with $2{\times}10^4$ SMARTA/Thy1.1+ CD4+ T cells at day -3 and then given LCMV-Armstrong. Spleen cells were analyzed for the presence of B cells and virus-specific T cells at the indicated times after infection: day 9 (**B–E**), day 40 (**F–I**), day 70 (**J–M**). (**B, F, J**) The number of B220+CD19+ B cells per spleen in mice given anti-CD20 (+) or isotype antibody (−). (**C, G, K**) The total number of SMARTA cells per spleen in mice given anti-CD20, or isotype control, or in μMT−/− (BCKO) mice. (**D, H, L**) The percentage of SMARTA CD4+ T cells that were double positive for IFNγ and IL-2 in an ICCS assay. (**E, I, M**) The number of CD8+ T cells specific for $GP_{33\text{-}41}$ based on ICCS. The data represent 3 experiments with 6–13 mice/group. A two-tailed Student's t-test was used to evaluate significance with $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

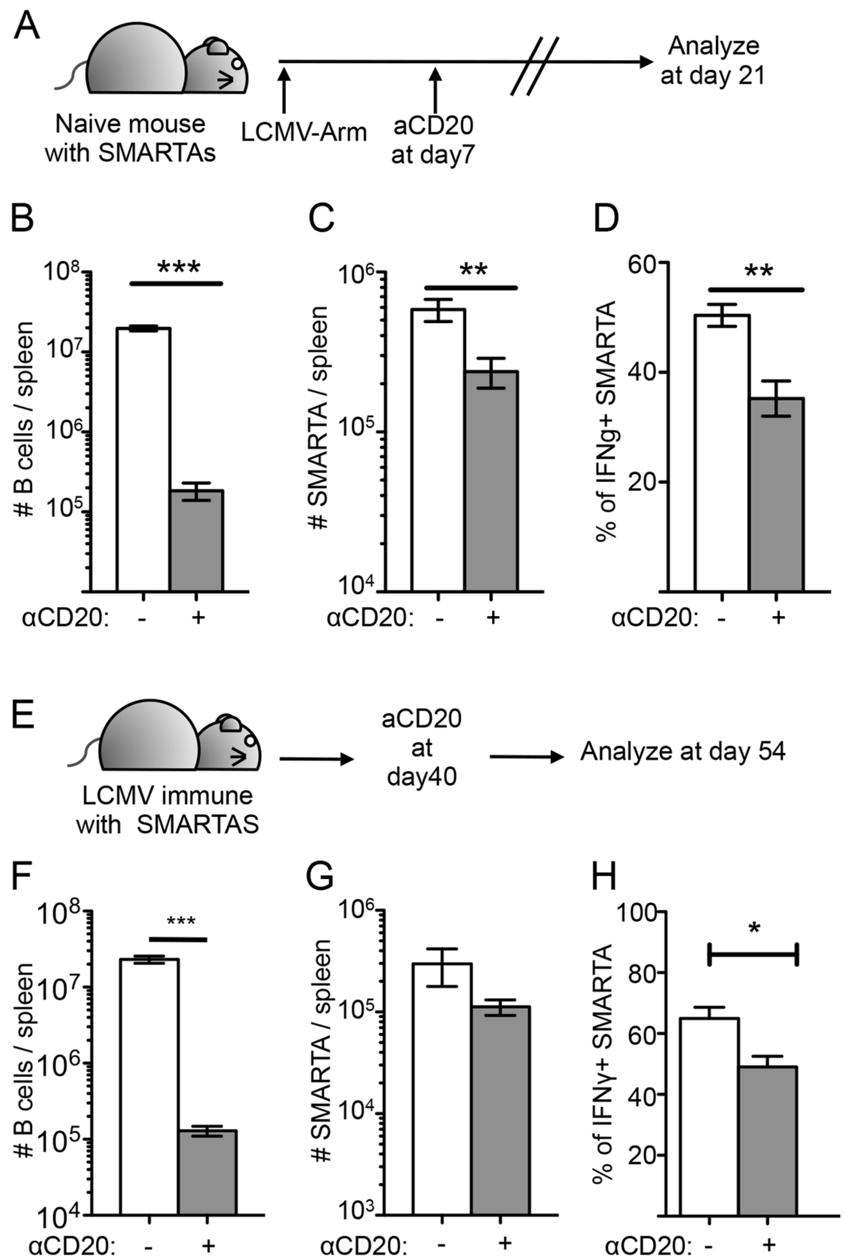

Figure 5. B cell depletion during contraction reduces CD4 memory but pre-existing CD4 memory is unaffected by B cell loss

