

CD8 T Cell Memory in B Cell-deficient Mice

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Summary

Antigen presentation by B cells and persistence of antigen-antibody complexes on follicular dendritic cells (FDC) have been implicated in sustaining T cell memory. In this study we have examined the role of B cells and antibody in the generation and maintenance of CD8⁺ cytotoxic T lymphocyte (CTL) memory. To address this issue we compared CTL responses to lymphocytic choriomeningitis virus (LCMV) in normal (+/+) versus B cell-deficient mice. The CTL response to acute LCMV infection can be broken down into three distinct phases: (a) the initial phase (days 3–8 after infection) of antigen-driven expansion of virus-specific CD8⁺ T cells and the development of effector CTL (i.e., direct ex vivo killers); (b) a phase of death (between days 10 and 30 after infection) during which >95% of the virus-specific CTL die and the direct effector activity subsides; and (c) the phase of long-term memory (after day 30) that is characterized by a stable pool of memory CTL that persist for the life span of the animal. The role of B cells in each of these three phases of the CTL response was analyzed. We found that B cells were not required for the expansion and activation of virus-specific CTL. The kinetics and magnitude of the effector CTL response, as measured by direct killing of infected targets by ex vivo isolated splenocytes, was identical in B cell-deficient and +/+ mice. Also, the expansion of CD8⁺ T cells was not affected by the absence of B cells and/or antibody; in both groups of mice there was an ~10,000-fold increase in the number of LCMV-specific CTL and a greater than 10-fold increase in the total number of activated (CD44hi) CD8⁺ T cells during the first week after virus infection. Although no differences were seen during the “expansion” phase, we found that the “death” phase was more pronounced in B cell-deficient mice. However, this increased cell death was not selective for LCMV-specific CTL, and during this period the total number of CD8⁺ T cells also dropped substantially more in B cell-deficient mice. As a result of this, the absolute numbers of LCMV-specific CTL were lower in B cell-deficient mice but the frequencies were comparable in both groups of mice. More significantly, the memory phase of the CTL response was not affected by the absence of B cells and a stable number of LCMV-specific CTL persisted in B cell-deficient mice for up to 6 mo. Upon reinfection, B cell-deficient mice that had resolved an acute LCMV infection were able to make accelerated CTL responses in vivo and eliminated virus more efficiently than naive B cell-deficient mice. Thus, CTL memory, as assessed by frequency of virus-specific CTL or protective immunity, does not decline in the absence of B cells. Taken together, these results show that neither B cells nor antigen-antibody complexes are essential for the maintenance of CD8⁺ CTL memory.

CD8⁺ CTL constitute an important defense mechanism against intracellular pathogens (1–4). Both effector and memory CTL play critical but distinct roles in antiviral immunity. Effector CTL control infection by killing infected cells and secreting cytokines such as IFN- γ (5–7). Memory CTL play an important role in protective immunity (1–4, 8–11); they do not prevent infection per se, but limit the severity of infection/disease by making accelerated responses upon reexposure (8–11). Memory CTL are qualitatively distinct from naive CTL and express higher levels of adhesion molecules (8–11). Upon reencounter with spe-

cific antigen, memory CTL proliferate rapidly, differentiate into effector cells, get to the site of infection, and eliminate infected cells.

Long-term CTL memory is seen in many different antigenic systems and under both natural and experimental situations (8–11). For example, mice that have undergone an acute infection with influenza virus, Sendai virus, or lymphocytic choriomeningitis virus (LCMV)¹ exhibit lifelong

¹Abbreviations used in this paper: FDC, follicular dendritic cell; LCMV, lymphocytic choriomeningitis virus.

CTL immunity (1, 12–16). A recent study has documented long-term CTL memory in humans, by isolating vaccinia virus-specific CTL from individuals vaccinated >30 yr earlier (17). Prolonged CTL memory is also seen in several “noninfectious” antigenic systems (i.e., H-Y, ovalbumin), and also after immunization with peptides (1, 8–11).

A question of fundamental importance is how this long lasting CD8⁺ T cell memory is maintained. The more conventional model has been that long-term memory is dependent on persistent antigenic stimulation (8, 14, 18). Several recent studies, however, have provided evidence that the original priming antigen is not essential for the maintenance of CD8⁺ T cell memory (13, 15, 16, 19). However, this issue still remains controversial and one of the arguments is that antigen-antibody complexes persisting on follicular dendritic cells (FDC) provide a stimulus for memory T cells (8). It is well established that FDC can trap antigen in the form of antigen-antibody complexes and retain it on their cell surface for long periods of time (8, 20, 21). Since FDC are inefficient in antigen processing, it has been postulated that B cells pick up this trapped antigen and present it to T cells, thereby sustaining long-term memory (8). Although this mode of antigen loading favors presentation through MHC class II molecules, there are now several examples of exogenous antigens being processed and presented by MHC class I molecules (22, 23). Thus, antigen depots on FDC could play a role in sustaining CD8⁺ T cell memory. The availability of B cell-deficient mice (μ MT/ μ MT) (24) now allows us to formally test this hypothesis and examine the role of B cells and antigen-antibody complexes in the generation and persistence of CD8⁺ T cell memory. In this study we have analyzed LCMV-specific CTL responses in B cell-deficient mice and compared them with responses seen in +/+ mice.

Materials and Methods

Mice. μ MT/ μ MT mice were made by targeted disruption of one of the membrane exons of the IgM H chain (24). μ MT (-/-) mice back-crossed on a C57BL/6 background were kindly provided by Dr. Herbert Virgin (Washington University School of Medicine, St. Louis, MO) or purchased from The Jackson Laboratory (C57BL/6-Igh; Bar Harbor, ME). Mice were then bred and maintained at the University of California, Los Angeles. C57BL/6 (+/+) mice were purchased from The Jackson Laboratory. The C57BL/6 LCMV carrier colony was established and bred at the University of California, Los Angeles as previously described (25).

