

CD40L-deficient Mice Show Deficits in Antiviral Immunity and Have an Impaired Memory CD8⁺ CTL Response

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Summary

The ligand for CD40 (CD40L) is expressed on the surface of activated CD4⁺ T cells and its role in T-B cell collaborations and thymus-dependent humoral immunity is well established. Recently, by generating CD40L-knockout mice, we have confirmed its previously described role in humoral immunity and defined another important function of this molecule in the *in vivo* clonal expansion of antigen-specific CD4⁺ T cells. Here, we investigated the potential *in vivo* role of CD40L in antiviral immunity by examining the immune response mounted by CD40L-deficient mice following infection with lymphocytic choriomeningitis virus (LCMV), Pichinde virus, or vesicular stomatitis virus. Humoral immune responses of CD40L-deficient mice to these viruses were severely compromised, although moderate titres of antiviral IgM and some IgG2a were produced by virus-infected CD40L-deficient mice by a CD4⁺ T cell-independent mechanism. By contrast, CD40L-deficient mice made strong primary CTL responses to all three viruses. Interestingly however, although memory CTL activity was detectable in CD40L-deficient mice two months after infection with LCMV, the memory CTL response was much less efficient than in wild-type mice. Together, the results show that CD40-CD40L interactions are required for strong antiviral humoral immune responses, and reveal a novel role for CD40L in the establishment and/or maintenance of CD8⁺ CTL memory.

The CD40 ligand (CD40L, gp39, TBAM)¹ is a type II membrane glycoprotein whose extracellular domain is homologous to tumor necrosis factor (TNF)- α and - β (1, 2). This protein is expressed transiently on CD4⁺ T cells following activation *in vitro* or *in vivo* (3–5), and weakly on CD8⁺ T cells following *in vitro* activation with anti-CD3 or PMA/ionomycin (1, 3, 4, 6, 7). CD40L binds to CD40, a 50-kD member of the TNF receptor superfamily expressed on several immune system cell types including immature and mature B lymphocytes, interdigitating cells in the T cell areas of secondary lymphoid organs, follicular dendritic cells, and thymic epithelium (8). On B cells, CD40 is an important triggering molecule through which mitogenic signals can be delivered (8).

Interactions between CD40L and CD40 on B cells play a key role in the development of thymus-dependent hu-

moral immune responses, as they mediate the cognate interactions between helper T cells and B cells that are essential for the induction of B cell activation and immunoglobulin (Ig) production (1, 2, 7, 8, 9). Not only primary, but also secondary humoral responses are dependent on CD40L-CD40 interactions: germinal center formation and the generation of B cell memory also fail to take place if CD40L-CD40 interactions do not occur (10–13). In addition to these roles in the generation and maintenance of humoral immunity to thymus-dependent antigens, it can be inferred from their sites of expression that CD40L-CD40 interactions likely also play other more diverse roles in the functioning of the immune system. For example, CD40L is expressed on thymocytes, and unpublished data reviewed in (14) suggests that here it may play a role in thymic selection. Moreover, our recent studies show a requirement of CD40L for *in vivo* priming of CD4⁺ T cells (15).

To allow further study of the potential roles of CD40L in immune responses, CD40L-deficient mice have recently been generated by gene targeting (16, 17). No significant alterations in lymphocyte development are apparent in these mice, and they possess normal distributions and per-

¹Abbreviations used in this paper: ASC, antibody-secreting cell; CD40L, CD40 ligand; GP, glycoprotein; LCMV, lymphocytic choriomeningitis virus; moi, multiplicity of infection; NP, nucleoprotein; pfu, plaque-forming unit; PNA, peanut agglutinin; PV, Pichinde virus; VSV, vesicular stomatitis virus; VV, vaccinia virus.

centages of B and T cell subpopulations. Although they have low levels of most circulating immunoglobulin isotypes, they do not exhibit the spontaneous hyper-IgM (HIM) syndrome seen in humans with mutations in their CD40L gene (16, 17). After immunization with inert antigens, the CD40L-deficient mice respond normally to thymus-independent antigens, but have a decreased IgM response and fail altogether to produce an antigen-specific IgG1 response to thymus-dependent antigens. Moreover, they do not develop germinal centers in response to thymus-dependent antigens, suggesting they may have an inability to develop memory B cell responses (16).

We examined the immune response mounted by CD40L-deficient mice after infection with different viruses, including the arenaviruses lymphocytic choriomeningitis virus (LCMV) and Pichinde virus (PV), and vesicular stomatitis virus (VSV), a rhabdovirus. All of these viruses elicit strong cellular and humoral immune responses in normal mice. However, while arenavirus clearance is primarily mediated by the CD8⁺ MHC class I-restricted antiviral cytotoxic T lymphocyte (CTL) response (18–21), in VSV-infected mice neutralizing antibody production plays a much more important role in protection (22–24). CD40L-deficient mice mounted strong primary CTL responses after infection with all of these viruses, and cleared infectious virus from the periphery with similar kinetics to non-deficient control animals. In addition to producing some antiviral antibody of the IgM isotype, CD40L-deficient mice also produced virus-specific IgG2a, although at lower levels than in non-deficient control animals; the latter occurred by a CD4-independent mechanism. The antiviral antibody production in virus-infected CD40L-deficient mice took place in the absence of germinal center formation and titres of virus-specific antibody decreased over time; further, B cell memory was not established. Interestingly, while memory CTL activity could be detected in LCMV-infected CD40L-deficient mice, it was not present at as high a level in these animals at 2 mo after infection as in CD40L-expressing controls, suggesting a novel role for CD40L in the establishment and/or maintenance of CD8⁺ CTL memory.

Materials and Methods

Mice. CD40L-deficient mice were initially constructed as described by Xu et al. (16). The animals used in this study were obtained by mating mice homozygous for the CD40L mutation; Southern blot analysis of DNA derived from tail biopsies, performed as described by Xu et al. (16), was used to confirm homozygosity in offspring. Mice with wild-type CD40L expression were bred in parallel with the CD40L-deficient animals for use as controls in all experiments. C57L mice and C57BL/6 mice used as recipients in adoptive transfer experiments were obtained from Jackson Laboratories (Bar Harbor, ME) and the closed breeding colony of The Scripps Research Institute (La Jolla, CA), respectively.

Viruses: Preparation of Stocks, Concentration, and Titration by Plaque Assay. Viruses used were (a) the Armstrong 53b strain of LCMV, a clone triple plaque-purified from ARM CA 1371 (25);

(b) the Mudd-Summers strain of the Indiana serotype of VSV, originally obtained from Dr. J. Holland (UCSD, La Jolla, CA); and (c) Pichinde virus strain AN3739 (26) obtained from Dr. M.J. Buchmeier (The Scripps Research Institute, La Jolla, CA). Stocks of each of these viruses were prepared by growth in baby hamster kidney cells. Virus stocks were free of mycoplasma contamination as judged by Hoechst staining of cells growing in antibiotic-free medium at 48 h after virus infection.

