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A Specific Role for B Cells in the Generation of CD8 T Cell Memory by Recombinant *Listeria monocytogenes*

Hao Shen, Jason K. Whitmire, Xin Fan, Devon J. Shedlock, Susan M. Kaech, and Rafi Ahmed

In this study, we investigated whether B cells play a role in the induction and maintenance of CD8 T cell memory after immunization with an intracellular bacterium, *Listeria monocytogenes*. Our results show that B cells play a minimal role in the initial activation and Ag-driven expansion of CD8 T lymphocytes. However, absence of B cells results in increased death of activated CD8 T cells during the contraction phase, leading to a lower level of Ag-specific CD8 T cell memory. Once memory is established, B cells are no longer required for the long-term maintenance and rapid recall response of memory CD8 T cells. Increased contraction of Ag-specific CD8 T cells in B cell-deficient mice is not due to impaired CD4 T cell responses since priming of epitope-specific CD4 T cell responses is normal in B cell-deficient mice following *L. monocytogenes* infection. Furthermore, no exaggerated contraction of Ag-specific CD8 T cells is evident in CD4 knockout mice. Thus, B cells play a specific role in modulating the contraction of CD8 T cell responses following immunization. Elucidation of factors that regulate the death phase may allow us to manipulate this process to increase the level of immunological memory and thus, vaccine efficacy. *The Journal of Immunology*, 2003, 170: 1443–1451.

*Listeria monocytogenes* (LM) is a Gram-positive, intracellular bacterium that has proven to be a useful model to investigate intracellular parasitism and cellular responses to bacterial infection. LM invades host cells, escapes from the endosome, multiplies within the cytosol, and spreads directly from cell-to-cell (2). Early control of LM infection critically depends on the innate responses by bactericidal neutrophils, IFN-γ-producing NK cells, and activated macrophages that produce TNF-α, IL-12, and free radicals (3–9). Clearance of a primary LM infection and protective immunity against reinfection requires participation of Ag-specific T cells, particularly CD8⁺ CTL (10–13).

The humoral immune response has been long thought to play no role in the control of LM infection (14–17). However, recent studies have revisited this dogma and found that B cells and Abs can directly influence the course of LM infection and confer protective immunity under certain experimental conditions. Edelson et al. (18, 19) have shown that passive transfer of mAb specific to a virulence factor, listeriolysin O (LLO), can provide resistance to LM infection by acting intracellularly to neutralize LLO and block LM escape from the phagosome. Using B cell-deficient mice, Ochsenbein et al. (20) have shown that natural Abs in naive animals may play a role in reducing early dissemination of LM into vital organs. By trapping bacteria and their Ags in secondary lymphoid organs where specific immune responses are initiated, B cells and Abs can facilitate the generation of the protective T cell response. Ag retention as Ag-Ab complexes on follicular dendritic cells has also been implicated in the maintenance of long-term T cell memory (21, 22), although this view has been challenged by recent experimental evidence (23–25). Furthermore, B cells can function as APCs and produce cytokines that modulate the T cell response (26–29). Thus, B cells and Abs may also contribute indirectly to anti-LM immunity by playing a role in the generation of the T cell response and memory.

The role of B cells in modulating T cell responses has been examined in various experimental models and appears to differ depending on the type of antigenic stimulation and infectious agent. B cells are known to take up, process, and present soluble Ags and are required for inducing CD4 T cell responses by some nonreplicating Ags (30–33), but not others (34–37). CD8 T cell responses in B cell-deficient mice are normal in response to acute infections with lymphocytic choriomeningitis virus (LCMV) or influenza virus, but defective during chronic LCMV infection (23, 35, 38). Clearance of *Plasmodium chabaudi* infection is dependent on CD4 lymphocytes and B cells are thought to augment CD4 T cell responses and sustain long-term control of this parasite (39–45). Resolution of *Leishmania major* infection is also T cell-dependent. Interestingly, B cell-deficient mice clear *L. major* infection quicker than wild-type mice, leading to the hypothesis that B cells may inhibit T cell responses and resistance to infection (46–49). B cell-deficient mice show reduced T cell responses and lower mortality rates after lung infection with *Chlamydia* (50); however, in a genital tract infection, no differences were observed between B cell-deficient and wild-type mice (51). Recently, Matsuzaki et al. (52) reported reduced T cell proliferative and IFN-γ responses to LM infection in the absence of B cells. Thus, B cells are clearly involved in modulating T cell responses in the context of many infections. However, the precise roles of B cells in supporting the T cell response to...
infection are yet to be fully defined. In most cases, the extent to which B cell deficiency may have on the T cell response has not been examined by quantitative analysis of Ag-specific T cells. It is unclear whether CD4 and CD8 T cell responses are differentially affected when B cells are absent.

