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Role of CD40 Ligand and CD28 in Induction and Maintenance of Antiviral CD8\(^+\) Effector T Cell Responses\(^1\)

Susanne Ørding Andreasen, Jeanette Erbo Christensen, Ole Marker, and Allan Randrup Thomsen\(^2\)

The primary aim of this report was to evaluate the immune responses of CD40 ligand-deficient (CD40L \(^{-/-}\)) mice infected with two viruses known to differ markedly in their capacity to replicate in the host. Lymphocytic choriomeningitis virus (LCMV) is a natural mouse pathogen that replicates widely and extensively, whereas vesicular stomatitis virus (VSV) spreads poorly. We found that the primary response of CD40L \(^{-/-}\) mice toward VSV is significantly impaired; proliferation of both CD4\(^+\) and CD8\(^+\) cells is reduced 2- to 3-fold, few CD8\(^+\) cells acquire an activated phenotype, and little functional activity is induced. Very similar results were obtained in VSV-infected, CD28-deficient mice. In contrast, neither CD40L nor CD28 was required for induction of a primary CD8\(^+\) response toward LCMV. Surprisingly, lack of CD4\(^+\) T cells had no impact on the primary immune response toward any of the viruses, even though the CD40 ligand dependence demonstrated for VSV would be expected to be associated with CD4 dependence. Upon coinfection of VSV-infected mice with LCMV, the requirement for CD40 ligand (but not CD28) could be partially bypassed, as evidenced by a 3-fold increase in the frequency of VSV-specific CD8\(^+\) T cells on day 6 postinfection. Finally, despite the fact that the primary LCMV-specific CD8\(^+\) response is virtually unimpaired in CD40L \(^{-/-}\) mice, their capacity to maintain CD8\(^+\) effector activity and to permanently control the infection is significantly reduced. Thus, our results demonstrate that the importance of CD40/CD40L interaction for activation of CD8\(^+\) T cells varies between viruses and over time. The Journal of Immunology, 2000, 164: 3689–3697.

Induction of an efficient CD8\(^+\) T cell response is required for clearance of many viral infections (1). CD8\(^+\) T cells mediate their effector function through cell contact-dependent destruction of target cells and release of proinflammatory cytokines. As a consequence, aberrant T cell responses constitute a serious threat to the integrity of normal tissue functions, and the activation of CD8\(^+\) T cells must therefore be strictly controlled. Robust activation of naive T cells requires two signals (2, 3). The first signal is delivered through binding of the TCR to peptide/MHC complexes on APCs. The second, costimulatory signal is delivered through interactions with molecules provided by professional APCs. The classical model of how costimulation works for CD8\(^+\) T cells describes a three-cell interaction, where a Th cell and a CD8\(^+\) T cell recognize their specific Ags on the same APC simultaneously. Theoretically, the Th cell becomes activated and secretes cytokines (e.g., IL-2) that provide the necessary costimulation for nearby CD8\(^+\) T cells triggered through their TCR (3, 4). However, this classical model has two apparent weaknesses. First, the three-cell interaction must be a rare event due to the low frequency of Ag-specific cells, particularly in the initial, critical phase of the immune response. Second, some CD8\(^+\) T cell responses can be mounted in the absence of Th cells (5–8). Recently, an alternative model has received substantial experimental support (9–11). According to the new model, the classical three-cell interaction is split into two steps. First, a Th cell recognizes Ag on an APC, whereby the Th cell becomes activated and consequently able to condition the APC. Activation of the APC is mediated through interactions of CD40 (APCs) and CD40L\(^3\) (activated Th cells). Second, following conditioning the APC acquire the capacity to activate CD8\(^+\) T cells directly. Up-regulation of B7.1/B7.2 on the APC is believed to constitute part of the mechanism underlying the increased stimulatory potential of the conditioned APC (9). Notably, incorporated into the new model, different stimuli are predicted to bypass the requirement for Th cells by directly conditioning the APC; Abs toward CD40 are one such stimulus (9–12). More important in the physiological setting, viral infection may directly condition APCs (9, 13). Based on this reinterpretation of the role of Th cells in activation of CD8\(^+\) T cells, it would be predicted that only viruses that induce Th-independent CTL responses would also induce CTL responses in a CD40L-independent fashion.