For use in coating plates, LCMV and PV were concentrated and partly purified from the culture fluid of infected cells by precipitation with 6.5% polyethylene glycol followed by centrifugation at 35,000 rpm in a Beckman SW41 rotor for 75 min on a discontinuous renografin gradient (27, 28). VSV was enriched by pelleting culture fluids from infected cells and then banding them on a 5–40% continuous sucrose gradient (29).

The titres of virus stocks, concentrated preparations of virus, and also viral titres in the sera and tissues of infected mice were determined by plaque assay on Vero cells as previously described (25).

Infection of Mice and In Vivo Depletion of CD4⁺ T Cells. Mice were infected as adults (at least 8 wk old) by intraperitoneal (i.p.) inoculation with 2×10^5 plaque forming units (pfu) of either LCMV or PV, or by intravenous (i.v.) inoculation with 5×10^5 PFU of VSV.

In some experiments mice were depleted of CD4⁺ T cells in vivo by treatment with the anti-CD4 rat monoclonal antibody YTS 191.1 (30). The antibody was partially purified from ascites by ammonium sulfate precipitation, dialyzed against phosphate-buffered saline (PBS), and adjusted to 10 mg/ml. Mice were inoculated i.v. with 1 mg of antibody on days -1, +3, and +7, relative to the time of virus infection (day 0). The efficiency of depletion of CD4⁺ T cells was checked by fluorescence-activated cell sorter (FACS[®]) analysis of peripheral blood mononuclear cells obtained by eye-bleeding mice just before administration of the third dose of anti-CD4 antibody on day seven after infection, and was >99%.

Assays for Virus-specific CTL Activity. Virus-specific CTL activity was quantitated in vitro in standard chromium-51 (⁵¹Cr) release assays as described by Byrne and Oldstone (18). In assays to measure primary CTL activity, effector cells were erythrocyte-depleted splenocyte suspensions from mice infected 7 d previously with LCMV, PV, or VSV. Target cells were ⁵¹Cr-labeled fibroblast cell lines MC57 (H-2^b i.e., syngeneic to the CD40L-deficient mice) and Balb C1 7 (H-2^d i.e., allogeneic to the CD40L-deficient mice) either uninfected, infected 48 h before ⁵¹Cr-labeling with LCMV or PV at a multiplicity of infection (moi) of 3 PFU per cell, or infected with VSV at a moi of 100 PFU per cell immediately before ⁵¹Cr-labeling so that they were used in the assay 2–3 h after infection. Target cells were plated at 10^4 /well, and effectors added to give effector/target (E:T) ratios between 100:1 and 12.5:1. All variables were set up in triplicate. The assay time was 5 h for the detection of arenavirus-specific CTL activity, and 6 h for VSV. Results are expressed as the percent specific ⁵¹Cr release, calculated as $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$.

LCMV-specific memory CTL activity was assessed by two different methods. In the first, splenocytes from mice infected with LCMV ~2 mo previously were restimulated in vitro as described by Byrne and Oldstone (18), then tested for cytolytic activity in an in vitro ⁵¹Cr-release assay. Target cells were ⁵¹Cr-labeled fibroblast cell lines either uninfected or infected with LCMV as used to test primary LCMV-specific CTL activity, or infected 10 h before ⁵¹Cr-labeling at a moi of 3 PFU per cell with recombinant vaccinia viruses (VV) obtained from Dr. J.L. Whitton (The

Scripps Research Institute, La Jolla, CA) encoding the nucleoprotein (VVNP) (31), full-length glycoprotein precursor (VVGp) (Dr. J. Lindsay Whitton, unpublished observation), glycoprotein 1 H-2^b CTL epitope (VVMG 3, referred to in this paper as VVGp1) (32), or glycoprotein 2 H-2^b CTL epitope (Vvg, referred to in this paper as VVGp2) (33) of LCMV, or β -galactosidase only (VVSC11) (31) as a control.

Alternatively splenocytes were adoptively transferred (without *in vitro* restimulation) into syngeneic recipient mice persistently infected with LCMV to determine whether they could mediate viral clearance *in vivo*. C57BL/6 mice persistently infected with LCMV were produced by inoculating neonatal mice (within 48 h of birth) with 10³ PFU of LCMV Armstrong intracranially (ic); persistently infected animals were used as adoptive transfer recipients when 2–4 mo old. Recipient mice were irradiated (300 rads), then the following day were inoculated *i.p.* with 4 × 10⁷ donor splenocytes, and subsequent virus clearance was assessed by measuring serum virus titres 14 d later.

Enzyme Linked Immunosorbent Assays (ELISAs) for Measurement of Antiviral Antibody Levels. Antiviral antibody levels were determined by ELISA, using a method based on that described by Ahmed et al. (34). Briefly, Falcon Microtest III 96-well flat-bottomed plates (Becton Dickinson and Co., Oxnard, CA) were coated overnight with partly purified LCMV, PV or VSV at 5 mg/ml in PBS. After a blocking step, primary antibodies were added: four-fold dilutions of each serum sample from 1/25 to 1/25,600,000 were tested. Antibody binding was detected using as secondary antibodies horseradish peroxidase-conjugated anti-mouse IgM (used at 1/2,000), anti-mouse IgG F_c (used at 1/50,000), anti-mouse IgG1 (used at 1/1,000), and anti-mouse IgG2a (also used at 1/1,000), all purchased from Cappel Research Products (Organon Teknika Corporation, Durham, NC), followed by an orthophenyldiamine-containing substrate. Plates were read at 492 nm using a Titertek Multiskan MCC/340 ELISA reader (Flow Laboratories, Inc., Santa Barbara, CA). Endpoint titres of virus-binding serum antibodies were determined as the last serum dilution giving an absorbance at 492 nm more than three standard deviations above the mean value of eight negative control wells on that plate. In some experiments results are expressed as endpoint titres of virus-specific serum antibody, *i.e.*, virus-binding serum antibody titre at a particular time post-infection minus the titre of virus-binding antibody (if any) in the pre-infection bleed serum sample from the same mouse.

The antigen specificity of the ELISA technique was shown by the finding that unlike post-infection sera from VSV-inoculated mice, post-infection sera from arenavirus-inoculated mice did not show any increase over pre-immune levels in binding to VSV-coated plates in this assay, and vice-versa. To demonstrate the specificity of the second antibodies used for the appropriate mouse immunoglobulin classes/subclasses, an ELISA was performed in which plates were coated with purified mouse IgM, IgG1, and IgG2a standards (Southern Biotechnology Associates, Inc., Birmingham, AL) at concentrations from 10–0.01 mg/ml in PBS, and the ability of the four different second antibodies (at the concentrations at which they were routinely used in the ELISAs for virus-specific antibody detection) to bind to these immunoglobulin standards was measured. Most importantly, this experiment demonstrated that there was a complete absence of cross-reactivity of the anti-total IgG and the two IgG subclass-specific second antibodies on IgM.