T cell responses can be viewed in four distinct phases (53). During the initial period, T cells are activated and driven to proliferate by specific Ags (the expansion phase). As the Ags are cleared, the majority of activated T cells undergo apoptosis, thereby restoring homeostasis of the T cell compartment (the contraction phase). However, a small pool of Ag-specific T cells survives the contraction and persists as memory T cells for a long period of time (the memory phase). Upon reencounter of specific Ags, these memory T cells are capable of mounting an accelerated recall response that provides protective immunity (the recall response). Factors that modulate these various phases of T cell responses are largely unknown. In this study, we examined each phase in detail by quantitative analysis of Ag-specific T cells in B cell-deficient mice (μMT−/−) following LM immunization. Our results show that 1) B cells are not required for initial activation and expansion of Ag-specific CD8 and CD4 T cell responses; 2) absence of B cells results in a more profound contraction phase leading to a lower level of Ag-specific CD8 T cell memory; and 3) once memory is established, B cells play a minimal role in the long-term maintenance and rapid recall response of memory CD8 T cells. Therefore, B cells play a specific role in modulating the contraction of CD8 T cell responses following LM immunization.

Materials and Methods

Mice

C57BL/6 (H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used as controls. C57BL/6-Jg-6m/cPln (B cell-deficient, also known as μMT−/−) and C57BL/6-Cd4−/− (CD4-deficient) strains were obtained from The Jackson Laboratory and bred in house. These mice have been described previously (54, 55).

Bacteria and virus

The rLM-gp33 strain is an rLM strain that expresses the gp33–41 epitope of LCMV, which was derived from the wild-type strain 10403s as described previously (56, 57). The LD50 of rLM-gp33 in C57BL/6 mice is similar to that of the wild-type 10403s (~5 × 105 CFU). Adult experimental mice (6–8 wk old) were infected i.v. with ~3 × 107 CFU for immunization or with ~5 × 105 CFU for challenge. LM CFU in the spleen and liver of infected mice was determined by homogenization of the tissues in sterile H2O containing 1% Triton X-100 followed by plating dilutions of homogenate onto brain heart infusion plates. Colonies were scored after 24 h of growth at 37°C.

The clone 13 strain of LCMV was used in this study. The isolation and phenotypic characterization of this strain has been described previously (58). Mice were given a dose of 2 × 108 PFU i.v., and infectious virus in the tissues of infected mice was measured by plaque assay on Vero cell monolayers as previously described (58).

IFN-γ-ELISPOT assay

gp33-specific CD8 T cell responses were measured by IFN-γ-ELISPOT assay as described previously (59, 60). The capture Ab, anti-mouse IFN-γ (clone R4-6A2), and the detection Ab, biotinylated anti-mouse IFN-γ (clone XM1G1.2) were purchased from BD PharMingen (San Diego, CA). The ELISPOT plates were purchased from Millipore (Bedford, MA). LCMV peptide, gp33–41, was used at 1 μM to stimulate CD8 T cells.