Several studies have examined the cellular immune response of CD40L-deficient (CD40L \(^{-/-}\)) mice toward infection with various viruses (14–17). The results presented by Borrow et al. (14) showing that CD40L \(^{-/-}\) mice mount an unimpaired CTL response toward both VSV and LCMV intrigued us, as these two viruses are known to have very different biological characteristics and have previously been found to differ substantially in their requirements for induction of a CTL response (18). LCMV is a natural mouse pathogen that replicates extensively and widely in several organs;

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\(^2\) Address correspondence and reprint requests to Dr. Allan Randrup Thomsen, Institute of Medical Microbiology and Immunology, University of Copenhagen, The Panum Institute, 3C Blegdamsvej, DK-2200, Copenhagen N, Denmark. E-mail address: a.r.thomsen@immi.ku.dk

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\(^3\) Abbreviations used in this paper: CD40L, CD40 ligand; CD40L \(^{-/-}\), CD40L deficient; VSV, vesicular stomatitis virus; LCMV, lymphocytic choriomeningitis virus; p.i., postinfection; BrdU, 5-bromo-2′-deoxyuridine; CD28 \(^{-/-}\), CD28 deficient; IFN-\(\gamma\) \(^{-/-}\), IFN-\(\gamma\) deficient; Cy, CyChrome; GP, glycoprotein; NP, nucleoprotein; VLA-4, very late Ag-4; DC, dendritic cell.
following an i.v. infection high virus titers are detectable in the spleen throughout the infection until approximately day 10 postinfection (p.i.) (19). In contrast, VSV is not a natural mouse pathogen, and it replicates poorly in the spleen. Virus can be detected on day 1 p.i., but by approximately day 2 live virus becomes undetectable (20). These differences in biological behavior have been linked to their different requirements in elicitation of efficient CTL responses. Thus, LCMV induces a Th-independent, CD28-independent CTL response, whereas generation of VSV-specific CTLs has been reported to require both Th cells and CD28 (20).

Consequently, the primary aim of this study was to re-evaluate and compare the immune responses of CD40L−/− mice following primary infection with these two viruses. Three parameters of activation were evaluated: cell cycle progression (in vivo incorporation of 5-bromo-2′-deoxyuridine [BrdU]), phenotypic conversion, and effector function (cytolytic activity and capacity to produce IFN-γ). Furthermore, based on the assumption that a primary function of CD40 signaling is to enhance the B7/CD28 interaction (24), we also wanted to compare the requirements for CD40L and CD28. This was done by assessing the same parameters in virus-infected CD40L−/− mice. As recent advances in technology have made it possible to directly visualize Ag-specific T cells and thus precisely quantitate and compare effector cell generation (25–27), we decided to apply one of these assays (intracellular staining for cytokine [IFN-γ]) following brief stimulation with specific peptide) to address the above questions. Because this methodology has not previously been used to analyze the VSV-induced CD8+ T cell response, initial experiments were performed to validate the specificity of our experimental approach and to define the kinetics of the VSV-specific CD8+ T cell response measured in this fashion.

Materials and Methods

Mice

CD40L−/− mice (C57BL/6, 129-Cd40tm1mn), CD28−/− mice (C57BL/6-Cd28tm1meh), and IFN-γ−/− mice (C57BL/6-Iγtm1) were the progeny of breeding pairs obtained from The Jackson Laboratory (Bar Harbor, ME). MHC class II-deficient (Ab2−/−) mice and their wild-type littermates, back-crossed five times onto a C57BL/6 background, were obtained from Taconic Farms (Germantown, NY). C57BL/6 were purchased from Bomholgaard (Ry, Denmark). Mice from outside sources were always allowed to rest for 1 wk before entering into experiments; by that time the animals were about 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 such infections were detected.

Virus infection

LCMV of the Armstrong strain (clone 53b) was provided by M. B. A. Oldstone (Scripps Clinic and Research Foundation, La Jolla, CA) (14). For i.v. infection of mice, 4800 PFU of LCMV in a volume of 0.3 ml was used. Inoculation of immunocompetent mice by this route is followed by transient, immunizing infection (19, 28). VSV of the Indiana strain, originally provided by M. B. A. Oldstone (Scripps Clinic and Research Foundation, La Jolla, CA) (14). For infection with VSV, mice were inoculated with 106 PFU i.v. This virus dose is nonlethal to immunocompetent mice. As recent advances in technology have made it possible to directly visualize Ag-specific T cells and thus precisely quantitate and compare effector cell generation (25–27), we decided to apply one of these assays (intracellular staining for cytokine [IFN-γ]) following brief stimulation with specific peptide) to address the above questions. Because this methodology has not previously been used to analyze the VSV-induced CD8+ T cell response, initial experiments were performed to validate the specificity of our experimental approach and to define the kinetics of the VSV-specific CD8+ T cell response measured in this fashion.

In vivo depletion of CD4+ cells

Depletion of CD4+ cells was obtained by treatment with clarified ascites fluid containing the CD4-specific mAb GK1.5. Mice were injected with 0.1 ml i.p. on days −1, 0, and +2 relative to virus inoculation, and the efficacy of CD4+ depletion was always verified by flow cytometry at the time of harvesting splenocytes for functional analysis (<1% CD4+ cells were detected).