ELISPOT Assay for Quantitation of Antiviral Antibody Secreting Cells (ASC). LCMV- and VSV-specific ASC in the spleen and bone marrow of infected mice were quantitated by an ELISPOT

method based on that detailed in Slifka et al. (35). Briefly, nitrocellulose-bottomed 96-well Multiscreen HA filtration plates (Millipore Corporation, San Francisco, CA) were coated overnight at 4°C with 50 μ l/well of concentrated, partly purified LCMV or VSV, or bovine serum albumin (BSA) at 10 mg/ml in PBS, then were washed and non-specific binding sites were blocked. Erythrocyte-depleted single cell suspensions were prepared from the spleen and bone marrow (from one femur) of LCMV- and VSV-infected mice, and resuspended in RPMI medium containing 7% FBS at 10⁷ cells/ml. Threefold dilutions of the cells were added to the plates in 100- μ l vol/well. Plates were then incubated at 37°C in a humid atmosphere with 5% CO₂ for 4–5 h, after which they were washed and specific antibody bound to the plates was detected by successive incubations with a biotinylated mouse anti-mouse IgG2a^b antibody (PharMingen, San Diego, CA), horseradish peroxidase-conjugated avidin D (Vector Laboratories, Burlingame, CA), and a 3-amino-9-ethylcarbazole (AEC)-containing substrate. Spots where antibody was present were enumerated using a large magnifying glass. The antigen specificity of this technique was demonstrated by the finding that no spots were observed when cells from LCMV-infected mice were tested on VSV-coated plates, and vice-versa; also no spots were observed when cells from mice infected with either virus were tested on BSA-coated plates.

Immunocytochemical Analysis of Germinal Centers. Spleens were removed from uninfected mice and mice infected with VSV 10 d previously (the peak time of the germinal center reaction), embedded in Tissue-Tek OCT compound (Miles Diagnostics Division, Elkhart, IN), snap frozen in isopentane-dry ice, and stored at –20°C. Immunohistochemical staining was performed on 10- μ m cryostat sections using a method based on that described by Borrow et al. (36). Briefly, sections were fixed for 10 min with acetone at 4°C, rinsed in PBS, blocked with avidin and biotin solutions (Vector Laboratories, Inc., Burlingame, CA), then incubated with biotinylated peanut agglutinin lectin (PNA) (Biomedica Corp., Foster City, CA; 2 mg/ml stock solution used at a 1/1,000 dilution) or PBS only as a control for 1 h at room temperature. After washing in PBS, slides were incubated for a further hour at room temperature with peroxidase-conjugated streptavidin (Jackson Immunoresearch Laboratories, Inc., West Grove, PA; used at a 1/500 dilution in PBS), washed again in PBS, and then staining was detected with diaminobenzidine (DAB) as a chromagen. Finally, sections were counterstained with Mayer's hematoxylin (Sigma, St. Louis, MO) and mounted in Aqua-Mount (Lerner Laboratories, Pittsburgh, PA).

Adoptive Transfer Experiments for Analysis of B Cell Memory. To determine whether memory B cells were developed following virus infection of CD40L-deficient mice, erythrocyte-depleted splenocyte suspensions were prepared from CD40L-deficient and wild-type mice infected 3 to 4 mo previously with VSV or LCMV, and also from uninfected wild-type mice, all of which were on the C57BL/6 background (H-2^b, Igh^b), and 5 × 10⁶ cells/mouse were adoptively transferred into irradiated (600 rads) C57L recipients (H-2^b, Igh^a), which had been primed with the homologous virus 6 wk previously. Recipient mice were challenged with the appropriate virus at the time of transfer. 7, 10, and 14 d later, the recipient mice were eye-bled, and serum titres of antiviral IgG2a^b assessed by ELISA employing a biotinylated IgG2a^b-specific secondary antibody (PharMingen; used at a 1/5,000 dilution in PBS plus 10% FBS) followed by peroxidase-coupled streptavidin (Jackson Immunoresearch Laboratories, Inc., West Grove, PA; used at a 1/2,000 dilution) to detect IgG2a^b binding.

Results and Discussion

Analysis of Primary CTL and Antibody Responses in Virus-infected CD40L-deficient Mice. To investigate potential roles CD40L may play in the functioning of the immune system in vivo, the immune response mounted following virus infection by CD40L-deficient mice, which were generated by gene targeting (16), was examined.

In initial experiments the primary cell-mediated and humoral immune responses made by CD40L-deficient mice infected with VSV, LCMV, and PV were compared to those mounted by wild-type animals. Groups of six to eight CD40L-deficient and wild-type mice were infected with each virus. Several animals from each group were sacrificed 7 d after infection, and the primary antiviral CTL activity mediated by splenocytes from individual mice was determined by ⁵¹Cr release assay. CD40L-deficient mice infected with VSV, LCMV, or PV mounted virus-specific, major histocompatibility complex (MHC)-restricted CTL responses that were indistinguishable from those of wild-type mice (Table 1 and data not shown), indicating that CD40L is not essential for the induction of primary CTL responses following virus infection. This result is consistent

with the fact that CD40L is absent or expressed at only very low levels on the majority of CD8⁺ T cells (1, 3, 4, 6, 7).

The remaining mice in each group were eye-bled at different times in the 2-mo period after virus infection, and their serum titres of virus-specific IgM, total IgG, IgG1, and IgG2a antibody were determined by ELISA using appropriate isotype-specific peroxidase-conjugated secondary antibodies. Fig. 1 *a* shows the ELISA results obtained in an assay measuring the levels of virus-binding total IgG in pre-infection and day 14 post-infection serum samples from a representative CD40L-deficient and wild-type mouse, and illustrates the difference in the endpoint titres of virus-binding serum antibody in the two day 14 samples. Fig. 1 *b* summarizes the mean endpoint titres of virus-specific serum IgM, total IgG, IgG1, and IgG2a in groups of CD40L-deficient and wild-type mice at different times after infection with VSV or LCMV. It can be seen that CD40L-deficient mice produced lower levels of virus-specific antibody of the IgM isotype than wild-type animals. Surprisingly, as well as producing virus-specific IgM, infected CD40L-deficient mice also made an antiviral IgG response, although again this was

Table 1. Primary CTL Responses Mounted by CD40L-deficient and Wild-type Mice after Infection with Different Viruses*

Source of effector cells	Target cells			
	H-2 ^b uninf	H-2 ^b + VSV	H-2 ^d uninf	H-2 ^d + VSV
VSV-infected wild-type mice				
W1	0/0	59/52	2/0	0/2
W2	12/4	65/49	ND/1	ND/4
W3	0/0	48/39	ND/0	0/0
VSV-infected CD40L-deficient mice				
D1	3/0	59/32	5/0	2/0
D2	9/7	49/34	5/0	10/0
D3	13/6	57/37	ND/4	8/0
	Target cells			
	H-2 ^b uninf	H-2 ^b + LCMV	H-2 ^d uninf	H-2 ^d + LCMV
LCMV-infected wild-type mice				
W4	7/0	77/49	8/4	13/6
W5	1/0	50/39	1/0	16/1
W6	0/0	71/57	3/2	5/5
LCMV-infected CD40L-deficient mice				
D4	2/0	81/67	3/2	15/9
D5	5/2	62/47	2/1	13/8
D6	1/1	78/78	10/5	13/15

