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Deficient CD4\(^+\) T Cell Priming and Regression of CD8\(^+\) T Cell Functionality in Virus-Infected Mice Lacking a Normal B Cell Compartment\(^1\)

Jan Pravsgaard Christensen, Susanne Ørding Kauffmann, and Allan Randrup Thomsen\(^2\)

In this study, we investigate the state of T cell-mediated immunity in B cell-deficient (B\(^{-/-}\)) mice infected with two strains of lymphocytic choriomeningitis virus known to differ markedly in their capacity to persist. In B\(^{-/-}\) C57BL mice infected with the more persisting virus, virus-specific CD8\(^+\) T cells are initially generated that are qualitatively similar to those in wild-type mice. However, although cell numbers are well sustained over time, the capacity to produce cytokines is rapidly impaired. In similarly infected B\(^{-/-}\) BALB/c mice, virus-specific CD8\(^+\) T cells are completely deleted, indicating that host genotype influences the severity of the T cell defect. In B\(^{-/-}\) C57BL mice infected with the less persisting virus, CD8\(^+\) T cell dysfunction was not as pronounced, although it was clearly present. Most importantly, the appearance of dysfunctional CD8\(^+\) T cells clearly precedes recrudescence of detectable virus, indicating that the T cell defect is not simply a secondary event due to virus buildup resulting from the failure of B\(^{-/-}\) mice to produce neutralizing Abs. In contrast with CD8\(^+\) T cells, which initially respond almost as in wild-type mice, the priming of virus-specific CD4\(^+\) T cells was markedly impaired in B\(^{-/-}\) mice infected with either virus strain. Thus, our results indicate that B cells play an important role in antiviral immunity not only as Ab producers, but also in promoting an optimal and sustained T cell response. The T cell defects are likely to contribute to the chronic course of viral infection in B\(^{-/-}\) mice. *The Journal of Immunology*, 2003, 171: 4733–4741.

CD8\(^+\) T cells are central to immune-mediated control of many primary virus infections. This fact has been clearly documented in several animal model systems, but also seems to hold true for the majority of viral infections in humans. Furthermore, sustained CD8\(^+\) T cell immune surveillance seems to be required for efficient control of infections caused by poorly cytopathic viruses capable of establishing lifelong chronic infections. These include important human pathogens such as hepatitis B and C, cytomegalovirus, EBV, and HIV (1–6). For this reason, understanding the requirements for induction and maintenance of virus-specific T cell responses in general, and of CD8\(^+\) T cell-dependent immune surveillance in particular, is very important.

Infection of mice with lymphocytic choriomeningitis virus (LCMV)\(^3\) offers an excellent model to study the cellular requirements for a sustained antiviral CD8\(^+\) T cell response, and using this model system, we and others have found the interplay between various antiviral effector cell subsets to be quite complex. Thus, it has been found that although the initial virus knockdown is mediated primarily by CD8\(^+\) effector T cells (7), both CD4\(^+\) T cells and B cells are needed for lifelong control of this infection, and absence of either subset leads to impaired CD8\(^+\) T cell memory (8–12). Moreover, because very similar results were subsequently obtained in CD40 ligand-deficient mice (13), focus was placed on T cell help to B cells as being the central phenomenon. It was assumed that the role of B cells was simply to produce neutralizing Abs, which would mop up free infectious virus particles otherwise escaping T cell-mediated immunity. Hence, the presence of Abs would prevent the virus load from building up to a level that would eventually lead to CD8\(^+\) T cell anergy and/or deletion. This interpretation was initially supported by the observation of impaired virus control in B cell-deficient (B\(^{-/-}\)) mice (11, 12). However, a number of more recent studies in B\(^{-/-}\) mice indicate that this explanation may be too simplistic. First, even before infection, the architecture of the secondary lymphoid organs in these mice is severely disturbed by the absence of B cells, with fewer CD4\(^+\) and CD8\(^+\) T cells present in spleen and lymph nodes (14). Second, impaired virus control is found even in B\(^{-/-}\) mice infected with virus isolates, which induces few or no neutralizing Abs (13). Third, various functional defects within both the CD4\(^+\) and CD8\(^+\) T cell compartments of these mice have been described recently (for LCMV-infected mice, see Ref. 15). However, using more classical cellular assays, it has been difficult to determine the precise nature and sequence of the observed defects, and in particular it is unclear to what extent the defects are quantitative, qualitative, or both. Therefore, to better characterize the T cell defects in LCMV-infected B\(^{-/-}\) mice, we have performed a reevaluation of virus-specific CD4\(^+\) and CD8\(^+\) T cell responses using contemporary techniques to visualize Ag-specific T cells, i.e., intracellular cytokine staining for function and MHC/peptide tetramers for numbers. Furthermore, to better understand how the virus load might influence CD8\(^+\) T cell function, key experiments were conducted using both a rapidly replicating LCMV strain, LCMV Traub, and a relatively slowly replicating LCMV strain, LCMV Armstrong (13, 16). When B\(^{-/-}\) mice are infected using the latter