(**A–D**) Mice engrafted with 2×10^3 SMARTA CD4+ T cells were infected with LCMV-Armstrong. T cells were allowed to differentiate normally until day 7, when the mice were given anti-CD20 (+) to deplete B cells through the contraction phase or treated with isotype control (−). Splenocytes were analyzed at day 21. (**B**) The number of B cells per spleen based on B220 and CD19 co-staining. (**C**) The number of SMARTA cells per spleen. (**D**) The percentage of SMARTA CD4+ T cells that made IFNγ and IL-2 in an ICCS assay. (**E–H**) Naïve mice with 2×103 naïve SMARTA CD4+ T cells were given LCMV-Armstrong infection and allowed to develop memory. At day 40, some of these immune mice were given anti-CD20 (+) or isotype-control antibody to evaluate the effect of B cell loss on existing memory cells. The mice were analyzed 14 days after antibody treatment. (**F**) The number of B cells at day 54. (**G**) The number of memory SMARTA CD4+ T cells per spleen. (**H**). The percentage of SMARTA CD4+ T cells that made IFNγ with IL-2 in an ICCS assay. The data in B–D represent 7–8 mice per group from one of two experiments. The data in **F–H** represent the average of 4 mice per group from one of two similar experiments. A two-tailed Student's t-test was used to evaluate significance with *P <0.05; **P <0.01; ***P <0.001.

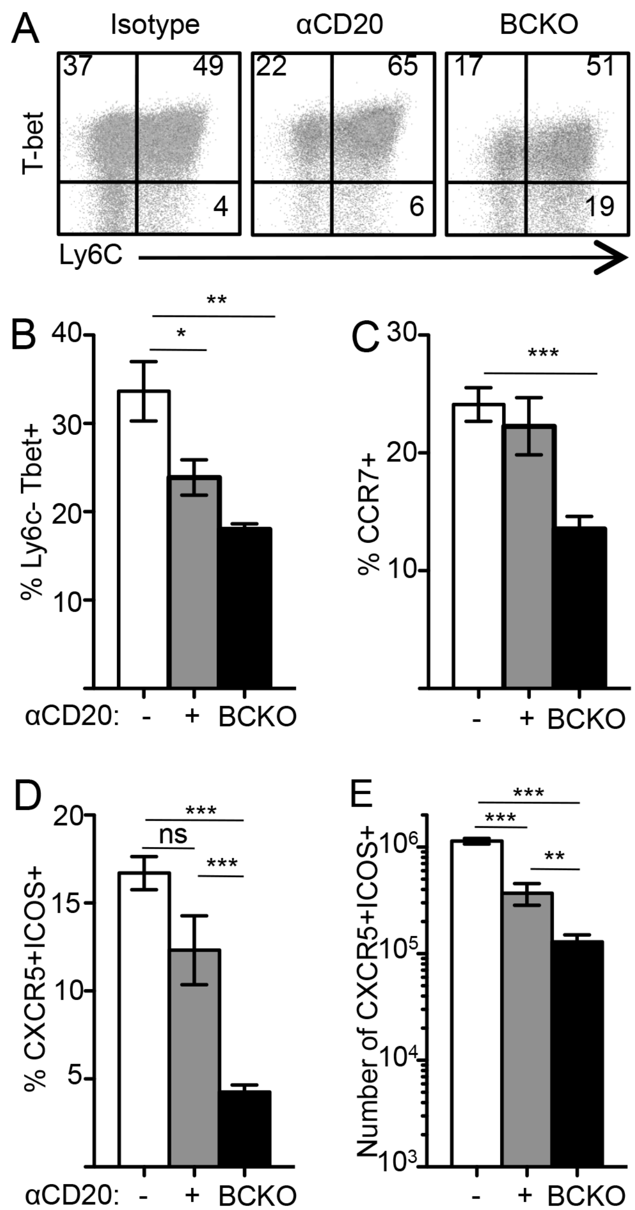

Figure 6. Reduced number of CD4+ memory precursors and T_{FH} cells in mice without B cells
(**A**) As in Figure 4, WT mice were depleted of B cells (+) or given isotype control antibody (−) and were engrafted with a small number of SMARTA CD4+ T cells and then infected with LCMV-Armstrong. μMT−/− mice were similarly engrafted with SMARTA cells followed by infection. At day 9, the SMARTA CD4+ T cells were identified and analyzed for T-bet and several surface markers by flow cytometry. (**A**) The representative dot plots are gated on the SMARTA cells and show their expression of Ly6C and T-bet. (**B**) The percentage of SMARTA CD4+ T cells that are Ly6C-negative and T-bet positive based on the gating in panels A. (**C**) The percentage of SMARTA cells that are CCR7-positive. (**D**) The percentage of SMARTA cells that co-express CXCR5 and ICOS. (**E**) The number of SMARTA CD4+ T cells that were CXCR5+ and ICOS+ per spleen. (**A–C**) The data represent 6–8 mice per group in one experiment. (**D–E**) The data represent the average (sem) of 6–9 mice per group. A two-tailed Student's t-test was used to evaluate significance with *P <0.05; **P <0.01; ***P <0.001.