*The ability of splenocytes from CD40L-deficient and wild-type mice infected 7 d previously with VSV or LCMV to mediate virus-specific MHC-restricted CTL lysis was tested by in vitro ⁵¹Cr-release assays. Target cells were MHC-matched (H-2^b) or mismatched (H-2^d) fibroblast lines either uninfected or infected with VSV or LCMV. The results shown are the mean (of triplicate wells) percent specific ⁵¹Cr-release (calculated as described in Materials and Methods) mediated by effector splenocytes from individual CD40L-deficient (D1-6) or wild-type (W1-6) mice at E:T ratios of 100:1/50:1 (experiment with VSV) or 25:1/12.5:1 (experiment with LCMV). The variance was always <10%.

profoundly reduced compared to the response mounted by wild-type animals. In addition, while wild-type mice produced antiviral IgG of both the IgG1 and IgG2a isotypes, infected CD40L-deficient mice did not produce a significant amount of virus-specific IgG1. Further, whereas in wild-type mice the serum titres of virus-specific IgG were sustained at two months post-infection at similar levels to those present 7–10 d after infection, in CD40L-deficient mice a 1–2 log decrease in serum IgG titres occurred over this time period. Very similar results were obtained with PV-infected mice (not shown). Thus while CD40L-mediated interactions are clearly involved in the generation of the primary humoral immune response following virus infection, and are essential for the production of antiviral antibody of the IgG1 isotype, modest levels of virus-specific IgM and a short-lived IgG response of the IgG2a isotype are generated in virus-infected mice by a CD40L-independent mechanism.

Mechanism of IgG Production in Virus-infected CD40L-deficient Mice. A further series of experiments investigated how antibody of the IgG isotype was being produced in the absence of CD40L-mediated interactions. Although CD40L-CD40L interactions are known normally to be required for thymus-dependent humoral immune responses (1, 2, 5, 7, 9, 11), there are reports of CD4⁺ T cells stimulating B cells to undergo activation and class switching by non-CD40L-mediated mechanisms including via membrane TNF- α or by CD2-CD58-dependent pathways (37–40). In addition, antibody production to T-independent antigens has been shown not to involve CD40L-mediated interactions (5, 10, 11, 16, 17). Whether the antiviral IgG production observed in infected CD40L-deficient mice was occurring by some CD4⁺ T cell-dependent mechanism or alternatively by a CD4⁺ T cell-independent pathway was thus determined. CD4⁺ T lymphocytes were depleted from CD40L-deficient and wild-type mice using a CD4-specific monoclonal antibody (30), then these and undepleted control animals were infected with VSV or LCMV, and the serum titres of virus-specific IgM, total IgG, IgG1, and IgG2a measured by ELISA on days 7 and 10 post-infection. The results obtained in the experiment using VSV are shown in Fig. 2; similar data were obtained with LCMV-infected mice (not shown). The antiviral IgG response mounted by CD4⁺ T cell-depleted CD40L-deficient mice was found to be just as strong as that made by CD40L-deficient mice from which the CD4⁺ T cells were not depleted, indicating that the virus-specific antibody production in CD40L-deficient mice was occurring by a CD4⁺ T cell-independent pathway. Further, the level of antiviral IgG production in wild-type mice depleted of CD4⁺ T cells before virus infection was reduced compared to that made by non-depleted wild-type animals to a similar level to that observed in CD40L-deficient animals, and again as in CD40L-deficient animals, although virus-specific IgG2a was produced by CD4⁺ T cell-depleted wild-type mice, almost no IgG1 production was observed. This demonstrates that in virus-infected wild-type mice too, there is a CD4⁺ T cell-independent component to the antiviral antibody response, although

this is supplemented by a CD4⁺ T cell-dependent component that is necessary for high titres of IgG to be produced, and for class switching to IgG1 to occur.

These results are in agreement with previous reports that the humoral immune response to VSV has a T cell-independent component (41, 42) in addition to neutralizing IgG being produced by a T_H-dependent mechanism (43). Thymus-independent antigens fall into two classes: type I antigens, which are B cell mitogens and cause polyclonal B cell activation; and type II antigens which have no intrinsic B cell stimulating activity, but possess highly repetitive structures and stimulate specific mature B cells by extensively cross-linking their surface immunoglobulin. There is some controversy as to whether VSV glycoproteins have mitogenic activity (44, 45); however as virions consist of highly organized arrays of viral proteins, they are certainly able to act as type II thymus-independent antigens (45). The fact that production of cytokines such as interferon- γ (IFN- γ) is stimulated at high levels after virus infection likely also played a part in the antibody generation observed in CD40L-deficient mice, as cytokines are potent regulators of B cell responses to thymus-independent antigens. IFN- γ , for example, regulates both B cell maturation to Ig secretion and Ig class switching in immune responses to thymus-independent antigens (46, 47). Treatment of CD40L-deficient mice with an antibody that blocks the activity of IFN- γ (48) in fact resulted in a slight increase in the level of virus-specific IgG produced following infection with VSV or LCMV (data not shown), suggesting that IFN- γ is probably only one of a network of cytokines that regulates antibody responses to type II thymus-independent viral antigens.

Control of Acute Virus Infection in CD40L-deficient Mice. Whether the primary humoral and cell-mediated immune responses mounted by CD40L-deficient mice were sufficient to confer protection against acute infection with LCMV and VSV was also investigated. Groups of 15 CD40L-deficient and wild-type mice were infected with each virus, and three animals from each group were killed 1, 3, 5, 7, and 10 d post-infection and infectious virus titres in the serum, spleen, and liver determined by plaque assay. Fig. 3 shows the LCMV titres in the spleen and liver of CD40L-deficient and wild-type mice over time post-infection: the CD40L-deficient mice cleared infectious virus from these tissues (and also the serum, data not shown) with similar kinetics to the wild-type animals. As LCMV clearance is primarily mediated by virus-specific CD8⁺ CTL (18–21), this finding was consistent with the data in Table 1 showing that as measured by in vitro ⁵¹Cr-release assay, the primary CTL response mounted by CD40L-deficient mice is equivalent to that mounted by wild-type animals.

After infection with VSV, mice exhibit a natural resistance to viral replication in the periphery, which is mediated by IFN- α/β (49, 50). In keeping with such an innate resistance mechanism, although low viral titres were observed in the spleen and serum of some CD40L-deficient and wild-type mice on the first day after infection with VSV (not shown), infectious virus was undetectable in the periphery of both groups of mice thereafter. Interestingly,

however, during the course of all the experiments performed with VSV-infected CD40L-deficient mice and wild-type controls, several CD40L-deficient mice were found to die 8–12 d after infection with VSV, whereas no similar deaths occurred in VSV-infected wild-type control mice. The deaths of the CD40L-deficient mice were likely due to VSV-induced meningoencephalitis (51). As the antiviral antibody response normally contributes to protection in VSV-infected mice (23, 24), these deaths may have occurred as a consequence of the reduced titres of antiviral antibodies produced by the CD40L-deficient animals (Fig. 1 *b*); central nervous system (CNS) antibody levels may have been insufficient to mediate control of virus replication in the brain.