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\(^{3}\)Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; B\(^{-/-}\), B cell deficient; LCMV ARM, LCMV of the Armstrong strain; BrdU, bromodeoxyuridine; np, nucleoprotein; MFI, mean fluorescence intensity; HJV, HJM strain of mouse hepatitis virus.
virus strain, the acute infection is controlled almost as rapidly as in wild-type mice, and no virus is detectable by sensitive assays for up to 3–4 mo; beyond this time point, low levels of infectious virus may again be detected in the organs of most B−/− mice (13). Therefore, perturbation of T cell numbers and function during the first 2 mo of infection with this virus strain is likely to reflect intrinsic defects of immune regulation in B−/− mice rather than being a result of a high viral antigenic load caused by lack of Ab-dependent virus control. Our results indicate that both CD4+ and CD8+ T cell priming are impaired in B−/− mice. However, whereas the primary CD8+ T cell response is only delayed and slightly impaired in B−/− mice, CD4+ T cell priming is markedly reduced. Furthermore, whereas the spectrum of cytokines produced by virus-specific CD8+ T cells initially is almost similar, CD8+ T cells from B−/− mice gradually lose the ability to produce the entire range of cytokines produced by matched cells from wild-type mice. Although the speed with which this difference develops is influenced by the virus strain used for infection, functional defects of virus-specific CD8+ T cells start developing even in the absence of a substantial viral burden. Therefore, we conclude that partial anergy of virus-specific CD8+ T cells may be an important contributing factor underlying the failure of B−/− mice to permanently control active virus replication.

Materials and Methods

Mice

Two sources of B−/− μMT/μMT mice were used. Part of the mice were the progeny of breeder pairs originally obtained from the National Institutes of Health (Bethesda, MD). These mice were bred using heterozygous females and homozygous males, and the offspring were selected by testing sera in a sandwich ELISA for the presence of IgM; heterozygous littermates were used as wild-type controls. Other μMT/μMT mice came directly from The Jackson Laboratory (Bar Harbor, ME) (B6.129S2-Igh-6-129/cR). In this case, normal C57BL/6 mice were used as controls. IFN-γ-deficient C57BL/6 mice were the progeny of breeder pairs obtained from The Jackson Laboratory. B−/− J1D mice (C129.B6-H2d−/−) were purchased from Taconic Farms (Germantown, NY), and wild-type C57BL/6 and BALB/c mice came from Taconic M&B (Ry, Denmark). Mice lacking both TNF-R1 (p55) and R2 (p75) (TNF-R1 + 2−/−) and matched wild-type controls (B6 × 129SFr) were obtained directly from The Jackson Laboratory. Mice from outside sources were always allowed to rest for at least 1 wk before entering into experiments; by that time the animals were ~7–8 wk old. Animals were housed under controlled (specific pathogen-free) conditions that included the testing of sentinels for unwanted infections according to Federation of European Laboratory Animal Science Association standards. No contaminant infections were detected.

Virus infection

LCMV Traub was produced, stored, and quantified as previously described (17). LCMV of the Armstrong strain (LCMV ARM, clone 53b) was kindly provided by M.B.A. Oldstone (Scirrps Clinic and Research Foundation, La Jolla, CA) (18). Mice to be infected with LCMV received 200 PFU (LCMV Traub) or 400 PFU (LCMV ARM) in an i.v. injection of 0.1 ml. Titrations of organ virus levels were performed by an immunological focus assay on MC57G cells (19).

In vivo bromodeoxyuridine (BrdU) labeling

Mice were given BrdU (Sigma-Aldrich, St. Louis, MO) at 0.8 mg/ml in their drinking water for a period of 3 (acutely infected mice) or 7 days (late memory) (20, 21). BrdU-containing water was protected from light and was changed daily.

Cell preparations

Single cell suspensions of spleen cells were obtained by pressing the organ through a fine steel mesh. When used for analysis by flow cytometry (except for intracellular staining), erythrocytes were lysed by 0.83% NH4Cl treatment (Gey’s solution).

MHc/peptide tetramers for flow cytometry

H-2D+gp33–41, H-2D+nuclieoprotein(np)396–404, and H-2D+np118–126 tetramers were obtained through the National Institute of Allergy and Infectious Disease Tetramer Facility and the National Institutes of Health AIDS Research and Reference Reagent Program.

Monoclonal Abs

The following mAbs were purchased from BD PharMingen (San Diego, CA) as rat anti-mouse mAbs: CyChrome-conjugated anti-CD4 and anti-CD8a; FITC-conjugated anti-CD49 days (α4 integrin); FITC-conjugated anti-CD44; FITC-conjugated anti-CD62 ligand (L-selectin); FITC- and PE-conjugated anti-IFN-γ; PE-conjugated anti-IL-2, anti-IL-4, anti-IL-5, anti-IL-10, anti-GM-CSF, and anti-TNF-α; and matched isotype controls. For BrdU staining, FITC-conjugated anti-BrdU (BD Biosciences, San Jose, CA) was used.

Fluorescence staining and flow cytometric analysis

Staining for flow cytometry was done as described previously (21–23). Briefly, 1–2 × 106 cells were incubated for 5 min in FACS medium (PBS containing 10% rat serum, 1% BSA, and 0.1% NaN3). Subsequently, cells were incubated with relevant Abs in the dark for 20 min at 4°C, after which they were washed three times in PBS with 0.1% NaN3 and fixed with 1% paraformaldehyde in PBS. In the case of biotin-conjugated Ab, cells were additionally incubated with Streptavidin-Tri-color (Caltag Laboratories, Burlingame, CA) for 20 min before fixation.