Virus. The Armstrong CA 1371 strain of LCMV was used in these studies for infection of mice (26). Spleen variant clones 13 or 28b were used in rechallenge experiments. These variants were isolated from the spleens of adult BALB/c carrier mice infected at birth with the Armstrong CA 1371 strain (26, 27). All LCMV stocks used in this study were triple plaque purified on Vero cells, and the stocks were grown in BHK-21 cells. Virus stocks at the passage one or two level were used in all experiments. Infectious virus was quantitated by plaque assay (26).

Antibodies. The numbers of CD8⁺ and CD4⁺ cells in the spleen, blood, and LN were determined by staining with specific mAbs followed by analysis on a FACScan[®] flow cytometer (Becton Dickinson & Co., San Jose, CA). The following antibodies

were used: R-phycoerythrin-conjugated rat anti-mouse CD4 (RM4-5), biotin-conjugated rat anti-mouse CD8a (53-6.7), FITC-conjugated rat anti-mouse CD44 (IM7) from PharMingen (San Diego, CA), and streptavidin r-phycoerythrin from Caltag Laboratories (South San Francisco, CA). All antibodies were used at concentrations recommended by the manufacturers.

Cytotoxicity Assay. LCMV-specific CTL activity of spleen and LN cells (pooled axillary, mesenteric, and inguinal) was measured by a standard 6-h ⁵¹Cr-release assay on uninfected and LCMV-infected MC57 (H-2^b) targets (26). To determine CTL recognition of the three major H-2^b-restricted LCMV epitopes, MC57 target cells were coated with selected LCMV peptides at a concentration of 2 μ g/ml for 1 h at 37°C. Effector cells were then added to prepared targets. Peptides were a gift from Cytel (San Diego, CA).

In Vitro Depletion of CD8⁺ Cells. Spleen cells were treated with anti-Ly 2.2 mAb (anti-CD8) purchased in the form of ascites fluid plus rabbit complement (low tox M; both from Cedarlane Laboratories Ltd., Hornby, ON, Canada) or complement alone. Antibody and complement were used at concentrations specified by the manufacturer. After depletion, cells were enumerated and used as effectors in the cytotoxicity assays.

Antibody Titrations. LCMV-specific antibody in serum was measured by a solid-phase ELISA as previously described (26).

Determination of the LCMV-specific CTLp Frequency. Spleen cells from LCMV-immunized mice were cultured in graded doses in 96-well flat bottom plates (12 wells per dose). 8×10^5 syngeneic feeder spleen cells from uninfected mice and 2×10^5 syngeneic stimulator spleen cells from LCMV carrier mice were irradiated (1,200 rad) and added to each well. Recombinant human IL-2 from *Escherichia coli* (specific activity 18×10^6 IU mg⁻¹ (Cetus Corp., Emeryville, CA) was added at a final concentration of 50 U ml⁻¹. After 8 d, the contents from each well were split to test CTL activity against LCMV-infected and uninfected MC57 targets in a 6-h ⁵¹Cr-release assay.

Results

Kinetics of the CD8⁺ CTL Effector Response in B Cell-deficient Mice. To determine whether B cells play a role during induction of the primary CTL response, we compared the kinetics of the CTL effector response in B cell-deficient and age-matched C57BL/6 (+/+) mice during an acute LCMV. Mice were infected with 2×10^5 i.p. PFU of LCMV Armstrong, and direct ex vivo cytotoxicity in the spleens was measured at various times after infection (Table 1).

On day 3 of infection, the majority of the cytotoxic response in the spleens was nonspecific, with lysis of both LCMV-infected and uninfected target cells. By day 5, LCMV-specific CTL activity was detectable in both the B cell-deficient and +/+ mice. Although there was still some lysis of uninfected target cells, there was less than that seen at day 3. Previous work has shown that the early nonspecific cytotoxic activity after LCMV infection is due to natural killer cells (28).

The magnitude of the CTL response increased dramatically by day 8 of infection, when spleen cells from both B cell-deficient and +/+ mice demonstrated high specific killing of LCMV-infected targets. Results from two separate experiments showed that the magnitude of the primary CTL effector response in the spleens of -/- mice was com-

Table 1. *B Cell-deficient Mice Generate a Primary LCMV-specific CTL Effector Response**

Mouse genotype	Days after infection	Percent specific ⁵¹ Cr-release from MC57(H-2 ^b) targets at indicated E/T ratio											
		LCMV-specific CTL in the spleen						LCMV-specific CTL in LN					
		LCMV Infected			Uninfected			LCMV infected			Uninfected		
		50:1	16.6:1	5.5:1	50:1	16.6:1	5.5:1	50:1	16.6:1	5.5:1	50:1	16.6:1	5.5:1
-/-	3	31	12	4	19	7	1	ND	ND	ND	ND	ND	ND
+/+	3	11	9	10	18	15	13	ND	ND	ND	ND	ND	ND
-/-	5	24	14	5	10	5	2	28	12	5	4	1	1
+/+	5	22	10	4	6	2	1	14	16	2	7	2	0
-/-	8	68	54	36	4	1	0	31	12	2	1	0	0
+/+	8	64	48	20	0	0	0	42	29	13	0	0	0
-/-	16	42	25	12	0	1	0	3	0	0	2	2	3
+/+	16	35	14	7	0	0	0	3	1	0	0	0	0

*6–8-wk-old mice were infected i.p. with 2×10^5 PFU of LCMV Armstrong. Direct ex vivo LCMV-specific CTL activity was measured by a 6-h ⁵¹Cr-release assay at the indicated days after infection. Data represent the average of two to four mice per group.

parable to that seen in +/+ mice. In B cell-deficient mice, depletion of CD8⁺ T cells using a mAb and complement before the cytotoxicity assay abrogated 90% of the measured CTL response at day 8, demonstrating that as with +/+ mice, primed CD8⁺ T cells were responsible for the direct ex vivo CTL response seen (data not shown). By day 16, CTL activity showed over a threefold drop in both the B cell-deficient and the +/+ mice; and by day 30 after infection, direct ex vivo cytotoxic responses had come down to baseline (data not shown). LN cells from B cell-deficient mice also showed direct ex vivo cytotoxicity, although responses were generally lower than that seen in the spleens. LCMV-specific responses were detected at days 5 and 8 after infection, and had come down to baseline by day 16. Similar results were seen in +/+ mice. These experiments demonstrated that the kinetics and magnitude of the primary CTL effector response in B cell-deficient mice is similar to +/+ mice.