Analysis of Long-term Production of Antiviral Antibody by CD40L-deficient Mice. Results shown in Fig. 1 illustrate that although CD40L-deficient mice did mount a humoral immune response after infection with VSV or LCMV, serum titres of virus-specific IgG were not sustained, but waned over the 2-mo period after infection. By contrast, serum titres of antiviral IgG in similarly infected wild-type mice remained high over this time period. It has been shown that whereas at early times after infection of wild-type mice with viruses such as LCMV, large numbers of cells secreting virus-specific antibody are found in the spleen, at later times post-infection antiviral plasma cell numbers in the spleen decline, and a population of virus-specific plasma cells appears in the bone marrow that constitutes the major source of long-term antibody production (35). Whether the waning titres of antiviral antibody in the serum of virus-infected CD40L-deficient mice correlated with a lack of population of the bone marrow with virus-specific ASC in these mice was thus investigated. CD40L-deficient and wild-type mice were infected with either VSV or LCMV, and the number of cells in the spleen and bone marrow secreting virus-specific IgG2a was quantitated by ELISPOT assay on days 14 and 64 post-infection. The results in Table 2 show that 14 d post-infection the number of cells actively secreting virus-specific IgG2a in CD40L-deficient mice was ~20-fold lower after LCMV infection, and three- to eightfold lower after VSV infection than in infected wild-type mice, consistent with the relative serum titres of virus-specific IgG2a shown in Fig. 1 *b*. Interestingly, however, in CD40L-deficient mice just as in wild-type mice, although the majority of the antiviral plasma cells were found in the spleen at this timepoint, some ASC could also be detected in the bone marrow. The ratio of spleen: bone marrow ASC in LCMV-infected CD40L-deficient mice was approximately equal to that in wild-type animals, while following VSV infection it was slightly higher in the CD40L-deficient mice. However, ASC did not persist in the bone marrow of CD40L-deficient mice: by 2 mo post-infection, virus-specific IgG2a-secreting cells could no longer be detected in either the spleen or the bone marrow of LCMV or VSV-infected CD40L-deficient animals (Table 2). By contrast, in similarly infected wild-type mice, although the number of antiviral plasma cells in the spleen had decreased ~50-fold, virus-specific ASC numbers in the bone marrow

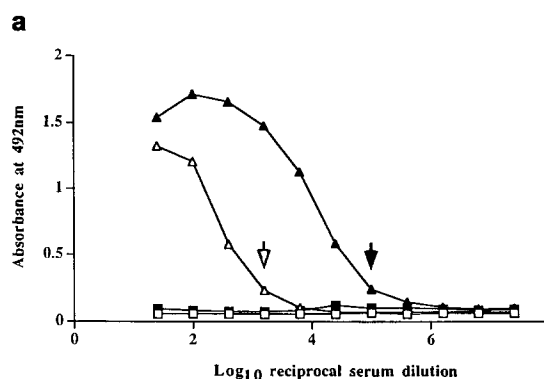


Figure 1. Comparison of the antiviral antibody response mounted by CD40L-deficient and wild-type mice following infection with VSV or LCMV. Groups of three to eight CD40L-deficient and wild-type mice were infected with VSV or LCMV, and the serum titres of virus-binding antibody of the IgM, total IgG, IgG1, and IgG2a isotypes in prebleed and serum samples obtained at the indicated times post-infection were determined by ELISA as detailed in Materials and Methods. (*a*) ELISA results obtained when prebleed (*squares*) and day 14 post-infection (*triangles*) serum samples from a representative CD40L-deficient (*open symbols*) and wild-type (*filled symbols*) mouse infected with VSV were tested for VSV-binding total IgG. The endpoint ELISA titres of VSV-binding antibody in the two day fourteen samples (i.e., last serum dilution which gave an absorbance value at 492 nm more than three standard deviations above the mean value of the negative control wells on the plate on which it was tested) are indicated by the arrows. (*b*) Mean \log_{10} reciprocal endpoint titres of virus-specific serum antibody of the IgM, total IgG, IgG1, and IgG2a isotypes in groups of CD40L-deficient (*open bars*) and wild-type (*shaded bars*) mice at the times shown following infection with VSV or LCMV. The vertical lines indicate one standard error above and below the mean titre for each group of animals.

remained much more constant at this timepoint (Table 2). These results demonstrate that population of the bone marrow by virus-specific ASC can occur in the absence of CD40L-mediated interactions, but that the ASC generated by CD40L-independent mechanisms do not persist *in vivo*.

Investigation of Whether Germinal Center Formation and the Development of B Cell Memory Occur after Virus Infection of CD40L-deficient Mice. In normal mice, plasma cell precursors are thought to be generated in germinal centers, regions which develop transiently within the primary follicles of secondary lymphoid organs following antigenic stimulation where B cells undergo antigen-driven oligoclonal proliferation, and isotype switching and affinity maturation occur (52, 53). Whether the lack of persistence of plasma cells producing virus-specific antibody observed in CD40L-deficient mice correlated with a defect in germinal center formation after virus infection of these mice was thus investigated. CD40L-deficient and wild-type mice were killed 10 d after infection with VSV, the peak time of the germinal center reaction, and their spleens were analyzed immunocytochemically for PNA binding, characteristic for germinal center B cells (Fig. 4). Although large numbers of germinal centers were seen to have formed in the spleen of wild-type mice after infection with VSV (compare the staining in Fig. 4 *b* with *a*), no germinal centers were seen in the VSV-infected CD40L-deficient mice (Fig. 4 *c*). This result was consistent with the previous finding that CD40L-