In vivo BrdU labeling

Mice were given BrdU (Sigma, St. Louis, MO) at 0.8 mg/ml in their drinking water for a period of 3 days (30). BrdU-containing water was protected from light and 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). Peritoneal cells were obtained by lavage with 5 ml of cold HBSS.

Monoclonal Abs

The following mAbs were purchased from PharMingen (San Diego, CA) as rat anti-mouse Ab: FITC-conjugated anti-CD49d (common α4-chain of LPAM-1 and VLA-4; R1-2), PE- and CyChrome (Cy)-conjugated anti-CD8α (Ly-2; 53-6.7, PE-conjugated anti-CD4 (L3T4; H129.19), biotinylated anti-L-selectin (CD62L, LECAM-1, and Ly-22; MEL-14), and PE-conjugated anti-IFN-γ. For BrdU staining, FITC-conjugated anti-BrdU (Becton Dickinson, San Jose, CA) was used.

Fluorescence staining and flow cytometric analysis

Staining for flow cytometry was performed as described previously (31–33). Briefly, 1 × 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 case of biotin-conjugated Abs, cells were additionally incubated with streptavidin-Tri-color (Caltag, Burlingame, CA) for 20 min before fixation.

For BrdU staining, cells were stained for surface markers as described above, resuspended in PBS and 1% NaN3, transferred to cold 0.15 M NaCl solution, and fixed by adding cold 96% ethanol drop by drop. After a 30-min incubation on ice, cells were washed once with PBS and resuspended in PBS, 0.01% Tween 20, and 1% paraformaldehyde. After a 1-h incubation at room temperature, cells were pelleted and resuspended in PBS, 0.15 M NaCl, and 4.2 mM MgCl2, pH 5, containing 50 Kunitz units/ml of DNase I (Sigma). After incubation for 15 min at 37°C, cells were washed once in PBS before adding the anti-BrdU Ab. After a 30-min incubation at room temperature, cells were washed in PBS and analyzed.

To detect intracellular IFN-γ, splenocytes were cultured at 37°C in 96-well round-bottomed plates at a concentration of 1 × 10⁶ cells/well in a volume of 0.2 ml of complete RPMI medium supplemented with 10% FCS (Sigma) and antibiotics (penicillin/streptomycin) for 5 days before fixation.

Cytotoxicity assays

The activity of CTLs was assessed in 51Cr release assays (26, 33). Targets for evaluation of LCMV-specific cytotoxicity were MC57G cells infected with LCMV for 48 h; uninfected MC57G cells served as control targets. VSV-specific cytotoxicity was evaluated using either VSV-infected or VSV-NP32-39 pulsed EL-4 cells as specific targets; unpulsed EL-4 cells served as control targets. The assay time was 5 h, and the percentage of specific lysis was calculated as previously described (26).

Assay for IFN-γ release

Cell culture supernatants of splenocytes (1 × 10⁶ cells/0.2 ml/well) incubated for 9 h with or without relevant peptides were assayed using kits purchased from Endogen (Cambridge, MA).
Results

High frequency of virus-specific Tc1 cells are generated during acute VSV infection

Recently, techniques to directly enumerate Ag-specific CD8\(^+\) T cells have been developed, e.g., staining with tetrameric peptide-MHC complexes and detection of intracellular IFN-\(\gamma\) following brief in vitro stimulation with relevant peptides (25–27). Several reports describing these methods primarily in the context of the LCMV infection have recently been published, but to our knowledge the T cell response of VSV-infected animals has not previously been analyzed by applying any of these techniques. In the present study staining for intracellular IFN-\(\gamma\) was selected as the primary means to quantitate CD8\(^+\) responses in virus-infected mice. Therefore, the aim of our initial experiments was to validate the use of this methodology under our experimental conditions. Groups of C57BL/6 mice were infected with either VSV or LCMV, and on day 6 (VSV) or 8 (LCMV) p.i., splenocytes were incubated in vitro without stimulation (no peptide) or with LCMV GP33–41 or VSV NP52–59. These time points were chosen based on previous analysis of maximal primary CTL responses to these two viruses (34–36). Flow cytometry was used to assess the frequencies of peptide-specific IFN-\(\gamma\)^+ CD8\(^+\) T cells (Fig. 1). Spleen cells from virus-infected animals made no IFN-\(\gamma\) in the absence of peptide stimulation; background frequencies were always \(<1\%\). IFN-\(\gamma\)-CD8\(^+\) cells were only detectable when splenocytes were incubated with the peptide with which the mice had been primed, thereby confirming the specificity of the assay and in addition demonstrating that no cross-reactivity exists at the CD8\(^+\) level. Spleen cells from uninfected mice did not produce any IFN-\(\gamma\) after stimulation with any of the viral peptides, and peptide stimulation did not induce IL-5 CD8\(^+\) in any group (data not shown), demonstrating that both viral infections were associated with the generation of Tc1 cells (37, 38).