Epitope Repertoire in B Cell-deficient Mice. To determine whether CTL from B cell-deficient mice recognize the same LCMV epitopes as those of +/+ mice, direct ex vivo cytotoxicity was measured using targets coated with peptides from three of the major LCMV epitopes. In H-2^b mice, the dominant LCMV CTL epitopes are nucleoprotein amino acids 396–404, and glycoprotein amino acids 33–42 and 278–286 (29–31). As seen in Table 2, spleen cells from B cell-deficient mice infected 8 d previously were able to lyse peptide-coated targets in an identical manner to spleen cells from +/+ mice. LN cells from B cell-deficient mice also showed a similar pattern of killing (data not shown). Thus, LCMV-specific CTL primed in the absence of B cells recognize the same CTL epitopes as those primed in the +/+ environment.

CD8⁺ T Cell Expansion and Activation. For further assessment of the CD8⁺ T cell response in B cell-deficient mice, we quantitated CD8 expansion and activation after LCMV infection. Six infected and six uninfected mice were evaluated per group. At baseline, uninfected B cell-deficient mice had significantly smaller spleens than uninfected, age-matched +/+ mice. Spleens from -/- mice were on average sixfold smaller than spleens of +/+ mice, and in addition to lacking B cells, also contained fewer CD4⁺ and CD8⁺ T cells. In -/- mice, the average number of CD4⁺ T cells was $5.5 \times 10^6 \pm 1.2 \times 10^6$ SD versus $1.8 \times 10^7 \pm 4.6 \times 10^6$ SD in +/+ mice. The average number of CD8⁺ T cells was $4.0 \times 10^6 \pm 1.2 \times 10^6$ SD in -/- mice versus $9.7 \times 10^6 \pm 3.2 \times 10^6$ SD in +/+ mice. Despite the difference in total numbers, CD8⁺ spleen cells from B cell-deficient and +/+ mice showed a similar degree of expansion in response to LCMV infection. B cell-deficient mice had a 10-fold increase in the number of CD8⁺ spleen cells by day 8, versus a ninefold increase in +/+ mice.

To assess CD8 activation, cells were double stained for CD8 and CD44, an adhesion molecule whose expression goes up in activated T cells (8–11). Representative FACS[®] analyses of the spleen and blood from B cell-deficient and +/+ mice are shown in Fig. 1. Comparison of uninfected and day 8-infected mice illustrates the tremendous increase in the overall percentage of CD8⁺ cells and the percentage of CD8⁺ cells expressing the activated (CD44^{hi}) phenotype. The total number of activated CD8⁺ cells in the spleens increased 20-fold in -/- mice and 10-fold in +/+ mice. Whereas the number of activated CD8⁺ cells increased dramatically after infection, the number of resting CD8⁺ cells remained relatively unchanged in both groups. This resulted in a large increase in the ratio of activated versus rest-

Table 2. CTL from B Cell-deficient Mice Recognize the Three Major CTL Epitopes of LCMV

Mouse genotype*	LCMV-specific CTL in the spleen											
	Percent specific ⁵¹ Cr-release from peptide-coated [‡] MC57 (H-2 ^b) targets at indicated E/T ratio											
	NP 396-404			GP 33-42			GP 276-286			Uninfected		
	50:1	16.6:1	5.5:1	50:1	16.6:1	5.5:1	50:1	16.6:1	5.5:1	50:1	16.6:1	5.5:1
-/-	50	33	20	36	35	18	10	4	1	0	0	0
+/+	42	21	8	38	23	18	15	6	0	0	0	0

*6–8-wk-old mice were infected i.p. with 2×10^5 PFU of LCMV Armstrong. Direct ex vivo LCMV-specific CTL activity was measured 8 d later. Data represent the average of two mice for each group.

[‡]MC57 (H-2^b) targets were coated with peptides representing the three major LCMV epitopes recognized by CTL from mice of the H-2^b background. LCMV nucleoprotein amino acids 396–404 and glycoprotein amino acids 33–42 and 278–286 were used.