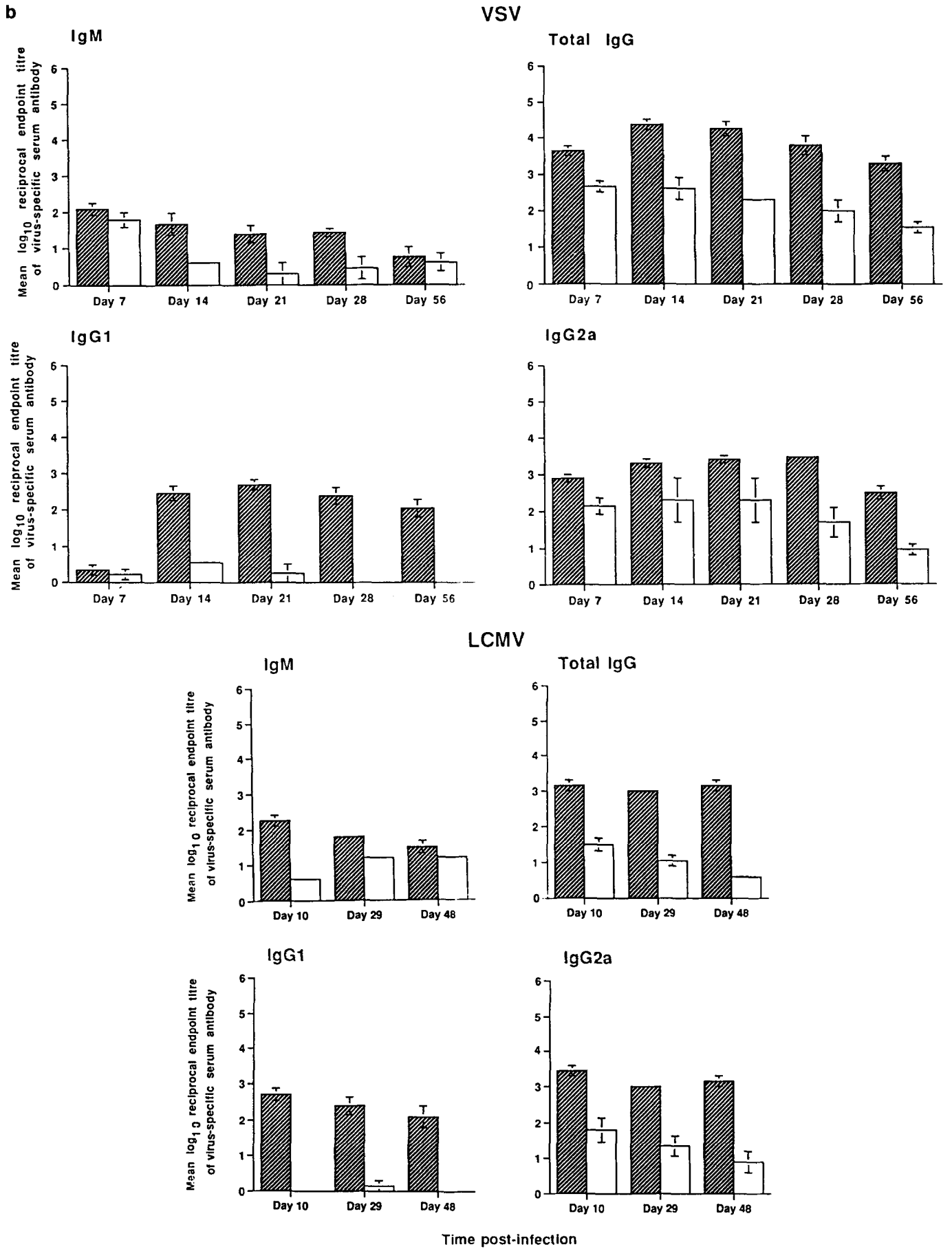


Figure 1. Continued

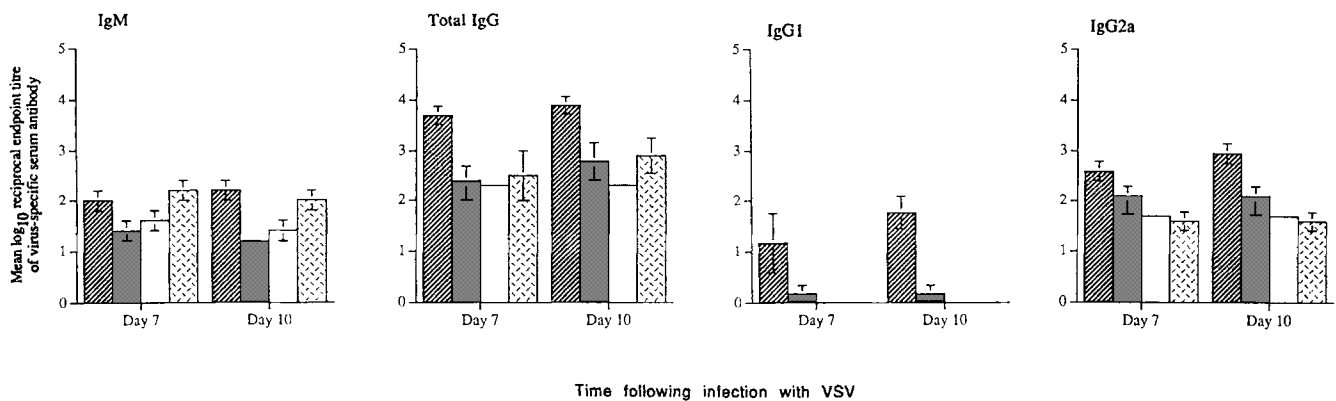


Figure 2. Effect of CD4⁺ T cell depletion on the antiviral antibody response mounted by CD40L-deficient and wild-type mice after infection with VSV. Groups of three wild-type and CD40L-deficient mice which were either depleted of CD4⁺ T cells by treatment with an anti-CD4 monoclonal antibody, or were undepleted, were infected with 5×10^5 PFU VSV iv, and their serum titres of VSV-specific antibody of the IgM, total IgG, IgG1, and IgG2a isotypes were determined on days 7 and 10 post-infection by ELISA as detailed in Materials and Methods. The results shown are the mean \log_{10} reciprocal endpoint titres of VSV-specific serum antibody of each isotype in each group of mice. The bars represent \square undepleted wild-type mice; \blacksquare CD4⁺ T cell-depleted wild-type mice; \square undepleted CD40L-deficient mice; \square CD4⁺ T cell-depleted CD40L-deficient mice. The vertical lines indicate one standard error above and below the mean titre for each group of animals.

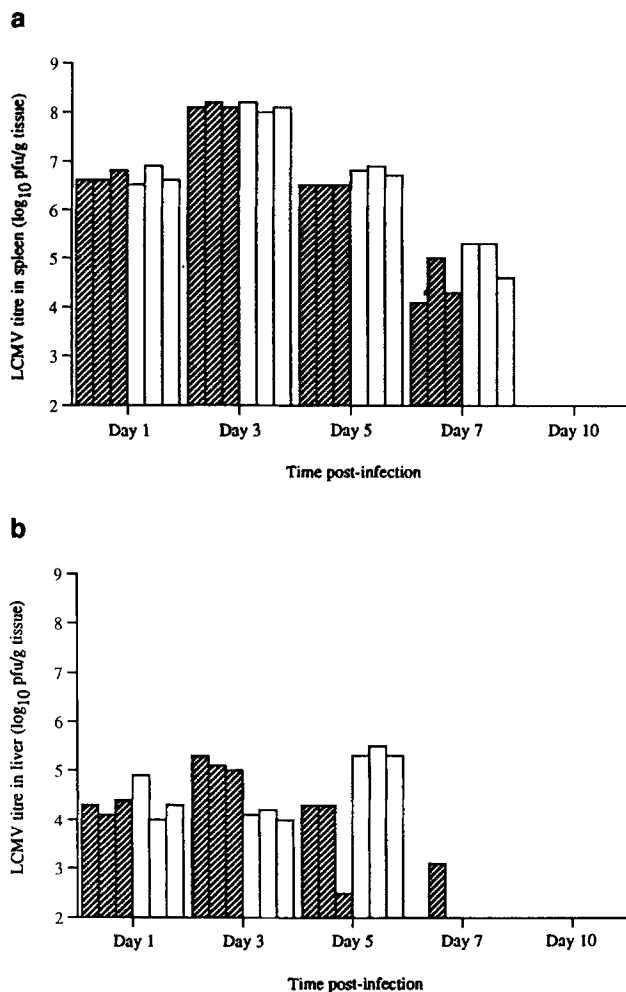


Figure 3. Virus clearance from the spleen and liver of wild-type and CD40L-deficient mice after infection with LCMV. Wild-type and CD40L-deficient mice were infected with 2×10^5 PFU LCMV ip, and 1, 3, 5, 7, and 10 d later three mice from each group were sacrificed and the

deficient mice do not form germinal centers after immunization with inert antigens such as sheep red blood cells (16), as well as with the observation that HIM patients lack detectable germinal centers (10).