Flow cytometry analysis

Spleen cells were surface stained with mAb from BD PharMingen that recognize CD8 (clone 53-6.7), CD4 (clone RM4-5), CD62L (clone MEL-14), or CD44 (clone IM7) using a concentration of 1 μg/ml. For intracellular IFN-γ staining, spleen cells were stimulated in vitro with 1 μM gp33–41 or 5 μM LLO190–201 peptide or with no peptide for 5 h in the presence of brefeldin A. They were surface stained with anti-CD8 and anti-CD44 mAb and then permeabilized using the Cytofix/Cytoperm kit from BD PharMingen per the manufacturer’s recommended protocol. Intracellular IFN-γ was detected by staining with anti-IFN-γ mAb (clone XMG1.2) and its isotype Ab (rat IgG1) as negative control, MHC class I (H-2Kb) tetramers complexed with LCMV-gp34–41 peptides were produced and used as previously described (61, 62).

CTL assay

To measure CTL responses following rLM-gp33 immunization, splenocytes (4 × 106/well) from rLM-gp33 immunized mice were stimulated in vitro for 7 days in a 24-well plate with 1 × 106 LCMV carrier mouse spleen cells (58). To measure CTL responses after LCMV clone 13 challenge, splenocytes from infected mice were used directly, without in vitro culture, in an ex vivo CTL assay. gp33-specific cytotoxic activity was determined in a standard 5-h 51Cr-release assay using peptide-coated or uncoated target cells as described previously (63).

T cell proliferation assay

Spleen cells from individual mice were plated in triplicate at 8 × 104 cells/well (4 × 106/ml) in 96-well plates and stimulated with heat-killed LM (HKLM; equivalent to 106 CFU or multiplicity of infection of 1) or left unstimulated. HKLM was prepared by incubating at 70°C for 45 min. In some experiments, CD4 T cells were purified from splenocytes by MACS using CD4 beads per the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA). CD4 and CD8 T cells were cultured in the presence of 5 × 105 normal splenocytes as feeders and 105 CFU equivalents of HKLM or 5 μM LLO190–201 peptide. The cultures were incubated for 48 h at 37°C, 6% CO2 and then pulsed with 50 mCi/ml [3H]thymidine for 24 h. At 72 h of culture, plates were harvested using a Tomtec harvester (Tomtec, Hamden, CT), and incorporation of [3H]thymidine was measured using a Microbeta TriLux scintillation counter (Wallac, Gaithersburg, MD).

Results

CD8 T cells become activated and expand normally in response to LM infection in B cell-deficient mice

To examine a possible role of B cells in the generation of T cell responses after rLM immunization, we infected C57BL/6 normal and B cell-deficient mice (μMT−/− on a C57BL/6 background) with a sublethal dose (1 × 106 CFU) of a rLM strain (rLM-gp33) that expresses an H-2D b restricted epitope (gp33–41) from LCMV. Although increased bacterial titers are observed in the peripheral organs (such as brain) of μMT−/− mice at 6 h after a lethal infection with 2 × 102 CFU (52), bacterial growth was limited to the spleen and liver following sublethal dose immunization with 3 × 104 CFU. On day 3 postimmunization, rLM-gp33 grew to a similar number (~106 CFU/g of spleen and liver) in the wild-type and μMT−/− mice, and by day 7 both strains of mice cleared rLM-gp33 infection (<100 CFU/g tissue). These results are in agreement with a previous study showing similar kinetics of bacterial growth in B cell-deficient mice and the littermate controls (52).

To assess CD8 T cell activation and expansion, we harvested spleens at the peak of T cell responses on day 7 after LM infection (64). Splenocytes were stained with Abs specific to CD8 and CD44, an adhesion molecule whose surface expression is up-regulated upon T cell activation. Surface expression of CD44 on CD8-positive cells was analyzed by FACS, and the results from a representative mouse in each group are shown in Fig. 1. Comparison of uninfected with rLM-infected mice revealed a tremendous increase in the percentage of CD8 T cells that expressed the activated (CD44high) phenotype. In wild-type mice, the CD8+ CD44high population rose from 35 to 89% after rLM immunization. Similarly, in μMT−/− mice, rLM immunization resulted in a substantial increase in the percentage of splenocytes that were CD8+CD44high (86% compared with ~30% in uninfected μMT−/− mice). Thus, rLM immunization induces extensive activation of CD8 T cells in both normal and μMT−/− mice.