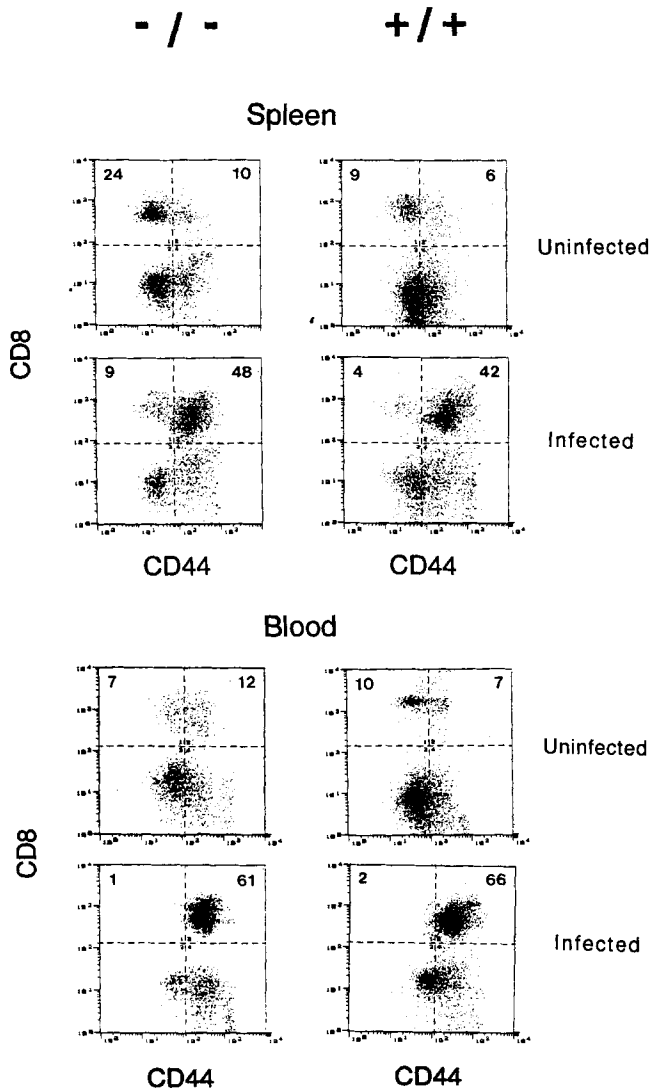


Figure 1. Expansion and activation of CD8⁺ T cells in B cell-deficient mice. Representative FACS[®] analysis of spleen and blood cells from uninfected and day 8-infected B cell-deficient and +/+ mice. B cell-defi-

ing CD8⁺ T cells (Fig. 2). In uninfected B cell-deficient mice, the ratio of activated to resting CD8⁺ spleen cells ranged from 0.5 to 0.6. By day 8 after infection, the ratio of activated to resting CD8⁺ cells had increased an average of 20-fold, and now ranged from 5 to 17. In uninfected +/+ mice, the ratio of activated to resting CD8⁺ spleen cells ranged from 0.6 to 1.2. After infection, the ratio ranged from 9 to 25, a 15-fold average increase.

These results demonstrated that CD8⁺ cells in B cell-deficient and +/+ mice show a similar degree of expansion and activation after LCMV infection, with a 9–10-fold increase in CD8 numbers and a 15–20-fold increase in the ratio of activated to resting CD8⁺ cells.

B Cell-deficient Mice Clear an Acute LCMV Infection. To evaluate the kinetics of viral clearance in B cell-deficient mice, we measured levels of infectious virus in various tissues on days 5 and 8 after LCMV Armstrong infection (Table 3). On day 5 of infection, infectious virus could be found in the serum, liver, lung, and spleen of both B cell-deficient and +/+ mice. Levels of infectious virus were slightly higher in the B cell-deficient mice at day 5, but by day 8, there was no detectable infectious virus in the serum, brain, kidney, liver, lung, or spleens of both the B cell-deficient and +/+ mice. The difference in viral titers at day 5 may be related to either the lack of B cells and/or antibody, or to the lower number of CD8⁺ cells in B cell-deficient mice. Overall, however, the kinetics of viral clearance in the B cell-deficient mice follows a similar pattern to that seen in +/+ mice.

cient and +/+ mice showed a significant increase in the percentage of CD8⁺ cells in the spleen and blood after infection, with a tremendous shift in the percentage of cells expressing the activated CD44hi phenotype. X- and Y-axes are in log₁₀ fluorescence. Numbers indicate percentage of cells in each quadrant. (The total number of CD8⁺ T cells in the spleens of uninfected -/- mice was about twofold less than in +/+ mice, although CD8 percentages were higher in -/- mice because of the absence of B cells).

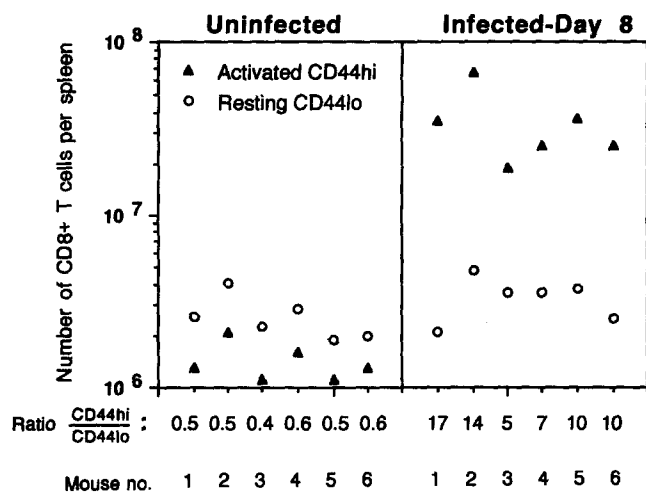


Figure 2. CD8⁺ T cells in B cell-deficient mice are activated in response to an acute LCMV infection. Spleen cells from six uninfected B cell-deficient and 6 B cell-deficient mice infected 8 d previously with LCMV Armstrong were double stained for CD8 and CD44. Total numbers of activated (CD44^{hi}) and resting (CD44^{lo}) CD8⁺ T cells in the spleens of individual mice are shown. In uninfected mice, the ratio of activated to resting CD8⁺ cells was 0.4–0.6. By day 8 after infection, there was a large shift in this ratio (ranging from 5 to 17), due to a greater than 20-fold increase in the number of activated CD8⁺ T cells.

Maintenance of CD8⁺ CTL Memory. To determine whether B cells or antibody are needed for maintaining CTL memory, we quantitated the number of LCMV-specific memory CTL in the spleens of $-/-$ and $+/+$ mice at various times after infection. The results of these limiting dilution analyses are shown in Fig. 3 A. In both B cell-deficient and $+/+$ mice, the CTLp frequency per spleen cell peaked at 1/30 at 8 d after infection. By day 16, the CTLp frequency began to fall in both groups. From days 35–170, CTLp frequencies in B cell-deficient mice ranged from 1/110 to 1/4,150. In $+/+$ mice, CTLp frequencies ranged from 1/120 to 1/1,180. This variation, however, showed no specific trend over time, illustrating that in B cell-deficient and $+/+$ mice, the CTLp frequencies are being maintained at relatively the same rate.