For tetramer staining, cells were incubated with the tetramers for 30 min at 4°C, followed by surface labeling as described above.

To detect intracellular cytokine, splenocytes were cultured at 37°C in 96-well round-bottom plates at a concentration of 1–2 × 106 cells/well in a volume of 0.2 ml of complete RPMI 1640 medium supplemented with 10% FBS and 20 U/ml recombinant IL-2 (R&D Systems Europe, Abingdon, U.K.) and 5 μM monensin (Sigma-Aldrich) either with or without peptide. The peptides used were as follows: LCMV gp33–41 and np396–404 (both immunodominant class I epitopes in H-2d mice), np118–126 (immunodominant class I epitope in H-2b mice), and gp61–80 (immunodominant class II epitope in H-2b mice). Peptide stimulation was conducted at a concentration of 1 μg/ml for class I restricted epitopes and at 1.0 μg/ml for the class II epitope. After 5 h of culture, cells were washed once in FACS medium (PBS containing 1% BSA, 0.1% NaN3, and 5 μM monensin) and subsequently were incubated with relevant surface Abs in the dark for 20 min at 4°C. Cells were washed twice in PBS with 3 μM monensin and were resuspended in 200 μl of 1% paraformaldehyde in PBS. After 30 min of incubation in the dark at 4°C, cells were washed in FACS medium and resuspended in PBS with 0.5% saponin. After 10 min of incubation in the dark at 20°C, cells were pelleted and resuspended in PBS with 0.5% saponin and relevant Abs. After incubation for 20 min at 4°C, cells were washed twice in PBS/saponin and were analyzed. Combined staining for BrdU and intracellular cytokine was performed using the BrdU Flow Kit from BD Biosciences.

Cell samples were analyzed with a FACSCalibur (BD Biosciences), and at least 10,000 mononuclear cells were gated using a combination of forward angle and side scatter. Data analysis was conducted by use of the CellQuest program, and results are presented as dot plots.

Results

Impairment of CD8+ T cell function in chronically LCMV-infected B−/− mice

B−/− mice infected with LCMV Traub never completely control the infection (11). To examine the CD8+ T cell response under these conditions, B−/− mice and matched heterozygous littermates (C57BL background) were infected with 200 PFU of LCMV Traub. Using this dose and strain of virus, we have previously found that, although some virus control is obtained, most B−/− mice are consistently viremic and viral titers tend to increase slightly with time (11). Eleven, 28, and 100 days after infection, numbers of virus gp33–41-specific CD8+ T cells were determined using tetramers (Fig. 1). In addition, the functionality of the cells was evaluated by their ability to synthesize cytokines normally produced by virus-specific CD8+ T cells: IFN-γ, TNF-α, GM-CSF, and IL-2 (Fig. 2). Generally the CD8+ T cell response is slightly delayed in B−/− mice (11, 24), but by day 11 postinfection, both the frequency and the absolute number of virus-specific
CD8\(^+\) T cells are similar in B\(^{-/-}\) mice and matched wild-type animals (Fig. 1). By this time point, the capacity to produce cytokines is also comparable. Thus, most gp33–41-specific cells produced IFN-\(\gamma\), whereas smaller subsets produced TNF-\(\alpha\), GM-CSF, and IL-2 (Fig. 2). Four weeks after infection, there is still no difference in frequency or absolute number of tetramer\(^+\) CD8\(^+\) T cells. Furthermore, tetramer\(^+\) cells from both genotypes have a primed phenotype, as evidenced by high expression of \(\alpha_4\) integrin (data not shown). However, CD8\(^+\) T cells from B\(^{-/-}\) mice are now clearly inferior to CD8\(^+\) T cells from wild-type mice regarding effector capacity. Fewer cytokine-producing cells are present in B\(^{-/-}\) mice, and even for those cytokines, which are produced by a substantial proportion of gp33–41-specific CD8\(^+\) T cells from B\(^{-/-}\) mice, mean fluorescence intensity (MFI) is markedly reduced compared with matched cells from wild-type mice (for IFN-\(\gamma\), see representative histograms in Fig. 2; double staining for IFN-\(\gamma\)/TNF-\(\alpha\) and IFN-\(\gamma\)/IL-2 can be found in Fig. 6). This reduced ability to produce type 1 cytokines does not reflect a switch toward a Tc2 or tolerant phenotype (25), in that no IL-4-, IL-5-, or IL-10-producing CD8\(^+\) T cells were found in B\(^{-/-}\) mice (<1%). Mice analyzed ~100 days after infection gave results similar to those analyzed 4 wk after infection (data not shown), indicating that the situation in B\(^{-/-}\) mice was stable over time. Therefore, if the dysfunctional cells were prone to undergo apoptosis, proliferation apparently can make up for the loss.