LM immunization resulted in not only extensive activation but also massive expansion of the CD8 T cell population as the percentage of splenic CD8 T cells increased substantially after rLM immunization (Fig. 1). To analyze the expansion quantitatively, we
The numbers of splenocytes were lower in uninfected mice (60 \times 10^6) than in wild-type mice (10 \times 10^6), consistent with previous reports that the spleens of \( \mu MT^{-/-} \) mice are smaller and have fewer T cells in addition to lacking B lymphocytes (23). On day 7 after rLM immunization, the number of total splenocytes expanded 2.8-fold to 167 \times 10^6 in wild-type mice and 3.8-fold to 45.6 \times 10^6 in the \( \mu MT^{-/-} \) mice. The total numbers of splenic CD8 T cells increased after infection from 10 \times 10^6 to 43 \times 10^6 in wild-type mice (4.3-fold) and from 2.5 \times 10^6 to 14.5 \times 10^6 in \( \mu MT^{-/-} \) mice (5.8-fold). As expected, almost all of CD8 T cell expansion was due to increases in the CD44^{high} populations, while the number of resting CD8 T cells remained relatively unchanged in both groups of mice. Thus, while \( \mu MT^{-/-} \) mice had fewer CD8 T cells, the fold increase in CD8 T cell expansion after rLM immunization was similar to, if not greater than, that in the wild-type mice.

**Immune response and B cell deficiency**

rLM immunization clearly induces massive CD8 T cell activation and expansion in \( \mu MT^{-/-} \) mice similar to that in the wild-type control mice. We next examined the induction of epitope-specific CD8 T cells by rLM immunization in the B cell-deficient host. Normal and \( \mu MT^{-/-} \) mice were immunized with a sublethal dose of rLM-gp33. On day 7 after immunization, the gp33-specific response in the spleen was measured using intracellular IFN-\( \gamma \) staining, IFN-\( \gamma \)-ELISPOT; limiting dilution assays, and MHC/peptide tetramer staining.

In immunized C57BL/6 mice, 0.51% of spleen cells (i.e., a frequency of 1/196) produced IFN-\( \gamma \) when stimulated with the gp33 peptide (Fig. 3A). A similar level (0.35% or 1/286 splenocytes) was detected in the \( \mu MT^{-/-} \) mice immunized with rLM-gp33. These IFN-\( \gamma \)-producing cells were specific to the gp33 epitope since the control cultures without the gp33 peptide stimulation had no IFN-\( \gamma \)-producing cells, nor did splenocytes from uninfected mice that were stimulated with the gp33 peptide. Interestingly, IFN-\( \gamma \)-producing cells all partially down-regulated their surface CD8 and had high levels of CD44 surface expression. These phenotypes are indicative of these cells have experienced the cognate Ag and thus are consistent with the notion that they are specific to the gp33 epitope. By gating on CD8 T cells, the frequency of gp33-specific cells among CD8 T cells were determined to be 2.2 and 1.0% (i.e., at the frequencies of 1/45 and 1/100) in immunized C57BL/6 and \( \mu MT^{-/-} \) mice (Fig. 3B), respectively. Similar results were observed by K\(^{d}\)/gp34 tetramer staining (Fig. 3C), by IFN-\( \gamma \)-ELISPOT assay, and by the traditional method of measuring CTL precursor frequency through limiting dilution (data not shown). By IFN-\( \gamma \)-ELISPOT assay, the number of gp33-specific cells among CD8 T lymphocytes were 1/91 and 1/109 in wild-type and \( \mu MT^{-/-} \) mice, respectively, and these numbers closely matched those of intracellular IFN-\( \gamma \) staining. The limiting dilution assay estimated that the frequency of gp33-specific CTL precursors among CD8 T cells was 1/580 for wild-type mice and 1/493 for \( \mu MT^{-/-} \) mice. These frequencies are ~50-fold lower than those from IFN-\( \gamma \)-ELISPOT and intracellular staining assays. The limiting dilution assay is known to underestimate the frequency of Ag-specific CD8 T cells by ~10-fold (59), and our results further support this conclusion. Nevertheless, the limiting dilution assay also revealed that \( \mu MT^{-/-} \) mice had similar levels of gp33-specific CD8 T cells as compared with wild-type mice. In addition, gp33-specific CD8 T cells in \( \mu MT^{-/-} \) mice were capable of killing target cells, as demonstrated in limiting dilution and secondary bulk CTL assay (data not shown).