As an additional control, IFN-\(\gamma\)-mice were infected with 10\(^6\) PFU of VSV, and on day 6 p.i. spleen cells were harvested and stimulated with VSV-NP52–59. As expected, no IFN-\(\gamma\)-cells could be detected in VSV-infected IFN-\(\gamma\)^−/− mice; this was due to lack of activation of CD8\(^+\) T cells, as about 50% expressed the activation marker VLA-4 (data not shown). About 15% of wild-type CD8\(^+\) T cells reacted with the immunodominant VSV-NP52–59 peptide (Fig. 1), and detailed phenotypic analysis of the induced CD8\(^+\)IFN-\(\gamma\)^+ cells characterized these as being VLA-4\(^\text{high}\), LFA-1\(^\text{high}\), CD44\(^\text{high}\), and mostly Mac-1\(^+\) (data not shown), the expected phenotype of recently activated CD8\(^+\) cells (31).

Kinetics of CD8\(^+\) T cell proliferation and differentiation in VSV-infected C57BL/6 mice

To study the kinetics of the clonal expansion of CD8\(^+\) T cells during a VSV infection, mice were infected with VSV on day 0 and given BrdU in their drinking water for a period of 3 days before analysis; on days 3, 6, 9, and 72 p.i., spleen cells were harvested, and CD8\(^+\)BrdU\(^+\) cells were quantified. As shown in Fig. 2, splenic CD8\(^+\) T cells were observed to proliferate above background during days 0–3 and days 6–9 p.i., but peak proliferation was observed between days 3–6 p.i., during which period about 50% of the CD8\(^+\) T cells had incorporated BrdU. The level of CD8\(^+\) T cell proliferation in uninfected mice is depicted on day 0. Proliferation of CD8\(^+\) T cells later after infection is identical to that observed in uninfected mice.

By staining for intracellular IFN-\(\gamma\)-the number of VSV-NP-specific CD8\(^+\) T cells was determined at different time points after VSV infection. No CD8\(^+\) IFN-\(\gamma\)-T cells could be detected in naive or day 3 infected mice. On day 6 p.i., when the response peaks, 10–15% of the CD8\(^+\) T cells were specific for the immunodominant VSV-NP52–59 peptide. The number of IFN-\(\gamma\)-T cells then decreased with time, but a small percentage (2–3%) of NP52–59-specific CD8\(^+\) cells was still detectable in the spleen after 2 mo. Notably, maximal T cell proliferation and differentiation were observed several days after infectious virus had disappeared from the spleen (approximately day 2 p.i.). We did not detect a substantial number of NP52–59-specific T cells in the peripheral lymph nodes (<1%). However, in both acutely infected and immune mice a relatively high percentage of VSV-specific cells was detected in the peritoneum (Fig. 3). This is in keeping with the expected circulation pattern of primed cells favoring patrolling of nonlymphoid organs.

Impaired immune response in VSV-infected CD40L\(^−/−\) mice

In the above experiments we have validated our methodology and defined the optimal time point to evaluate the CD8\(^+\) T cell response to VSV regarding both proliferation and differentiation. We subsequently applied this approach to investigate the immune response of CD40L\(^−/−\) mice.
CD40L<sup>−/−</sup> and wild-type mice were infected with 10<sup>6</sup> PFU of VSV and given BrdU in their drinking water for a period of 3 days before analysis. On day 3, 6, 9, or 72 p.i., splenocytes were surface stained with PE-conjugated anti-CD8, permeabilized, and stained with FITC-conjugated anti-BrdU; the percentage of BrdU<sup>+</sup> cells of CD8<sup>T</sup> cells is depicted. Effector cell generation was evaluated through staining for intracellular IFN-γ at the indicated time points. Splenocytes were stimulated in vitro with VSV-NP<sub>52-59</sub> for 6 h, surface stained with Cy-conjugated anti-CD8 and FITC-conjugated anti-VLA-4, permeabilized, and stained with PE-conjugated anti-IFN-γ. Gates were set for CD8<sup>T</sup> cells, and medians and ranges of VLA<sup>high</sup>/IFN-γ<sup>+</sup> cells are shown. Spleen virus titers were: day 1 p.i., 7×10<sup>2</sup> (6.5×10<sup>2</sup> to 12×10<sup>2</sup>) PFU/g of organ, day 2 p.i., at or below the detection level.