To determine whether the failure to maintain normal CD8\(^+\) T cell function resulted from a change in the threshold for activation, we compared the amount of peptide required to activate cytokine production in CD8\(^+\) T cells from B\(^{-/-}\) mice and matched wild-type mice infected 28 days earlier. CD8\(^+\) T cells from mice of both genotypes were incubated with decreasing amounts of peptide (gp33–41), and the frequency of IFN-\(\gamma\)-producing CD8\(^+\) cells as a function of peptide concentration was determined by intracellular staining (26). When compensation was made for the lower frequency of IFN-\(\gamma\)-producing cells in B\(^{-/-}\) mice (by normalizing to the maximal response obtained in each genotype), identical titration curves were obtained (data not shown), indicating that there is no difference in functional avidity. Thus, impaired cytokine production does not reflect a changed threshold for activation, but rather a reduced response subsequent to activation.

**FIGURE 1.** Virus-specific CD8\(^+\) T cells in B\(^{-/-}\) mice infected with LCMV Traub. Wild-type (WT) and B\(^{-/-}\) C57BL (\(\mu\)MT) mice were infected i.v. with 200 PFU of LCMV Traub, and at various times after infection, frequencies (upper panel) and absolute numbers (lower panel) of gp33–41-specific CD8\(^+\) T cells were measured using tetramers. Data represent the median and range of four to eight mice per group.

**FIGURE 2.** Cytokine production by virus-specific CD8\(^+\) T cells in B\(^{-/-}\) mice infected with LCMV Traub. Wild-type (WT) and B\(^{-/-}\) C57BL (\(\mu\)MT) mice were infected i.v. with 200 PFU of LCMV Traub, and at 11 (upper panel) and 28 (middle panel) days after infection, the frequencies of gp33–41-specific CD8\(^+\) T cells producing cytokines were examined by intracellular staining. Data represent the median and range of eight mice per group. * \(p < 0.05\) relative to wild type. Representative histograms for production of IFN-\(\gamma\) by activated (\(\alpha_4\) integrin\(^+\)) CD8\(^+\) T cells are also presented (lower panel).

**Absence of LCMV-specific CD8\(^+\) T cells in B\(^{-/-}\) mice on a BALB/c background**

It has recently been reported that B cell deficiency may affect T cell responsiveness in a genetically restricted manner. Thus, although B\(^{-/-}\) mice on a C57BL background generate a diminished T cell response in response to infection with the JHM strain of mouse hepatitis virus (JHMV), T cell expansion and function is unimpaired in similarly infected B\(^{-/-}\) mice on a BALB/c background (JHM-D mice) (27, 28). Consequently, it was pertinent to ask whether the CD8\(^+\) T cell defect in LCMV-infected mice would also be restricted to mice with a C57BL background or if it represented a more general phenomenon. To test this, JHM-D mice and matched BALB/c wild-type mice were infected as above, and T cell numbers and function were assessed 4 wk later (Fig. 3). Unlike the situation in JHMV-infected mice, we found that the T cell defect was even more pronounced in LCMV-infected BALB/c mice than in C57BL mice. Thus, very few primed (\(\alpha_4\) integrin\(^{high}\)) CD8\(^+\) T cells and no tetramer\(^+\) (np118–126) or cytokine\(^+\) (IFN-\(\gamma\)/TNF-\(\alpha\)) CD8\(^+\) T cells were recovered from the spleen of B\(^{-/-}\)
BALB/c mice, indicating that virus-specific CD8\(^+\) T cells in this case were physically deleted from B\(^{-/-}\) hosts and were not just dysfunctional as in their C57BL counterparts. This correlates with much higher virus levels in B\(^{-/-}\) mice on a BALB/c background (Fig. 4). Most importantly, this observation revealed that the impairment of the antiviral CD8\(^+\) T cell response in LCMV-infected B\(^{-/-}\) mice was not restricted to mice of a particular genotype.

**Impaired cytokine expression but increased homeostatic proliferation by Ag-specific CD8\(^+\) T cells in virus-infected B\(^{-/-}\) mice with a low viral burden**

The capacity to permanently control LCMV replication in B\(^{-/-}\) mice varies markedly with the virus strain used for infection. If C57BL mice are infected with the more slowly replicating strain Armstrong instead of Traub, the virus infection is initially well controlled (Fig. 4), and only after a period of several months can low levels of virus be found in the organs of most infected B\(^{-/-}\) mice (13). To evaluate the antiviral CD8\(^+\) T cell response in B\(^{-/-}\) mice under these conditions, both gp33–41- and np396–404-specific CD8\(^+\) T cells were evaluated as described above. Ten days after infection, results were essentially similar to those obtained in LCMV Traub-infected mice for both epitopes in either genotype (data not shown). However, by 28 days after infection, a slightly different pattern was revealed. Similar frequencies of gp33–41-specific, tetramer\(^+\), and IFN-\(\gamma\)-producing CD8\(^+\) T cells were found in LCMV ARM-infected B\(^{-/-}\) mice and wild-type controls (Fig. 5), but as was found in Traub-infected mice, MFI was lower for IFN-\(\gamma\)-producing CD8\(^+\) T cells from B\(^{-/-}\) mice, and the ability of CD8\(^+\) cells to produce additional cytokines (TNF-\(\alpha\) and IL-2) was reduced (Fig. 6). For np396–404-specific CD8\(^+\) T cells, the qualitative analysis yielded similar results; however, in this case the frequency of virus-specific cells was increased in B\(^{-/-}\) mice, leading to significant skewing of the immunodominance hierarchy (ratio np396–404:gp33–41 specific cells) in these mice (Fig. 5). This was evident whether np396–404-specific cells were visualized using tetramers or by intracellular staining for IFN-\(\gamma\). Due to the lower number of CD8\(^+\) T cells in the spleens of B\(^{-/-}\) mice (average 1.3 \(\times\) 10\(^7\) compared with 1.9 \(\times\) 10\(^7\) in matched wild-type mice), the observed frequencies translate into roughly similar absolute numbers of np396–404-specific cells independently of genotype, whereas the number of gp33–41-specific cells was slightly reduced (1.5- to 2-fold) in B\(^{-/-}\) mice. Overall, this pattern was repeated if mice were analyzed 2–4 mo after infection.