Table 3. B Cell-deficient Mice Are Able to Control an Acute LCMV Infection

Mouse genotype*	Days after infection	LCMV titer (Log ₁₀ PFU/ml of serum or gram of tissue)					
		Serum	Brain	Kidney	Liver	Lung	Spleen
$-/-$	5	3.2	ND	ND	5.2	4.3	2.8
$+/+$	5	2.2	ND	ND	4.5	4.0	2.5
$-/-$	8	<1.7	<2.7	<3.0	<2.7	<3.0	<2.3
$+/+$	8	<1.7	<2.7	<3.0	<2.7	<3.0	<2.3

*6–8-wk-old mice were infected i.p. with 2×10^5 PFU of the Armstrong strain of LCMV, and virus titers were checked 5 and 8 d after infection. Data represent the average of four $-/-$ mice and two $+/+$ mice for each time point.

At day 8 after infection, the total number of CTLp per spleen was approximately threefold higher in $+/+$ mice compared with B cell-deficient mice. By day 16, both groups showed a rapid decrease in CTLp numbers and then a more gradual decline until day 50. During this time, when apoptotic T cell death occurred, B cell-deficient mice showed a greater decline in CTLp numbers compared with $+/+$ mice. B cell-deficient mice had over a 60-fold decrease in LCMV-specific CTLp cells compared with a 13-fold decrease in $+/+$ mice. From day 80 and beyond, the total number of CTLp cells remained lower in the B cell-deficient mice compared with $+/+$ mice. Although there was some variation in the total CTLp numbers during this time, both groups then maintained a relatively stable population of LCMV-specific CTL for up to 6 mo after infection.

Fig. 3 B shows the number of activated CD8⁺ spleen cells (CD44^{hi}) in B cell-deficient and $+/+$ mice from days 3 to 170 after infection. Uninfected B cell-deficient mice had approximately half the number of CD8⁺ T cells as the uninfected $+/+$ mice (with both CD44^{hi} and CD44^{lo} cells being two- to threefold lower). By day 8 after LCMV infection, the number of activated CD8⁺ cells in both $-/-$ and $+/+$ mice were nearly equal (less than 1.5-fold difference). Numbers of activated CD8⁺ cells began to decrease thereafter, with $-/-$ mice showing a greater drop compared with $+/+$ mice. From days 8 to 50, B cell-deficient mice had a 23-fold drop in activated CD8⁺ cells versus a sevenfold drop in $+/+$ mice. Although B cell-deficient mice showed greater cell loss during the “death” phase, the number of activated CD8⁺ cells stabilized from day 50 and beyond (in the maintenance phase). Numbers of activated CD8⁺ cells then remained two- to fivefold lower in B cell-deficient mice compared with $+/+$ mice, returning close to preinfection levels for each group. Fig. 3 C shows that in contrast to the changes seen in the CD44^{hi} population, the number of CD8⁺ CD44^{lo} cells remained relatively stable throughout the time period studied. At day 8 after infection, there was less than a twofold increase in the CD8⁺ CD44^{lo} population in both groups and no dramatic drop in CD8⁺ CD44^{lo} cells during the death phase. The absolute number of CD8⁺ CD44^{lo} cells remained two- to fivefold lower in $-/-$ compared with $+/+$ mice.

Thus, in the absence of B cells and/or antibody, there is a higher rate of death of activated CD8⁺ cells and LCMV-specific CD8⁺ CTL memory cells after virus infection. The increased cell death results in lower absolute numbers of activated CD8⁺ cells and LCMV-specific CTLp in $-/-$ compared with $+/+$ mice. Lower numbers of LCMV-specific CTL in B cell-deficient mice, however, was not due to selective loss of CTL memory cells, since CTLp frequencies in the spleens of $-/-$ mice were comparable with that seen in $+/+$ mice and remained relatively constant over time.

Long-term Protective Immunity in B Cell-deficient Mice. LCMV-specific memory CTL are responsible for mediating protective immunity against reinfection (13, 32). As an in vivo test of CTL memory, previously immunized B cell-deficient mice were rechallenged with LCMV Armstrong