Taken together, data from four independent assays demonstrated that rLM immunization induced elevated frequencies of epitope-specific CD8 T cells in \( \mu MT^{-/-} \) mice to ~50% of that found in wild-type mice on day 7 after infection. Therefore, B cells appear to play a minimal role in the initial activation and Ag-driven expansion of CD8 T cells after rLM infection.

**B cell deficiency results in increased depletion of Ag-specific CD8 T cells and consequently a lower level of immunological memory**

The initial Ag-driven expansion is typically followed by a contraction phase in which the majority of activated CD8 T cells are deleted, leaving a small stable pool responsible for immunological...
memory. We next asked if rLM immunization could establish similar levels of Ag-specific CD8 T cell memory in the absence of B cells. To this end, we measured the number of gp33-specific CD8 T cells using the IFN-γ-ELISPOT assay at different time points after infection.

As shown above, expansion of the CD8 T cell population and the frequency of gp33-specific cells were similar between wild-type and μMT−/− mice on day 7 after rLM immunization. However, during the ensuing contraction phase from days 7 to 30, μMT−/− mice behaved very differently from the normal control mice. In the wild-type mice, gp33-specific CD8 T cells contracted 2-fold, decreasing from a frequency of 1/91 on day 7 to 1/170 on day 30. Strikingly, the deletion of gp33-specific CD8 T cells in μMT−/− mice was much more pronounced, a 17-fold reduction from a frequency of 1/91 on day 7 to 1/1845 on day 30 (Fig. 4A). Staining with tetramers showed similar results and confirmed a much lower frequency of gp33-specific memory CD8 T cells in μMT−/− mice (data not shown). This conspicuous contraction in μMT−/− mice was even more evident when the total number of gp33-specific CD8 T cells per spleen was compared (Fig. 4B). Cell-deficient mice had a 50-fold contraction from 1 × 10^5 per spleen on day 7 to 2 × 10^3 per day 30. This is in contrast to a 4-fold reduction in the wild-type mice, from 4 × 10^5 per spleen on day 7 to 9 × 10^4 by day 30.

As a result of the increased deletion of Ag-specific CD8 T cells, μMT−/− mice had a much lower level of Ag-specific CD8 T cell memory. Therefore, B cells play an important role in modulating the death of Ag-specific CD8 T cells during the contraction phase and consequently in setting the level of immunological memory.

B cells are not required for the long-term maintenance and rapid recall response of memory CD8 T cells

The above results show that B cell-deficient mice exhibit a pronounced deletion of Ag-specific CD8 T cells during the contraction phase after rLM immunization. This raises the interesting question of whether a small pool of Ag-specific CD8 T cells will survive and persist as long-term memory in rLM-immunized μMT−/− mice. As shown in Fig. 4, the frequency of gp33-specific CD8 T cells in μMT−/− mice did not continue to drop precipitately as it did during the contraction phase. Instead it was relatively stable between days 30 and 150, and this was also true when the total numbers of gp33-specific CD8 T cells per spleen were compared. Similarly, the level of gp33-specific CD8 T cells was maintained during this period in wild-type mice. Thus, B cells do not play a major role in sustaining memory CD8 T cells. Importantly, the reduced number of Ag-specific CD8 T cells in μMT−/− mice was confirmed by tetramer staining (data not shown). This demonstrates that the reduced memory was not due to the development of Ag-specific cells that lack cytotoxic or IFN-γ function as seen following certain chronic viral infections (65, 66). These results are consistent with findings from other systems, demonstrating that memory CD8 T cells can persist for a long period of time after clearance of the pathogen in the absence of persisting Ag-Ab complexes on follicular dendritic cells (23–25).