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**FIGURE 3.** Deletion of virus-specific CD8\(^+\) T cells in LCMV-infected B\(^{-/-}\) BALB/c mice. Wild-type (WT) and B\(^{-/-}\) (JhD) mice were infected i.v. with 200 PFU of LCMV Traub, and 4 wk after infection np118–126-specific CD8\(^+\) T cells were visualized using tetramers and intracellular staining for cytokine (results for IFN-\(\gamma\) are presented, but similar results were obtained for production of TNF-\(\alpha\)). Data are representative of four mice per group; similar results were obtained 45 and 90 days after infection.

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**FIGURE 4.** Lung virus titers as a function of virus strain (Armstrong or Traub) and genetic background (C57BL or BALB/c). Wild-type (C57BL and BALB/c) and B\(^{-/-}\) (\(\mu\)MT or JhD) mice were infected i.v. with 200 PFU of LCMV Traub or 4800 PFU of LCMV ARM, and 4 wk later virus titers in the lungs were assayed. Points represent individual mice; dotted line denotes detection limit.

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**FIGURE 5.** Virus-specific CD8\(^+\) T cells in B\(^{-/-}\) mice infected with LCMV ARM. Wild-type (WT) and B\(^{-/-}\) C57BL (\(\mu\)MT) mice were infected i.v. with 4800 PFU of LCMV ARM, and 4 wk after infection frequencies of gp33–41- and np396–404-specific cells were determined using tetramers and intracellular staining for IFN-\(\gamma\) (upper panel). Data represent the median and range of four mice per group. Lower panel shows representative dot plots of np396–404-specific CD8\(^+\) T cells.
Although occasional B⁻/⁻ mice (2 of 19) had a CD8⁺ T cell response profile not much different from that of wild-type mice. Thus, even in the absence of a substantial viral burden (cf Fig. 4), virus-specific CD8⁺ T cells in B⁻/⁻ mice become functionally impaired.

In a previous study, we found that infection with LCMV ARM was associated with increased proliferation of phenotypically activated CD8⁺ T cells in the memory phase (24). To better understand the dynamics of virus-specific CD8⁺ T cells in B⁻/⁻ mice, we compared the homeostatic proliferation of virus-specific CD8⁺ T cells in LCMV ARM-infected B⁻/⁻ mice and matched littermates. About 3 mo after infection, mice were given BrdU in their drinking water for 1 wk, and splenocytes were costained for CD8⁺ and IL-2. Representative plots of four to eight mice per group. 

**FIGURE 6.** Impaired cytokine production in LCMV-infected B⁻/⁻ mice as a function of virus strain. Wild-type (WT) and B⁻/⁻ C57BL (μMT) mice were infected i.v. with 200 PFU of LCMV Traub or 4800 PFU of LCMV ARM, and 4 wk after infection the ability of gp33–41-specific CD8⁺ T cells to synthesize IFN-γ, TNF-α, and IL-2 was examined. 

**FIGURE 7.** Unimpaired proliferation of virus-specific CD8⁺ T cells in LCMV-infected B⁻/⁻ mice. Wild-type (WT) and B⁻/⁻ C57BL (μMT) mice were infected i.v. with 4800 PFU of LCMV ARM, and 3 mo after infection the mice were given BrdU in their drinking water for 1 wk. Frequencies of gp33–41 and np396–404-specific CD8⁺ T cells were determined using tetramers and intracellular staining, and BrdU-positive cells were detected through costaining with anti-BrdU. Total number of Ag-specific cells; lower panel, number of Ag-specific cells positive for BrdU. Data represent the average and SD of mice per group. *p < 0.05 relative to wild-type mice.