CD40L<sup>−/−</sup> and wild-type mice were infected with 10<sup>6</sup> PFU of VSV and given BrdU in their drinking water from days 3–6. On day 6 p.i., cell proliferation, expression of surface markers, and effector cell differentiation were evaluated. Naïve C57BL/6 mice served as controls and provided cut-off values in the flow cytometric analyses. As shown in Fig. 4, the response of CD40L<sup>−/−</sup> mice was significantly impaired for all four parameters investigated. Proliferation of both CD4<sup>+</sup> and CD8<sup>T</sup> T cells was reduced 2-fold in CD40L<sup>−/−</sup> mice compared with that in wild-type mice. Almost half the CD8<sup>T</sup> T cell population in wild-type mice had acquired an activated phenotype by day 6 p.i., whereas only a minor subset of CD8<sup>T</sup> cells in CD40L<sup>−/−</sup> mice shifted from a naïve phenotype (VLA-4<sub>low</sub>/L-selectin<sub>high</sub>) to an activated phenotype (VLA-4<sub>high</sub>/L-selectin<sub>low</sub>). Regarding effector cell differentiation, evaluated through detection of intracellular IFN-γ, the response in CD40L<sup>−/−</sup> mice was also significantly reduced (∼3-fold).

Because costimulation through CD28/B7 interaction is assumed to be interrelated with the CD40/CD40L pathway (9, 24), the T cell response of CD28<sup>−/−</sup> mice was similarly evaluated. Basically, the results in CD28<sup>−/−</sup> mice were similar to those obtained in CD40L<sup>−/−</sup> mice, perhaps with a trend for CD28 deficiency to be associated with more pronounced impairment of the immune response.
FIGURE 5. Proliferation of CD4+ and CD8+ cells and effector cell differentiation in CD40L+/-, CD28−/−, and C57BL/6 mice infected with 4800 PFU of LCMV. For cell proliferation analyses, mice were given BrdU in their drinking water for 3 days before analysis. On day 8 p.i., cells were surface stained with PE-conjugated anti-CD4 or CD8, permeabilized, and stained with FITC-conjugated anti-BrdU. Gates were set for either CD4+ or CD8+ cells, and medians and ranges of BrdU+ cells are presented. For detection of intracellular IFN-γ, cells were stimulated in vitro with LCMV-GP 33–41 for 5 h, surface stained with Cy-conjugated anti-CD8 and FITC-conjugated anti-VLA-4, permeabilized, and stained with PE-conjugated anti-IFN-γ. Gates were set for CD8+ cells, and medians and ranges of IFN-γ+ cells are shown (n = 3–6/group).

Unimpaired immune response in LCMV-infected CD40L−/− mice

It has previously been reported that the primary CTL response of CD40L−/− mice toward LCMV is unimpaired (14–17). Consequently, we examined the response of LCMV-infected CD40L−/− mice to confirm that the impaired response observed with VSV-infected CD40L−/− mice was virus related and not due to general immune impairment of our CD40L−/− mice.

Proliferation of CD4+ and CD8+ cells and the frequency of CD8+ IFN-γ+ cells were evaluated. CD40L−/−, CD28−/−, and wild-type mice were infected with 4800 PFU of LCMV and given BrdU in their drinking water from days 5–8, and on day 8 p.i. splenocytes were analyzed by flow cytometry. As shown in Fig. 5, proliferation of both cell subsets in LCMV-infected CD40L−/− mice was comparable to the proliferative response observed in wild-type mice. Furthermore, no impairment in the production of intracellular IFN-γ in CD40L−/− mice was observed. Essentially similar results were obtained in LCMV-infected CD28−/− mice, except that CD4+ T cell proliferation was significantly impaired in CD28−/− mice (Fig. 5).

Analysis of primary CTL responses in CD40L−/− mice infected with LCMV or VSV

The results previously published regarding the responses of CD40L−/− mice to VSV and LCMV have been obtained primarily by analyzing the virus-specific CTL response (14). To enable a comparison between our results obtained by use of flow cytometry with those from the literature obtained by CTL assays, we also measured the virus-specific and VSV-NP-specific cytotoxicities of VSV-infected animals examined. The results presented in Fig. 6 support the results obtained by flow cytometry (Figs. 4 and 5); LCMV-infected CD40L−/− mice showed no impairment in cytotoxic activity, whereas a significant ~3-fold reduction in cytotoxicity was noted in VSV-infected, CD40L−/− mice. Notably, similar results were obtained whether total or NP-specific CTL activity was assayed, demonstrating that the impaired CD8+ response did not involve only one particular epitope.