To further examine the function of memory CD8 T cells in the absence of B cells, we challenged rLM-immunized mice with LCMV clone 13 and assessed the recall response and protective immunity, two gold standards for measuring CD8 T cell memory in vivo. On day 8 after LCMV challenge, activation and expansion of total CD8 T cells and the gp33-specific response were analyzed (Fig. 5). LCMV clone 13 infection in unimmunized wild-type and μMT−/− mice induced a small increase in the percentage of total CD8 T cells and a shift of CD8 T cells from the resting (CD44low) toward the activated (CD44high) phenotype, a response that is routinely seen in animals after LCMV clone 13 infection (67). In contrast, both wild-type and μMT−/− mice previously immunized with rLM-gp33 had a much greater increase in the overall percentage of CD8 T cells and the increase was even more pronounced in the percentage of CD8 T cells of the activated (CD44high) phenotype. To assess the gp33-specific response, we measured the number of epitope-specific cells by IFN-γ-ELISPOT assay (Fig. 5B). Unimmunized mice infected with LCMV clone 13 had low levels of gp33-specific T cells (5.2 × 10^4/spleen in wild-type and 9.5 × 10^3/spleen in μMT−/− mice). In contrast, mice immunized with rLM-gp33 and then challenged with LCMV had much higher levels of gp33-specific cells in the spleen (4.3 × 10^7 in wild-type and 1.6 × 10^8 in μMT−/− mice). These represent expansions of almost 10-fold in wild-type mice and >100-fold in μMT−/− mice from gp33-specific memory cells that were present before LCMV challenge (Fig. 4B). We further tested gp33-specific cytolytic activity by direct ex vivo CTL assay (Fig. 5C). There was minimal lysis of gp33-coated targets by splenocytes from unimmunized wild-type
and μMT−/− mice infected with LCMV clone 13. In contrast, comparably high levels of specific lysis were observed in splenocytes from both wild-type and μMT−/− mice that were previously immunized with rLM-gp33. This enhanced recall response resulted in clearance of LCMV clone 13 infection so that both wild-type and μMT−/− mice immunized with rLM-gp33 had no detectable virus in the serum 30 days postchallenge, whereas the unimmunized mice still harbored a high level of virus in their sera (>10^4 PFU/ml, Fig. 5D). These data show that B cells play little or no role during a CTL recall response against a viral challenge after rLM immunization.

To test whether B cells are required for protective immunity to LM reinfection, wild-type or μMT−/− mice that were immunized with 3 × 10^4 CFU rLM-gp33 were challenged with 5 × 10^5 CFU rLM-gp33 38 days later. Three days after challenge, bacterial numbers in the spleen and liver were measured (Fig. 6). Although unimmunized mice had high levels of bacteria at this time point, immunized wild-type and μMT−/− mice had reduced bacterial loads and controlled the infection to a similar extent. This result shows that long-term LM-specific immunity is also intact in the absence of B cells.

In summary, B cells are not required for the initial induction and Ag-driven expansion of CD8 T cells after rLM immunization. However, B cells play an important role in the contraction phase since the absence of B cells resulted in increased death of Ag-specific T cells, and consequently a lower level of Ag-specific CD8 T cell memory. Once memory is established, B cells are no longer required for the long-term maintenance or for the rapid recall response of memory CD8 T cells to viral or LM challenge.

Epitope-specific CD4 T cell responses to LM infection are relatively normal in B cell-deficient mice

B cells have been shown to be important for the priming of CD4 T cells in several systems including LM infection (31, 32, 52, 68, 69). Thus, exaggerated contraction of the CD8 T cell response in B cell-deficient mice could be due to an impaired CD4 T cell response in B cell-deficient mice. This is a plausible hypothesis since activated CD4 T cells are likely to provide necessary help such as cytokines that might rescue activated CD8 T cells from.
apoptosis. To examine this possibility, we analyzed CD4 T cell responses to LM infection in B cell-deficient mice (Fig. 7). Infection of wild-type mice with rLM resulted in extensive activation of CD4 T cells, with >80% of CD4+ T cells becoming activated and expressing the CD44^{high} phenotype (Fig. 7A). In contrast, only ~47% of CD4 T cells became activated and expressed the CD44^{high} phenotype in rLM-infected μMT−/− mice. Thus, unlike CD8 T cells that are activated to a similar level in both groups (Fig. 1), CD4 T cells appear to be activated to a lower extent in μMT−/− mice.