regard to np396–404-specific CD8⁺ T cells, the number of functional (i.e., IFN-γ-producing) cells was roughly similar, independent of genotype; however, B⁻/⁻ mice had significantly more np396–404-specific cells when evaluated in terms of tetramer⁺ cells. As documented above, IFN-γ-producing CD8⁺ T cells from B⁻/⁻ mice were qualitatively inferior in cytokine production based on evaluation of MFI (data not shown). Taken together, these findings indicate that not only are virus-specific CD8⁺ cells from B⁻/⁻ mice generally impaired in their capacity to produce cytokine, but the capacity to produce cytokine varies depending on genotype; however, B⁻/⁻ mice had significantly more np396–404-specific cells when evaluated in terms of tetramer⁺ cells. As documented above, IFN-γ-producing CD8⁺ T cells from B⁻/⁻ mice were qualitatively inferior in cytokine production based on evaluation of MFI (data not shown). Taken together, these findings indicate that not only are virus-specific CD8⁺ cells from B⁻/⁻ mice generally impaired in their capacity to produce cytokine, but part (approximately one-third) of the expanded subset of np396–404-specific CD8⁺ T cells present in LCMV ARM-infected B⁻/⁻ mice appear to have completely lost the capacity to synthesize IFN-γ, based on comparison of the number of IFN-γ⁺ and tetramer⁺ cells (Fig. 7). As opposed to cytokine production, homeostatic proliferation was not impaired in B⁻/⁻ mice, and for np396–404-specific cells the absolute number of proliferating (BrdU⁺) cells was actually increased. Interestingly, the number of proliferating cells detected was markedly higher when np396–404-specific cells were determined using tetramers rather than through staining for intracellular cytokine. This finding points to the presence in B⁻/⁻ mice of an expanded subset of np396–404-specific CD8⁺ T cells characterized by active cycling, but lacking the capacity to produce cytokine.

**Immunodominance of np396–404-specific CD8⁺ T cells reflects chronic Ag stimulation**

To determine whether the increase in frequency of np396–404-specific cells and the associated skewing of the immunodominance hierarchy in B⁻/⁻ mice can be interpreted simply as a compensation for the reduced per cell effector capacity of virus-specific CD8⁺ T cells in these mice leading to a chronic infection, we studied the immunodominance hierarchy in similarly infected mice genetically deficient in the capacity to produce IFN-γ. We have previously found that these mice become chronically infected much like B⁻/⁻ mice (29) and, similar to the situation in the latter
mice, we now also find a preferential increase in tetramer+ np396–404-specific CD8+ T cells in IFN-γ-deficient mice (Fig. 8).

**Preferential impairment of CD4+ T cell priming in LCMV-infected B−/− mice**

Because the pattern of CD8+ T cell responsiveness in LCMV-infected B−/− mice resembled what we have recently observed in CD4+ T cell-deficient MHC class II−/− mice (30), we next evaluated LCMV-induced CD4+ T cell priming in B−/− mice. Repeated analysis revealed that CD4+ T cell priming was substantially impaired in B−/− mice when evaluated at the peak of the primary response (day 10 after infection), and this was seen independently of whether a rapidly invasive (Traub) or a slowly invasive (Armstrong) LCMV strain was used for infection (Table I). Because B cells are the dominant MHC class II-expressing splenocytes, we considered the possibility that the failure to detect normal virus-specific CD4+ T cell activity represented an in vitro artifact caused by lack of a sufficient number of MHC class II+ APCs in the cultures during the period of in vitro peptide stimulation. However, adding nude splenocytes rich in B cells to the cell cultures used for induction of cytokine expression only slightly increased the frequency of cytokine-producing CD4+ T cells (Table I). Hence, the lower frequency of cytokine-producing CD4+ T cells in B−/− mice was due to impaired in vivo priming and not to a lack of appropriate ex vivo stimulation. Supporting this conclusion, we also found overall CD4+ T cell expansion to be impaired in B−/− mice (fewer BrdU+ cells), and much fewer activated (α4 integrinhiL-selectinhv) CD4+ T cells were generated (Fig. 9A). With regard to absolute numbers of gp36–80-specific CD4+ T cells generated in B−/− mice and wild-type controls, a difference of approximately sevenfold was observed (Fig. 9B). For comparison, the difference in the number of gp33–41-specific CD8+ T cells present in the same B−/− and wild-type mice was less than twofold.

**Impaired CD4+ T cell priming in mice lacking B cell follicles and germinal centers**

The preferential impairment of CD4+ T cell priming in B−/− mice could reflect either that B cells themselves were essential as APCs for this T cell subset or that the perturbation of normal lymphoid architecture with lack of normal B cell zones and germinal centers indirectly influenced activation and/or survival of activated CD4+ T cells. To differentiate between these possibilities, we used TNF-R1+ 2−/− mice. These mice have normal B cells, but resemble B−/− mice in that they lack splenic B cell follicles, follicular dendritic networks, and germinal centers (31, 32). TNF-R1+ 2−/− mice and matched wild-type controls (B6 × 129F2) were infected with LCMV ARM as above, and 10 days after infection, we evaluated LCMV-specific CD4+ and CD8+ T cell responses. As in B−/− mice, the CD4+ T cell response was significantly impaired, whereas no defect in CD8+ T cell responsiveness was observed (Fig. 10).

**Discussion**

Acute viral infections are typically controlled by a cell-mediated immune response, and in many cases functional redundancy secures that either CD4+ or CD8+ T cells suffice for immune control (33). In contrast, the immunological mechanisms required to control chronic viral infections tend to be more elaborate, often relying on complex interactions between several components of the adaptive immune system. In the case of systemic LCMV infection, CD8+ T cells mediate the initial virus control (7). However, when mice are infected with rapidly replicating LCMV strains, virus is either not controlled or recrudescences, unless CD4+ T cells and B cells are also present (8, 9, 11). In a recent report, we obtained evidence indicating that CD8+ T cells lose functional capacity in absence of signals from CD4+ T cells (30), and the findings in that study prompted us to reevaluate the status of LCMV-specific CD8+ T cells in B−/− mice using a similar approach.