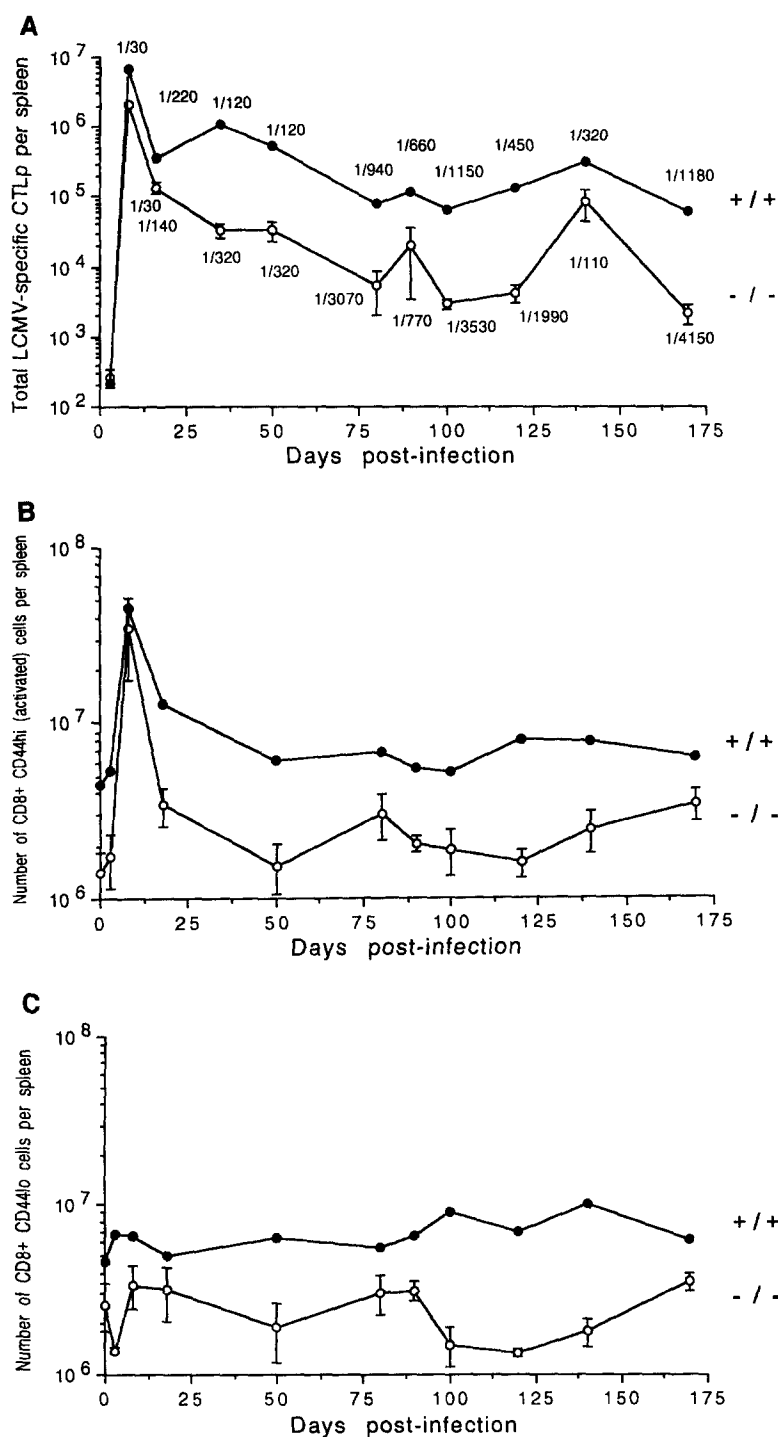


Figure 3. Maintenance of CD8⁺ CTL memory in B cell-deficient mice. (A) B cell-deficient and +/+ mice were infected with LCMV and the LCMV-specific CTLp frequency in the spleens was quantitated by limiting dilution assay. In both groups, CTLp frequencies were maintained at relatively the same rate over time, peaking at day 8, showing a significant decline by day 16, and stabilizing from day 50 and beyond. Expansion of LCMV-specific CTL in the two groups was comparable, with both B cell-deficient and +/+ mice demonstrating up to a 10,000-fold increase in the number of LCMV-specific CTL in the spleen by 8 d after infection. During the cell death phase, -/- mice showed a greater decrease in LCMV-specific CTL compared with +/+ mice. Between days 8 and 50, there was a 60-fold drop in -/- mice versus a 13-fold drop in +/+ mice. From day 50 and beyond, both groups maintained a stable population of LCMV-specific CTL, with absolute numbers in -/- mice remaining lower than in +/+ mice. (B) By 8 d after infection, the total number of activated CD8⁺ T cells increased 20-fold in -/- mice and 10-fold in +/+ mice. During the death phase, -/- mice showed greater loss of activated CD8⁺ cells. Activated CD8⁺ cells stabilized during the maintenance phase, returning close to preinfection levels for both groups of mice. (C) In both -/- and +/+ mice, the number of CD8⁺ CD44^{lo} cells remained relatively stable throughout the time period studied, although total numbers in -/- mice were two- to fivefold lower than in +/+ mice.

and evaluated for their ability to generate an accelerated CTL response. B cell-deficient mice immunized 2 mo previously were re-challenged with 10⁶ PFU of LCMV Armstrong. Naive (previously uninfected) age-matched B cell-deficient mice were similarly infected for comparison. Direct ex vivo CTL activity in the spleens was measured at days 3 and 5 after challenge. Previously immunized -/- mice generated an accelerated LCMV-specific CTL response in the spleen, with virus-specific CTL activity evi-

dent by day 3 after challenge (Fig. 4 A). Naive -/- mice showed no measurable LCMV-specific CTL activity at this time. By day 5 after challenge, virus-specific CTL activity increased even further in the previously immunized group, with 65% LCMV-specific target cell lysis at the 50:1 E/T ratio compared with 15% in the naive mice (data not shown).

Levels of infectious virus were measured on day 3 after challenge. In immunized -/- mice, levels of infectious virus