To assess the Ag-specific CD4 T cell response, we stimulated splenocytes from immunized mice with HKLM in vitro and measured [3H]thymidine incorporation, a method traditionally used to measure the presence of LM-specific CD4 T cells. In this assay, the proliferative response stimulated by HKLM is exclusively due to LM-specific CD4 T cells since in vitro depletion of CD4 T cells but not CD8 T cells abolishes the proliferation and B cells do not proliferate under these conditions (70, 71) (data not shown). No proliferation above background levels was detected when splenocytes from uninfected control mice were stimulated with HKLM. Splenocytes from rLM-immunized wild-type mice proliferated to high levels in response to HKLM stimulation, whereas those from μMT−/− mice had no detectable levels of proliferation (Fig. 7B). These results are consistent with previous findings by Matsuzaki et al. (52) and indicate a defective CD4 T cell response in μMT−/− mice. However, it is still possible that B cells themselves may be important APCs during in vitro stimulation and that they are required for CD4 T cell proliferation (52). To control for this effect, we purified CD4 T cells from infected wild-type and μMT−/− mice and stimulated them in the presence of naive wild-type spleen cells as feeders. Under these conditions, CD4 T cells from LM-immunized μMT−/− mice proliferated in response to HKLM stimulation (Fig. 7C). These results indicate that CD4 T cell responses to LM infection are not severely impaired in μMT−/− mice, contrary to previous findings (52). To confirm this new finding, we measured epitope-specific CD4 T cell responses by taking advantage of a recently identified LM CD4 T cell epitope, LLO190–201 (72). Our results showed that CD4 T cells from LM-immunized wild-type and μMT−/− mice proliferated similarly in response to in vitro stimulation with the LLO190–201 peptide (Fig. 7C). Furthermore, LM infection induced similar frequencies of LLO190–201-specific CD4 T cells in wild-type and μMT−/− mice, as determined by intracellular IFN-γ staining (Fig. 7D). Together, these data demonstrate that Ag-specific CD4 T cell responses are not defective in B cell-deficient mice.

B lymphocytes but not CD4 T cells play a role in modulating the contraction of Ag-specific CD8 T cell responses

Our results above indicate that an impaired CD4 T cell response is unlikely to be the reason for increased contraction of CD8 T cell
responses in B cell-deficient mice. To further examine the possible involvement of CD4 T cells, we compared contraction of gp33-specific CD8 T cell responses following rLM immunization in wild-type, μMT−/−, and CD4-deficient (CD4−/−) mice (Fig. 8). CD4−/− mice were fully capable of resolving primary LM infection (12), and the kinetics of bacterial growth were similar between CD4−/− and μMT−/− mice following low dose sublethal immunization (data not shown). Immunization with rLM-gp33 induced gp33-specific responses that peaked at normal levels on day 7, indicating that CD4 T cells are not required for inducing CD8 T cell responses in this system. In all three mouse strains, the CD8 response fully contracted by day 30, leaving a pool of memory T cell responses in this system. In all three mouse strains, the CD8 response had similar contractions of 3.2- and 5.1-fold, respectively (Fig. 8A). Thus, CD4 T cells play a minimal role in modulating the contraction of Ag-specific CD8 T cells, and the profound depletion of Ag-specific CD8 T cells during the contraction phase in B cell-deficient mice is unlikely due to an impaired CD4 T cell response.