Coinfection of CD40L−/− mice augments the response to VSV

According to recent theories regarding induction of CD8+ T cell responses, some viruses activate APCs directly, thereby bypassing the need for Th help that is normally mediated through CD4/CD40L interaction (9, 13). This prediction appeared to hold true regarding LCMV, as the immune response of CD40L−/− mice to this virus was indistinguishable from that of wild-type mice. Consequently, we wanted to examine whether coinfection of VSV-infected CD40L−/− mice with LCMV would normalize the response to VSV. This was performed by coinfected CD40L−/− and wild-type mice with VSV and LCMV and enumerating VSV-NP-specific IFN-γ+ CD8+ T cells on day 6 p.i. As depicted in Fig. 7, coinfection with LCMV augmented the response of both CD40L−/− and wild-type mice. In wild-type mice the effect was primarily seen as a higher production of IFN-γ on a per cell basis, as evidenced by higher mean fluorescence intensity. However, in CD40L−/− mice a significant, 2- to 3-fold increase in the frequency of VSV-specific IFN-γ+ T cells was observed (Figs. 7 and 8).

From the results presented in Fig. 1, it may be concluded that the augmentation of the VSV response following coinfection with LCMV is not due to cross-reactivity at the CD8+ level. However, cross-reactivity at the CD4+ level could not be ruled out. Therefore, to test whether CD4+ T cells were critically involved in the augmented response of coinfected mice, we evaluated the response of CD4-depleted and coinfected CD40L−/− mice. Efficient depletion of CD4+ cells did not impair the help provided by coinfection with LCMV (Fig. 8), demonstrating that cross-reactivity on the CD4+ level was not the mechanism underlying the augmented VSV-NP response seen in VSV-infected CD40L−/− mice coinfected with LCMV.

To test whether the B7/CD28 interaction was a prerequisite for LCMV to augment the VSV-specific response, CD28−/− mice were infected with both viruses, and the VSV-NP specific response was measured using either VSV-infected or VSV-NP52–59-pulsed EL-4 cells as targets; untreated EL-4 cells served as control targets. LCMV-specific CTL activity was evaluated on day 8 p.i., using LCMV-infected MC57G cells as specific targets; uninfected MC57G cells served as control targets. Results of individual mice are depicted; filled symbols represent lysis of specific target cells, and open symbols represent control target cells.
The above results clearly demonstrate that the CD8+ T cell response of VSV-infected animals is impaired when CD40L is absent (Figs. 4 and 6–7). This could lead to the conclusion that VSV-infected mice require CD4+ help to mount a CD8+ T cell response, and mice made deficient of CD4+ T cells would therefore be expected to resemble CD40L−/− mice. To examine whether this was the case, both CD4-depleted wild-type C57BL/6 mice and MHC class II−/− mice were infected with 10⁶ PFU of VSV, and their CD8+ T cell responses were compared that of normal mice. As shown in Fig. 9, deficiency of CD4+ T cells, regardless of whether it is acute (mAb depletion) or chronic (MHC class II−/−), was associated with little or no reduction in the CD8+ response to VSV.

CD40L, but not CD28, is pivotal for maintenance of CD8+ T cell effector capacity in LCMV-immune mice: evidence for a qualitative CD8+ T cell defect in CD40L−/− mice

Having found nearly parallel requirements for CD40L and CD28 during the induction phase of the antiviral CD8+ response (Fig. 5), we wanted to test whether this was also the case during the late immune phase in LCMV-infected mice. Using CD40L−/− mice we and others have recently demonstrated a requirement for CD40L in maintenance of virus-specific T cell memory and long term immune surveillance (14, 16). To evaluate whether a similar requirement existed for CD28, and a number of reports suggest that this could be the case (39–42), CD28−/−, CD40L−/−, and wild-type mice were infected with LCMV, and 2 and 4 mo later virus-specific CD8+ cells were enumerated. In addition, spleens and lungs were assayed for persistent infection at 4 mo (Fig. 10). As previously reported, virus could be detected in the organs of all CD40L−/− mice (n = 6), whereas little or no virus was detected in CD28−/− and wild-type mice (n = 4–6). As an underlying mechanism for this failure to permanently control the infection in CD40L−/− mice, we found that although similar numbers of splenic LCMV-specific CD8+ cells could be found in CD40L−/− and wild-type mice at 4 mo p.i., LCMV-specific CD8+ T cells from CD40L−/− mice suffered a functional impairment, as evidenced by a lesser capacity to synthesize IFN-γ. A similar trend was observed after 2 mo p.i. (data not shown), at which time CD40L−/− mice still controlled the infection (16); this is consistent with the assumption that impairment of CD8+ T cell function is the cause, and not an effect, of deficient virus control. As confirmation that the observed reduction in mean fluorescence intensity was functionally relevant, Ag-induced secretion of IFN-γ in vitro was also evaluated. Following 9 h of stimulation of splenocytes from CD40L−/− and wild-type mice with LCMV GP33–41 and NP396–404, we found that wild-type cells produced about 10-fold more IFN-γ than did cells from CD40L−/− mice. In contrast to the situation in CD40L−/− mice, slightly lower frequencies of virus-specific CD8+ T cells were detected in
Discussion