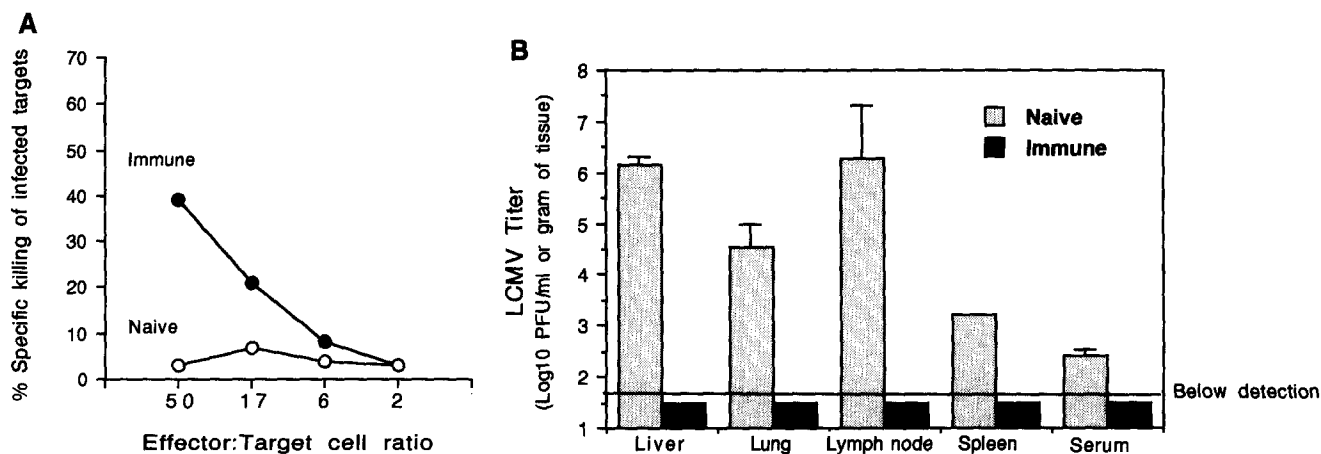


Figure 4. Immunized B cell-deficient mice show accelerated memory CTL responses in vivo upon rechallenge with LCMV. B cell-deficient mice immunized 2 mo earlier were re-challenged with 10^6 PFU of LCMV Armstrong. Naive (uninfected) B cell-deficient mice received the challenge dose for comparison. (A) Direct ex vivo CTL activity in the spleens 3 d after challenge. Percent specific killing of infected targets [(% ^{51}Cr -release from LCMV-infected targets) - (% ^{51}Cr -release from uninfected targets)] is shown for the indicated E/T ratios. Previously immunized mice generated an accelerated LCMV-specific CTL effector response by day 3. Naive mice had no LCMV-specific response at this time. (B) Level of infectious virus in the serum and tissues of challenged mice. Naive mice had high levels of infectious virus in the serum and tissues, whereas levels were below detection in previously immunized mice. Thus, B cell-deficient mice were able to generate a LCMV-specific CTL memory response in vivo after viral challenge that was capable of quickly controlling viral spread. Data represent the average of two mice per group. Lines on bar graph indicate range for each group.

in the serum, liver, lung, LN, or spleen, were below detection, whereas naive $-/-$ mice had high levels of infectious virus in both serum and tissues at this time (Fig. 4 B). Previously immunized $+/+$ mice also had no detectable infectious virus in the serum and tissues, whereas naive $+/+$ mice had high levels of infectious virus that were comparable with those in the naive $-/-$ mice (data not shown). Thus, previously immunized B cell-deficient mice generated an accelerated CTL response after LCMV Armstrong challenge and quickly controlled viral infection, demonstrating the presence of CTL memory in vivo.

As a more stringent in vivo test of CTL memory, challenge experiments were also performed using LCMV variant clone 13 or 28b. Clones 13 and 28b are virulent strains of LCMV that establish persistent infections in naive adult mice characterized by low LCMV-specific CTL responses and high levels of infectious virus in several tissues (26, 27). B cell-deficient mice immunized between 3 and 6 mo earlier with LCMV Armstrong were challenged with 1.5×10^6 i.v. PFU of clone 13 or 28b. Naive, age-matched B cell-deficient mice were challenged for comparison. Mice were killed 7 d after infection and direct ex vivo CTL activity in the spleen was measured. CTL activity in immune $-/-$ mice was 10–30-fold higher than in the naive $-/-$ mice (Table 4). In addition, there were striking differences in levels of infectious virus in serum and tissues between the two groups. Immune $-/-$ mice had undetectable levels of infectious virus in the serum, brain, liver, lung, and spleen. In sharp contrast, naive $-/-$ mice had very high levels of infectious virus in serum and tissues. Therefore, in two different types of challenge experiments (Armstrong or clones 13/28 b), B cell-deficient mice demonstrated accelerated LCMV-specific CTL memory responses

in vivo that resulted in faster clearance of virus. Thus, CTL-mediated protective immunity does not wane (for at least 6 mo) in the absence of B cells or antigen-antibody complexes.

Discussion

In this study we have examined the role of B cells in the generation and maintenance of CD8^+ CTL memory. To address this issue we compared CTL responses to LCMV in normal ($+/+$) versus B cell-deficient mice. The CTL response to acute LCMV infection can be broken down into three distinct phases. (a) The initial phase (days 3–8 after infection) is the phase of antigen-driven expansion of virus-specific CD8^+ T cells and the development of effector CTL (i.e., direct ex vivo killers). Most of the viral antigen is cleared during this period. (b) This is followed by a death phase (between days 10 and 30 after infection) during which >95% of the virus-specific CTL die and the number of direct effector cells falls below the level of detection. (c) The third phase is the memory phase (>30 d after infection) and is characterized by a stable pool of memory CTL that persist for the life span of the animal. The role of B cells in each of these three phases was analyzed. We found that B cells were not required for the expansion and activation of virus-specific CTL but that the death phase was more pronounced in B cell-deficient mice. A greater proportion of activated CD8^+ T cells died in these mice, resulting in lower absolute numbers of virus-specific CTL. However, the memory phase was not affected by the absence of B cells, and a stable number of LCMV-specific memory CTL persisted in B cell-deficient mice. Upon reinfection, the B cell-deficient mice that had resolved an acute LCMV infection

Table 4. Long-term Protective Immunity in B Cell-deficient Mice

Group	Days after infection	LCMV-specific CTL in spleen Percent specific ⁵¹ Cr-release from MC57 (H-2 ^b) targets at indicated E/T ratio						LCMV titer (Log ₁₀ PFU/ml of serum or gm of tissue)				
		LCMV infected			Uninfected			Serum	Brain	Liver	Lung	Spleen
		50:1	16.6:1	5.5:1	50:1	16.6:1	5.5:1					
Immune Mice*												
-/-	105	65	46	22	2	0	0	<1.7	<2.7	<2.7	<2.7	<2.7
-/-	135	75	66	34	11	5	2	<1.7	<2.7	<2.7	<2.7	<2.7
-/-	175	78	58	29	10	5	2	<1.7	<2.7	<2.7	<2.7	<2.7
Naive mice												
-/-	—	26	13	6	5	1	0	5.5	5.6	8.2	8.1	5.9
-/-	—	11	6	2	2	0	0	4.3	4.1	7.5	7.9	7.1

*6–8-wk-old B cell-deficient mice were infected with 2×10^5 PFU of LCMV Armstrong. 105–175 d later, mice were challenged with 1.5×10^6 PFU of LCMV clone 13 or 28b. 7 d after challenge, mice were killed and direct ex vivo CTL activity and virus titers were measured. Age-matched naive -/- mice were challenged for comparison. LCMV-specific serum antibody was below detection in both immune -/- and naive -/- mice. Data represent the average of two to four mice per group.

were able to make accelerated CTL responses in vivo and eliminated virus more efficiently than naive B cell-deficient mice. Taken together, these results show that neither B cells nor antigen-antibody complexes are essential for the maintenance of CD8⁺ memory CTL.