The generation of antigen-specific memory B cell clones is another process that is thought to occur within germinal centers (53), and which has been shown to be inhibited in mice treated with antibody specific for CD40L (12) or a soluble CD40 fusion protein that blocks CD40L-CD40 interaction (13). Engagement of CD40 by CD40L prevents the apoptosis of germinal center B cells, likely via *bcl-x* up-regulation, thus contributing to antigen-driven selection of memory B cells (54, 55). Whether CD40L-deficient mice thus failed to develop memory B cells after virus infection was investigated in adoptive transfer experiments. Splenocytes from CD40L-deficient and wild-type mice infected 3–4 mo previously with VSV or LCMV, or from uninfected mice, all on the C57BL/6 background (H-2^b, Igh^b) were adoptively transferred into C57L (H-2^b, Igh^a) mice which had been immunized with the homologous virus 6 wk previously, and would thus provide syngeneic memory helper T cells. The recipient mice were reinoculated with the appropriate virus at the time of adoptive transfer; and the levels of virus-specific IgG2a antibody of the donor (Igh^b) type in the recipient sera were measured by ELISA 7, 10, and 14 d post-transfer. As shown in Fig. 5, although increasing titres of virus-binding IgG2a^b were observed in the sera of mice which received cells from wild-type mice

titres of infectious LCMV in the spleen (a) and liver (b) of individual animals were determined by plaque assay on Vero cells. Each bar represents the virus titre (\log_{10} pfu/g tissue) in the spleen or liver of an individual wild-type (shaded bars) or CD40L-deficient (open bars) mouse killed at the indicated time post-infection. Where no bar is shown, virus was not detected by the plaque assay (the sensitivity of which was ~ 200 pfu/g tissue) in that tissue sample.

Table 2. Number of Virus-specific IgG2a-secreting Cells per 10^6 Leucocytes in the Spleen and Bone Marrow of CD40L-deficient and Wild-type Mice on Days 14 and 64 Post-infection

Mice	ASC/ 10^6 cells in:	
	Spleen	Bone marrow
WT, day 14 after LCMV	570 \pm 163	32 \pm 20
D, day 14 after LCMV	29 \pm 5	2 \pm 1
WT, day 64 after LCMV	16 \pm 7	31 \pm 5
D, day 64 after LCMV	0 \pm 0	0 \pm 0
WT, day 14 after VSV	1375 \pm 275	176 \pm 33
D, day 14 after VSV	583 \pm 87	22 \pm 6
WT, day 64 after VSV	27 \pm 5	53 \pm 10
D, day 64 after VSV	0 \pm 0	0 \pm 0

The number of leucocytes in the spleen and bone marrow of CD40L-deficient (*D*) and wild-type (*WT*) mice infected 14 or 64 d previously with LCMV or VSV which were actively secreting IgG2a antibody specific for the infecting virus was quantitated by ELISPOT assay, as described in Materials and Methods. The results shown are the mean \pm standard error number of ASC per 10^6 leucocytes (ASC/ 10^6 cells) in the spleen and bone marrow of groups of 3–5 mice.

previously infected with VSV over the 14-d period examined, the level of virus-reactive IgG2a^b detected in the sera of mice that received cells from CD40L-deficient mice previously infected with this virus was consistently very low, comparable to that found in the sera of mice that received cells from previously untreated donors. Very similar results were obtained using LCMV (not shown). These results demonstrate that although virus-specific memory B cells were present in the spleen of wild-type mice infected 3–4 mo previously with VSV or LCMV, similarly-infected CD40L-deficient mice did not appear to have developed memory B cells specific for their previous virus infection. Although B cell memory was not developed following virus infection of CD40L-deficient mice, the B cells in these animals were also not tolerized to the infecting virus, as comparable (low) levels of virus-specific IgM^b were detected in the se-

rum of recipients of cells from all three types of donor mice (virus-infected wild-type or CD40L-deficient mice, or previously uninfected mice) (data not shown).

In summary, after infection with viruses such as VSV and LCMV, CD40L-deficient mice mount a primary antiviral antibody response (Fig. 1) by a CD4⁺ T cell-independent mechanism (Fig. 2), likely caused by virion proteins cross-linking the membrane immunoglobulin of specific B cells sufficiently to directly trigger them to produce antiviral antibody of the IgM and IgG2a isotypes. However, in the absence of CD40L-mediated interactions, germinal center formation does not occur (Fig. 4), and processes which are believed normally to occur within germinal centers: further isotype switching, generation of long-lived plasma cells, and production of antigen-specific memory B cells, do not take place (Fig. 1, Table 2, Fig. 5). These results in vivo infection systems are consistent with conclusions from previous studies of the in vivo role of CD40L-CD40 interactions in the generation of humoral immune responses to inert antigens (5, 11–13, 16, 17). A number of interesting questions arise from these findings. For example, how many CD40L-mediated interactions normally occur in each of the processes that fail to take place in CD40L-deficient mice, and between what cell types? Does the lack of an initial CD40L-mediated interaction between B cells and CD4⁺ T cells account for the failure of germinal center formation, which in turn results in B cells not being in a suitable microenvironment to undergo isotype switching and development into long-lived ASC and memory B cells; or are a series of CD40L-mediated interactions, perhaps involving B cells, CD4⁺ T cells, follicular dendritic cells in the spleen and lymph nodes, and some cell type in the bone marrow involved in these processes? The latter seems likely in view of the large number of different cell types on which expression of CD40 or its ligand has been documented (8, 14), but precise definition of the steps involved requires further studies.