Less than 1 mo into infection with LCMV Traub, CD8+ T cells in B−/− mice are either absent (BALB/c mice) or functionally impaired (C57BL mice). Why there is this difference between JH D and μMT mice is not clear, but it is interesting to note that the pattern in LCMV-infected mice is the reverse of that found regarding responsiveness to JHMV (27, 28). This suggests that the degree of T cell dysfunction is related to the specific infection or even the involved epitopes and does not reflect a general difference due to the distinct gene segments targeted to cause the B cell defect.

In C57BL mice, the LCMV-specific CD8+ T cells in B−/− mice resemble those present in similarly infected chronically infected CD4-deficient mice (30), with an inability to produce IL-2 and a reduced capacity to synthesize IFN-γ and TNF-α. This observation is important for two reasons. First, because IFN-γ has been found to be essential for complete and sustained virus control (29), impaired capacity to produce this cytokine would clearly by itself reduce the ability of B−/− mice to prevent recrudescence of virus.

Second, with regard to the mechanism underlying the lack of a sustained CD8+ T cell response in B−/− mice, an important question is whether the T cell defect is merely the result of a high viral burden (reflecting lack of Ab neutralization), leading to anergy/deletion. This is particularly pertinent because recent studies by ourselves and others indicate that Ag by itself can be a driving force for loss of function (13, 30, 34). In this respect, the present analysis using two different virus strains with different biological properties provides important information. By comparing the kinetics of functional impairment within the CD8+ T cell subset with the established time course of virus levels in LCMV ARM-infected mice (13), which for a long time control the infection below the detection limit, we are led to conclude that if T cell dysfunction should exclusively represent a secondary phenomenon, then even a very low Ag load must suffice for induction of dysfunctional CD8+ T cells, because in this case the virus only becomes detectable subsequent to the time point at which there is evidence for impaired CD8+ T cell function. However, in that there is evidence indicating that LCMV also persists at low levels in wild-type mice.
infected with more rapidly replicating virus strains (e.g., Traub) (35, 36) yet no functional defect evolves, our findings are more in line with the alternative conclusion, namely that CD8$^+$ T cell function is less efficiently sustained in the absence of a normal B cell compartment. However, it is also evident from the difference in CD8$^+$ T cell response patterns of mice infected with LCMV Traub vs LCMV ARM and the difference between similarly infected C57BL and BALB/c mice that a high virus burden will lead to exacerbation of this basic defect.

Although the primary CD8$^+$ T cell response is slightly impaired in B$^{-/-}$ mice, this is primarily in the way of a quantitative defect, probably related to the reduced CD8$^+$ T cell pool present in these mice before infection. Thus, by day 10–11 postinfection, no substantial qualitative difference between CD8$^+$ T cells generated in wild-type and B$^{-/-}$ mice was detected. However, similar to the situation in CD4-deficient mice (30), primed CD8$^+$ T cells in B$^{-/-}$ mice gradually lose functional capacity evaluated in terms of cytokine production. Based on the fact that virus-primed CD8$^+$ T cells are initially similar in B$^{-/-}$ mice and wild-type controls, we find it unlikely that there is any intrinsic qualitative defect of naive CD8$^+$ T cells in B$^{-/-}$ mice. In contrast with CD8$^+$ T cell priming, the CD4$^+$ T cell response is substantially impaired already in the initial phase of clonal expansion/differentiation. Given the close similarity in outcome of infection in CD4-deficient mice and B$^{-/-}$ mice, it would be tempting to infer that defective CD4$^+$ T cell priming is the primary reason for impaired long-term CD8$^+$ T cell responsiveness. However, the number of LCMV-specific CD4$^+$ T cells in B$^{-/-}$ mice is reduced by about one order of magnitude, which is more than the reduction found in CD28 knockout mice, and the latter mice do not develop the CD8 deficiency observed in B$^{-/-}$ mice (26). Therefore, additional mechanisms have to be invoked. One possibility is that B cells somehow are involved in sustaining Ag-primed CD8$^+$ T cells. B cells are rich in CD70, and mice lacking the receptor for CD70, CD27, have deficient CD8$^+$ T cell memory (37). Alternatively, the Ab deficiency per se or the disrupted lymphoid architecture may play a role.