B cells were not required for the priming and activation of virus-specific CTL responses. The kinetics and magnitude of the effector CTL response, as measured by direct killing of infected targets by ex vivo isolated splenocytes (or LN cells), were almost identical in B cell-deficient and +/+ mice. Also, the overall expansion of activated CD8⁺ T cells was not affected by the absence of B cells; in both +/+ and B cell-deficient mice there was over a 10-fold expansion of activated CD8⁺ T cells and a ~10,000-fold increase in the number of LCMV-specific CTL during the first week after virus infection. In addition, we found that virus-specific CTL from B cell-deficient mice recognized the same LCMV epitopes as did CTL from +/+ mice, and the same hierarchy for the three CTL epitopes (NP 396 and GP 33 > GP 276) was seen in both +/+ and -/- mice. Taken together, these results show that B cells are not involved in the priming of virgin CD8⁺ T cells. In the past, this issue has been somewhat controversial, and in some studies, B cells were found to be essential for T cell priming in vivo (33–36). However, several other studies (37–40) have shown that B cells are unable to activate naive T cells and a recent paper (41) has shown that B cell-deficient mice do not have any defects in making primary CD4⁺ or CD8⁺ T cell responses to a variety of antigens. Our finding, along with these other reports, provides strong evidence that B cells are not needed for activating T cells. It is worth pointing out that there was massive expansion and activation of CD8⁺ T cells in B cell-deficient mice after

LCMV infection. These results show that not only are B cells not required for antigen presentation to naive CD8⁺ T cells, but that B cells play a minimal to no role in providing any necessary signals and/or cytokines for proliferation and differentiation of CD8⁺ CTL.

B cell-deficient mice were able to eliminate LCMV and at 8 d after infection there was no detectable infectious virus in any of the tissues tested (spleen, liver, lung, blood, brain, or kidney). This provides formal proof that antibody does not play an essential role in resolving LCMV infection. Earlier studies have shown that CD8⁺ CTL are the major players in controlling LCMV infection (5, 6, 25, 42–45).

Although no differences were seen during the expansion phase of the CTL response between B cell-deficient and +/+ mice, we found that the death phase was more pronounced in -/- mice. Between days 8 and 50 there was a 60-fold drop in the number of LCMV-specific CTL in B cell-deficient mice compared with a 13-fold drop in +/+ mice. During this period the total number of activated CD8⁺ T cells also dropped substantially more in the B cell-deficient compared with +/+ mice (23- versus 7-fold). The net result of this increased T cell death was a lower absolute number of LCMV-specific CTL in B cell-deficient mice (4–20-fold lower). However, it is worth noting that the difference in the frequency of LCMV-specific CTL/spleen cells or CD8⁺ T cells between the two groups of mice was much less (three- to fourfold), and in some experiments the frequency of virus-specific CTL was the same in +/+ and B cell-deficient mice. Why was there more overall T cell death in the absence of B cells? Activated T cells die by apoptosis and there is evidence implicating both *fas* and TNF in this process (46, 47). It is currently not well understood what type of signals and cellular interactions can rescue activated T cells

from undergoing apoptosis *in vivo*, but two recent studies have shown that LPS or TGF- β 1 can prevent apoptosis of activated T cells (48, 49). Our results suggest that B cells might deliver signals for rescuing peripheral T cells from death. B cells express a variety of adhesion/costimulatory molecules (B7-1, B7-2, CD40, etc.) on their cell surface that are potential candidates for mediating interactions with activated T cells (50). Some of these interactions may be involved in preventing cell death in peripheral T cells. In this context, it is worth noting that uninfected B cell-deficient mice have lower numbers of both CD4 and CD8⁺ T cells in their spleens compared with age- and sex-matched +/+ mice (an average of 5.5×10^6 CD4⁺ T cells in -/- mice versus 1.8×10^7 in +/+ mice; 4×10^6 CD8⁺ T cells in -/- mice versus 9.7×10^6 in +/+ mice).

A question of substantial interest was determining if CD8⁺ T cell memory would persist in the absence of B cells. The results of this study show that CTL memory, as assessed by frequency of virus-specific CTL or protective immunity, does not decline in the absence of B cells. The frequency of LCMV-specific CTL remained relatively constant in B cell-deficient mice over a 6-mo period, and these mice were

able to make an accelerated CTL response *in vivo* upon rechallenge with virus. This accelerated CTL response was able to confer protection against a virulent strain (clone 13 or 28b) of LCMV. These LCMV variants cause a persistent infection in naive adult mice but are cleared by immune mice. Ability to control LCMV clone 13 or 28b is a definitive *in vivo* marker for CTL memory, and immunized B cell-deficient mice were able to eliminate this infection efficiently.

It has been proposed that B cells sustain T cell memory by picking up antigen-antibody complexes trapped on FDC, processing this trapped antigen, and then presenting it to T cells (8, 20, 21). Our results show that this pathway does not play an essential role in sustaining CD8⁺ T cell memory. Our studies also show that once a stable pool of memory CTL has developed, these cells do not require any signals/help from B cells. Taken together, these results show that memory CD8⁺ T cells require neither antigen-antibody complexes nor B cells for their survival. It will be of interest to determine if the same rules apply for maintenance of CD4⁺ T cell memory.

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