In this study we show that VSV, despite a very brief period of detectable replication in the spleen, induces extensive CD8\(^{+}\) T cell activation as evidenced by BrdU incorporation and phenotypic analysis. Based on previous results indicating that VSV infection is associated with limited bystander activation (33), it may be inferred that most activated CD8\(^{+}\) T cells are probably VSV specific. Direct visualization of Ag-specific CD8\(^{+}\) T cells through analysis of IFN-γ production at the single-cell level clearly demonstrates that about one-third of the activated CD8\(^{+}\) T cells are specific for a single immunodominant epitope (NP\(_{52-59}\)), and memory cells with this specificity can be detected in low numbers even at 2 mo after infection. The effector cells generated are of the Tc1 subtype (IFN-γ\(^{-}\)IL-5\(^{+}\)) (35, 40, 43) and are phenotypically identical to the effector cells induced by LCMV infection (44). However, despite these similarities, the requirements for costimulation are significantly different.

Many viral infections are known to be capable of eliciting a strong CD8\(^{+}\) T cell response in the absence of CD4\(^{+}\) T cells, thus somehow bypassing the need for CD4-mediated activation of APCs (5–8). This activation is generally assumed to be mediated through CD40/CD40L interaction. Recently published data from Borrow et al. (14 and Bachmann and coworkers (45) indicate that the immune response of CD40L\(^{-}\)/2 mice toward LCMV as well as VSV is unimpaired. However, it is known that these two viruses differ significantly with respect to their capacity to spread in the host, and evidence exists suggesting that these viruses differ markedly in their requirements for costimulation (18). Thus, LCMV induces a Th-independent, CD40L- and CD28-independent CTL response, whereas generation of a VSV-specific CTL response has been reported to depend on Th help and be CD28 dependent (18, 45). Consequently, it would be expected that the requirement for CD40/CD40L interaction would also differ.

In contrast to previous reports (14, 45) we find that the generation of VSV-specific Tc1 cells is impaired, although not entirely absent, in CD40L\(^{-}\)/2 mice. A possible explanation for the discrepancy between our results and those published could have been...
that previous reports solely evaluated CTL activity. For this reason, we have investigated several parameters of activation (cell proliferation, activation markers, and IFN-γ production) in addition to cytotoxic capacity and found both clonal expansion and differentiation of VSV-specific CD8+/ T cells in CD40L−/− mice to be impaired. In agreement with results from other groups (14, 15, 17), we found the response of CD40L−/− mice to LCMV to be indistinguishable from that of wild-type mice, demonstrating that there was no general immune impairment of these mice. Thus, we are without any obvious explanation for the discrepancy observed. However, the difference in CTL activity is on the order of 3-fold, which may easily be overlooked if not backed by more precise assays. Furthermore, in one study conclusions on CTL responsiveness were based on comparison with +/+ mice, not +/+ mice, and with a molecule such as CD40L, a gene-dose effect is very likely. Indeed, a reassessment of the latter report reveals that +/+ mice had a CTL response less than half that in wild-type mice.

Notably, the dependence upon CD40L observed in VSV-infected animals did not reflect an absolute requirement for CD4 + T cells. This finding is in variance with the results of a recent study (5). However, differences in the environment that surrounded the infected mice may provide an explanation to this apparent discrepancy. Thus, it has previously been observed that while MHC class II-deficient mice respond optimally to influenza virus when bred under specific pathogen-free conditions, the CTL response in these mice is impaired under conventional conditions (46). Most important, under our experimental conditions CD4+ T cells are not essential, since neither acute depletion of CD4+ T cells nor a chronic deficiency such as that found in MHC class II−/− mice had a substantial impact on the response. Therefore, the results indicate that CD8+ T cells may suffice to deliver the necessary signals. Given that we found a significant role for CD40L in the context of the VSV-specific CD8+ response, this provides indirect evidence that CD8+ cells may generate their own help through this ligand. Apparently this may be critical during VSV, but not LCMV, infection. Perhaps the underlying reason for this difference is to be found in the extremely transient nature of viral replication following i.v. inoculation of VSV.