Why the generation of virus-primed CD4$^+$ T cells is preferentially reduced in B$^{-/-}$ mice is not clear at present. The role of B cells in induction and maintenance of CD4$^+$ T cell responses is a controversial subject. In some reports, no CD4$^+$ T cell deficiency has been demonstrable in B$^{-/-}$ mice (38–40). However, CD4$^+$ T cell responsiveness was also found to be impaired initially during ocular HSV infection (41) and in JHMV-infected mice (27, 28), and more recently an impaired CD4$^+$ T cell response in influenza-infected mice has been reported (42). Interestingly, CD4$^+$ T cell memory, but not the primary Th response, was impaired in B$^{-/-}$ mice immunized with keyhole limpet hemocyanin (43). In the latter report, detailed analysis indicated that direct Ag presentation by B cells was not required but that this cell subset was needed to provide an environment essential for a sustained CD4$^+$ T cell response. Because LCMV ARM at least does replicate in B cells (44), it seems rather unlikely that these should play a major role as presenters of viral Ag. Furthermore, that it is the environment of the B cell zones rather than the B cells themselves that is critical is supported by the observation that the LCMV-specific CD4$^+$ T cell response is selectively impaired in TNF-R1$^-$ mice, which do have B cells, but lack the normal organization of splenic B cell zones (31, 32). Hence, the impaired CD4$^+$ T cell priming in LCMV-infected B$^{-/-}$ mice could very well reflect the same underlying mechanism as in keyhole limpet hemocyanin-primed B$^{-/-}$ mice. However, in the case of LCMV that is a self-replicating virus, the distinction between initial expansion and survival of primed cells may be meaningless, because the clonal expansion during initial exposure is much more extensive, also depending to a high degree on division of progeny cells. For this reason, defects relating to, e.g., survival signals, may be exposed even during first encounter.

One additional observation of interest in relation to our understanding of the immunology of persistent viral infections is the marked difference in the behavior of CD8$^+$ T cells specific for gp33–41 and np396–404 in LCMV ARM-infected B$^{-/-}$ mice. While gp33–41-specific CD8$^+$ T cells are only partially impaired

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**Table 1. Deficient CD4$^+$ T cell priming in LCMV-infected B$^{-/-}$ (μMT) mice**

<table>
<thead>
<tr>
<th>Virus Strain$^a$</th>
<th>Background$^b$</th>
<th>IFN-γ</th>
<th>IL-2</th>
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<td></td>
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<tr>
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<td>0.9</td>
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<tr>
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<td>B6</td>
<td>10.4</td>
<td>1.2</td>
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<tr>
<td>Armstrong 2.exp</td>
<td>B6</td>
<td>8.1</td>
<td>1.1</td>
</tr>
</tbody>
</table>

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$^a$ Mice were infected with 200 PFU of LCMV Traub or 4800 PFU of LCMV ARM 10 days before analyses.

$^b$ B10, Wild type (WT) equals C57BL/10 μMT; B6, WT equals C57BL/6 $^{+/+}$.

$^c$ Percentage of CD4$^+$ T cells producing cytokine in response to gp61–80 stimulation; results of individual mice are presented.
in functionality (lower MFI), a substantial fraction of np396–404-specific cells eventually lose the capacity to produce cytokine altogether. Differences in the behavior of CD8+ T cells directed toward these two epitopes have been reported before, and np396–404-specific cells are more completely deleted in mice infected with very high doses of rapidly replicating virus, possibly due to a higher level of presentation of MHC/np396–404 Ag complexes in chronically infected mice (34). The present findings could be a reflection of the same basic difference, albeit at a lower level of chronic Ag stimulation. Thus, whereas gp33–41-specific cells are only slightly affected by the low-grade persistent infection in LCMV ARM-infected mice, np396–404-specific cells are more severely perturbed, resulting in an expanded population of which a significant fraction eventually becomes completely anergic. That gp33–41-specific cells can be driven to a similar state of functional exhaustion is indicated by the response pattern seen in Traub-infected mice, which also carries a higher viral burden. Interestingly, np396–404-specific cells in LCMV ARM-infected B−/− mice proliferate at least as frequently as do memory CD8+ T cells in wild-type mice or gp33–41-specific memory cells in the same hosts. Thus, under the influence of chronic Ag stimulation at a level that does not suffice to induce deletion, CD8+ T cells may retain the ability to proliferate and expand, but either effector cell differentiation may be impaired or the resulting cells may rapidly lose functional capacity. To balance virus replication under these conditions, compensatory expansion of effector cell numbers is to be expected, and this is exactly what we observe. Chronic Ag stimulation resulting from the reduction in cytokine production at the level of the individual cell (lower MFI) suffices to explain the skewed immunodominance hierarchy, because the same epitope preference can be observed in similarly infected IFN-γ-deficient mice.

In conclusion, our findings strongly indicate that it is not solely the lack of neutralizing Abs that leads to impaired virus control in LCMV-infected B−/− mice. The generation of virus-specific CD4+ T cells is markedly reduced, and CD8+ T cells rapidly become impaired in their ability to produce cytokines. Together, these defects may lead to impaired T cell-mediated virus control. Because there are no Abs to serve as a backup defense (45, 46), the result with a noncytopathic virus will be a general increase in virus load, which will then further impair CD8+ T cell responsiveness. Depending on the virus/host combination and the epitope studied, anything from chronic dysfunction to complete deletion may be observed. The implications of these findings are twofold. First, our results strongly indicate that B cells play an important role in antiviral immunity not only as Ab producers, but also in providing an environment essential for an optimal and sustained T cell response. Second, our data underscore that great caution should be exerted when interpreting results obtained in knockout mice. Thus, results from B−/− mice should be carefully evaluated, and functional defects should not immediately be taken to reflect a direct involvement of B cells and/or Abs.
Acknowledgments
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References