Discussion

Our finding of a relatively normal CD4 T cell response to LM infection in μMT−/− mice contrasts with results from other systems that demonstrate a requirement for B cells in the priming of CD4 T cells (31, 32, 68, 69). A recent study by Matsuzaki et al. (52) also reported a defective primary T cell response following LM infection of B cell-deficient mice, based on analysis of proliferative responses and IFN-γ production after in vitro stimulation with HKLM. These results are interpreted to indicate a defective CD4 T cell response since HKLM is presented mainly through the MHC class II pathway in the in vitro assays (70, 71). A caveat of these assays is that B cells may play a supporting or Ag-presenting role during in vitro HKLM stimulation. Thus, it is unclear whether the results from this early study reflect a totally defective CD4 T cell response in vivo or were partly due to poor stimulation of CD4 T cells by HKLM in the absence of B cells during in vitro culture. We also observed that proliferative responses by splenocytes from LM-immunized μMT−/− mice were largely defective during in vitro stimulation with HKLM (Fig. 7B). However, purified CD4 T cells from LM-immunized μMT−/− mice proliferated in response to HKLM stimulation when we included naive wild-type splenocytes including B cells as feeder cells to normalize the number of APCs in the in vitro assay (Fig. 7C). This result indicates that B cells are important during in vitro HKLM stimulation while in vivo CD4 T cell responses to LM infection are not impaired in B cell-deficient mice. This conclusion is further supported by our analysis of epitope-specific CD4 T cell responses, made possible by the recent identification of MHC class II-restricted epitopes of LM (72). Our results showed that LM infection induced similar frequencies of CD4 T cells specific to the LLO190–201 epitope in wild-type and μMT−/− mice as determined by proliferation and intracellular IFN-γ staining after in vitro stimulation with the LLO190–201 peptide.

Another major finding of our study is that the absence of B cells results in profound depletion of activated CD8 T cells during the contraction phase. Thus, B cells play an important role in modulating homeostasis of Ag-specific CD8 T cell responses. Whether B cells play a role in modulating the contraction of CD4 T cell responses remains to be investigated. B cells may play such a role and in doing so, indirectly modulate the contraction of CD8 T cell responses. This is a plausible hypothesis since CD4 T cells are likely to provide necessary help including cytokines that might rescue activated CD8 T cells from apoptosis. However, our results showed that CD4 T cells were not involved in modulating the contraction of CD8 T cell responses, as CD4−/− mice exhibited a contraction phase similar to normal mice. Thus, B cells/Ab must be directly involved and the exact mechanism(s) by which B cells participate in this process is not known. Recent evidence strongly suggests that dendritic cells are the major APC that activate naive CD8 T cells. Our finding of normal CD8 T cell responses during the initial expansion phase indicate that B cells are unlikely to play an important role in presenting Ag to naive CD8 T cells. However, Ag presentation by B cells could play a role in providing sustained antigenic and costimulation to effector CD8 T cells during the contraction phase. In this case, Ag trapping and retention by follicular dendritic cells may play a role in rescuing activated CD8 T cells from death by curtailing the rapid withdrawal of Ags, although persisting Ag is not required for the survival of memory T cells. In addition to Ag presentation, activated B cells make a number of cytokines, including lymphotoxin, IL-1, IL-6, IL-10,
TNF, and TGFβ, that are involved in the development of lymphoid architecture and regulation of T cell activation and death. It is possible that these cytokines and/or lymphoid microenvironments are needed during the contraction phase to provide proper signals to rescue activated T cells from apoptosis.

The level of CD8 T cell memory is determined by a combination of the magnitude of the initial expansion driven by Ag and the extent of contraction following the clearance of Ag. The goal of vaccination is to induce high levels of immunological memory that ultimately determines the level of immunological memory and thus vaccine efficacy.

Once memory is established, B cells are no longer required for the long-term maintenance of memory CD8 T cells. These results reinforce the notion, for the first time in the context of a bacterial infection, that memory CD8 T cells can persist for long periods after pathogen clearance and in the absence of persisting Ag-Ab complexes on follicular dendritic cells. More importantly, memory CD8 T cells induced by rLM were capable of mounting an enhanced recall response and providing protective immunity in B cell-deficient mice. Thus, B cells are not required for the maintenance or the recall response of CTL memory. These results suggest that rLM-based vaccines may represent an attractive means of immunological memory and thus vaccine efficacy.

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