The observation that neither Th cells nor CD40/CD40L interaction are required for induction of CD8+ responses to some viruses indicate that either 1) CD8+ T cells activate APCs also through CD40L-independent mechanisms; or 2) APCs may be activated directly by viral infection. Recent data from Ruedl et al. (45) show that naive CD8+ T cells following antigenic stimulation are capable of activating dendritic cells directly in the absence of CD40 and CD4+ T cells. Apparently this activation does not require direct interaction between CD8+ T cells and dendritic cells (DCs), but nevertheless leads to up-regulation of costimulatory molecules on the DCs. However, these results were obtained using TCR transgenic mice, in which the percentage of virus-specific CD8+ T cells is very much higher than that under normal physiologic conditions. Whether this CD8-mediated activation of DCs is relevant during the initiation of an immune response in the natural setting, i.e., when the frequency of virus-specific CD8+ T cells is low, has not been proven. More likely this mechanism could play a role later in the infection, when virus-specific CD8+ T cells have undergone clonal expansion and have become frequent.

It appears more reasonable to focus on the APCs, especially the DCs. It is known that viral infection in vitro may activate DCs directly (9), thereby up-regulating costimulatory molecules such as B7.1, B7.2, and CD40. Recently, it has been found that VSV and LCMV infections lead to very different levels of activation of DCs in vivo, as LCMV induces a much stronger up-regulation of costimulatory molecules than VSV (45). This further stresses the differences previously observed between the two viruses and may be a reflection of the difference in their capacity to replicate in the murine host. Although other types of APCs may participate in activation of virus-specific CD8+ T cells (21, 47), there is every reason to assume that virus-induced activation should function in a parallel manner for all major subtypes of APCs. Hence, the observation that infection with LCMV more efficiently induces the up-regulation of costimulatory molecules on DCs readily explains our results regarding coinfection with LCMV and VSV. Thus, we obtained evidence indicating that in CD40L−/− mice, LCMV was able to provide help for the response toward VSV in an Ag-non-specific, CD4-independent manner. The stronger, and probably prolonged, activation of APCs associated with LCMV infection (perhaps due to a more potent and prolonged IFN-α/β response (48, 49)) would cause DCs and other APCs to become more efficient in activating VSV-specific CD8+ T cells. In contrast, coinfection did not bypass the requirement for CD28. The latter finding is consistent with previous reports indicating that CD28 is directly involved in T cell activation, and that the importance of this molecule is inversely related to the strength of the TCR-dependent signal 1 (23). Because coinfection does not change the basic behavior of VSV, the transient replication of this virus may limit Ag presentation and thus explain the stringent requirement for costimulation in this case.

The finding that certain viral infections may provide non-specific help, thus bypassing control mechanisms normally perceived to be involved in preventing autoaggression, supports the theory put forward by Ruedl et al. (45) concerning autoimmune diseases involving self-reactive T cells not cross-reactive with viral epitopes. They propose that certain self-Ags are present at low amounts at all times, but due to an immature state of DCs are ignored by the T cells. However, during a viral infection DCs may become non-specifically activated and up-regulate costimulatory molecules, resulting in activation of self-reactive T cells, thus shifting the balance from tolerance to autoimmunity.

Notably, despite the fact that CD28 and CD40L appear to play almost parallel roles during initiation of the CD8+ T cell response, consistent with the idea of the interrelationship of these signaling pathways, the importance of these molecular interactions is very different during the memory phase. Thus, LCMV-infected CD40L−/− mice show evidence of impaired CD8+ T cell function and do not permanently control the infection. Notably, this is observed even following infection with an LCMV strain (Armstrong) that has limited potential for causing persistent infection in wild-type mice. In contrast, no evidence for failure of immune control was found in CD28−/− mice, even though the frequency of virus-specific CD8+ cells tended to be slightly lower. This may indicate that CD28/B7 interactions play little role once a significant population of primed T cells has been established (40). In contrast, CD40/CD40L interaction may be needed to enable APCs to maintain the activated state of the memory cell population required for continued immune surveillance (50); during this phase of the infection viral replication is too limited to contribute significantly to conditioning of APCs.

In conclusion, the results demonstrate that the requirement for CD40/CD40L interaction to elicit a CD8+ T cell response varies considerably between viruses and over time. CD40L is critical for generation of VSV-specific Tp1 cells regarding clonal expansion as well as differentiation, whereas the response to LCMV initially is CD40L independent. In the long term, CD40L seems to be required to maintain the functional capacity of primed CD8+ T cells even when the virus load is very low (low virus dose, virus strain with little capacity to persist (51)). Interestingly, coinfection with a CD40L-independent virus may augment the response to a
CD40L-dependent virus/Ag. The latter finding may have implications for understanding the relationship between viral infections and autoimmune disease.

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