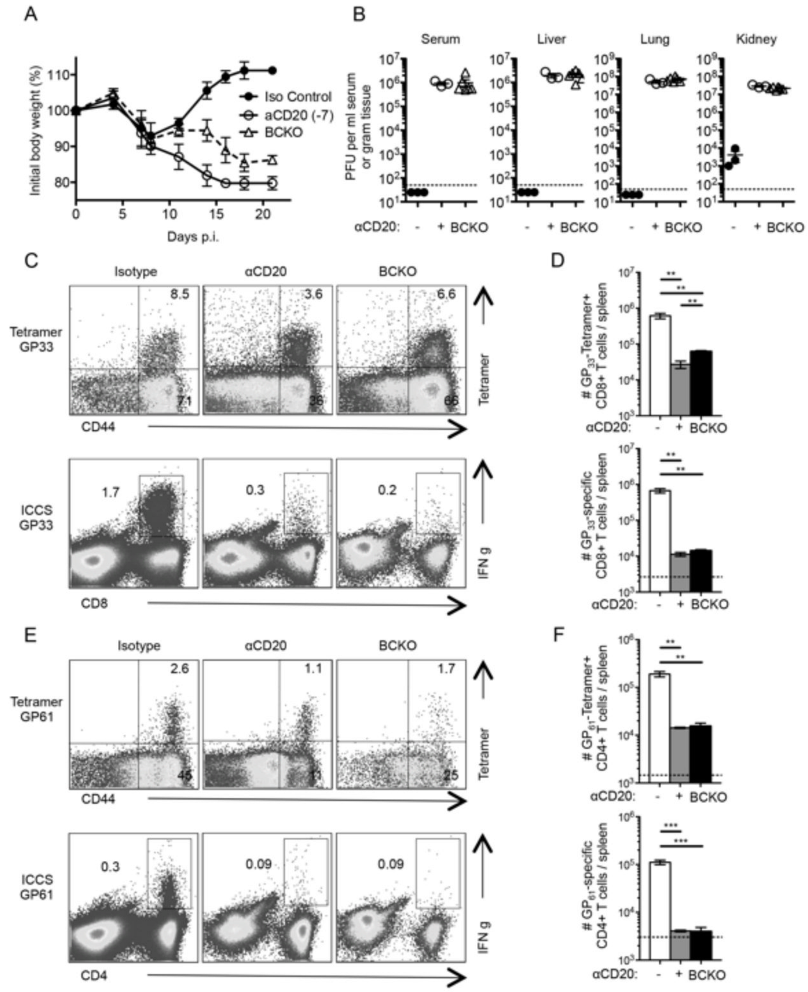

Figure 7. B cell depletion diminishes immunity to disseminating LCMV-t1b infection

C57BL/6 mice (with or without αCD20 treatment one week before infection) and μMT−/− mice were infected intravenously with 2×10^6 PFU of LCMV-t1b. (**A**) Weight loss during LCMV-t1b infection (mean±sem with 3–6 mice per group per day). (**B**) The viral burden in the mice was measured at day 21 in the serum or indicated tissues. The dotted line indicates the limit of detection the plaque assay. (**C–F**) Spleen cells from infected mice were analyzed on day 21 p.i. by tetramer staining or ICCS. (**C**) GP_{33}-specific CD8 T cell responses in the spleen were quantified by tetramer staining (top). The dot plots are gated on CD8+ cells (top) and show examples of GP_{33}-tetramer positive CD44+ CD8 T cells. The bottom dot plots are gated on all splenocytes and show CD8 T cell production of IFNγ as measured by ICCS assay. The numbers indicate the percentage of cells in each region. (**D**) The bar graphs show the average (± sem) number of GP_{33}-tetramer positive CD44+ CD8 T cells (top) and IFN-γ-producing GP_{33}-specific CD8 T cells per spleen in each group of mice. (**E**) GP_{61}-specific CD4 T cell responses in the spleen were quantified by tetramer staining (top) or ICCS (bottom). The upper dot plots are gated on all CD4+ T cells and show examples of GP_{61}-tetramer positive CD44+ CD4 T cells. The bottom plots are gated on all splenocytes and show CD4 T cell production of IFNγ. The numbers indicate the percentage of cells in each region. (**F**) The bar graphs show the average (± sem) number of GP_{61}-tetramer positive CD44+ CD4+ T cells (top) and IFN-γ-producing GP_{61}-specific CD4+ T cells (bottom) per spleen. For (**B–F**), the data represent with 3–6 mice/group. Asterisks indicate statistical significance (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) by a two-tailed Student's t-test.