Study of CTL Memory in CD40L-deficient Mice. A final series of experiments examined memory CTL activity in CD40L-deficient mice which had previously undergone an acute infection with LCMV. LCMV-specific memory CTL activity was initially assessed by restimulating splenocytes

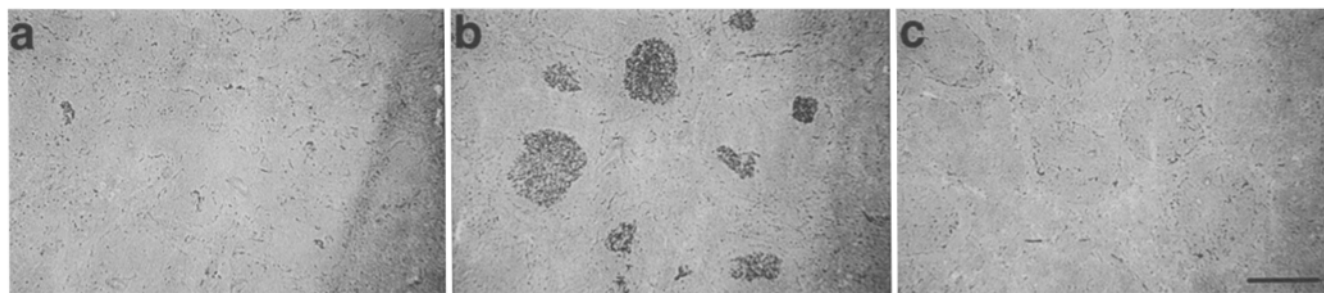


Figure 4. Germinal center formation occurs in wild-type but not CD40L-deficient mice after infection with VSV. Spleens were cryopreserved from uninfected wild-type mice (*a*) and wild-type (*b*) and CD40L-deficient (*c*) mice infected 10 d previously with 5×10^5 pfu VSV iv. 10- μ m cryosections were stained as described in Materials and Methods by incubation with biotinylated PNA followed by peroxidase-conjugated streptavidin, and staining was detected with DAB as a chromagen, which gives a brown product. Hematoxylin was used as a counterstain. Germinal centers are readily apparent in the spleen of wild-type mice infected with VSV (*b*), but not similarly-infected CD40L-deficient animals (*c*). Bar, 333 μ m.

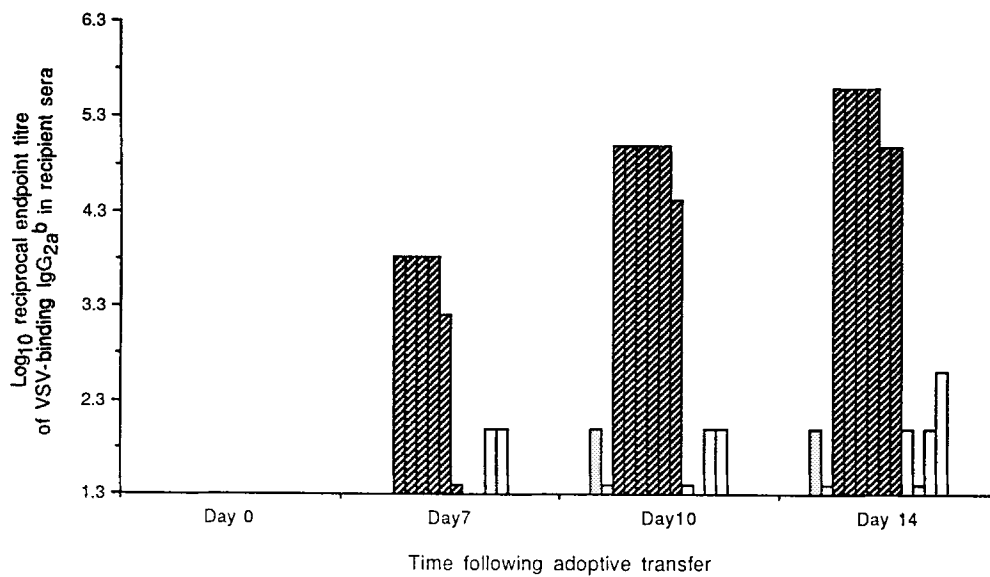


Figure 5. Adoptive transfer experiment comparing B cell memory in wild-type and CD40L-deficient mice after infection with VSV. Splenocytes from uninfected wild-type mice and wild-type and CD40L-deficient mice which were infected 3–4 mo previously with VSV all on the C57BL/6 background ($H-2^b$, Igh^b), were adoptively transferred into wild-type C57L recipients ($H-2^b$, Igh^a) that had been primed with VSV 6 wk previously. Recipient mice were challenged with VSV at the time of transfer. At the indicated times after the adoptive transfer, the titres of antiviral IgG_{2a} of the donor type (IgG_{2a}^b) in the recipient sera were determined by ELISA as detailed in Materials and Methods. The bars represent the \log_{10} reciprocal endpoint titres of antiviral IgG_{2a}^b in the serum of individual mice which received cells from uninfected donors, virus-infected wild-type donors, and virus-infected CD40L-deficient mice.

from CD40L-deficient and wild-type mice infected 2 mo previously with LCMV for 6 d in vitro, and then testing their ability to mediate virus-specific MHC-restricted CTL lysis in a ⁵¹Cr-release assay. Results from a representative experiment shown in Table 3 illustrate that although splenocytes from CD40L-deficient mice did mediate MHC-restricted CTL lysis of LCMV-infected target cells, the level of CTL activity they exhibited was lower than that of splenocytes from wild-type animals. The LCMV-specific CTL response in $H-2^b$ mice is known to be directed against three epitopes, one in the viral nucleoprotein (NP), one in glycoprotein 1 (GP1), and one in glycoprotein 2 (GP2); of these, the NP epitope is the most dominant (56). In accord

with the expected relative frequencies of memory CTL precursors (CTL_p) for these three epitopes, memory CTL from wild-type mice lysed target cells infected with a recombinant vaccinia virus expressing LCMV NP more efficiently than those infected with recombinant vaccinia viruses expressing LCMV GP or the GP1 or GP2 $H-2^b$ CTL epitopes (Table 3); similarly while NP-specific CTL activity could readily be detected in CD40L-deficient mice, GP-specific CTL activity was so low as to be virtually undetectable by this assay (<10% specific ⁵¹Cr release) (Table 3). CD40L-deficient mice did possess CTL_p specific for all three of the known $H-2^b$ -restricted LCMV CTL epitopes, however, as when splenocytes from CD40L-deficient mice

Table 3. Memory CTL Activity in CD40L-deficient and Wild-type Mice 2 mo after Infection with LCMV, as Measured by an In Vitro ⁵¹Cr Release Assay*

Source of Effectors	E:T Ratio	Target cells							
		Uninf	$H2^b$						$H-2^d$
			LCMV	VVNP	VVGP	VVGP1	VVGP2	LCMV	
Wild-type mice	25:1	2	69	53	41	31	26	3	
CD40L-deficient mice	25:1	0	44	25	8	0	5	1	

*Splenocytes pooled from groups of CD40L-deficient or wild-type mice infected 2 mo previously with LCMV were restimulated by in vitro culture with irradiated syngeneic LCMV-infected peritoneal macrophages as detailed in Materials and Methods. They were then tested for LCMV-specific CTL activity in an in vitro ⁵¹Cr-release assay. Target cells were MHC-matched ($H-2^b$) or mismatched ($H-2^d$) fibroblast lines either uninfected, infected with LCMV, or infected with recombinant vaccinia viruses encoding the nucleoprotein (VVNP), full-length glycoprotein precursor (VVGP), GP1 $H-2^b$ CTL epitope (VVGP1), or GP2 CTL epitope (VVGP2) of LCMV. The results shown are the mean (of triplicate wells) percent specific ⁵¹Cr-release (calculated as described in Materials and Methods) mediated by splenocyte cultures at the E:T ratios shown. The variance was always <10%.

were cultured *in vitro* for a 13-d period before testing in a ⁵¹Cr-release assay, to allow more expansion of virus-specific CTL to occur, CTL activity specific for all three epitopes could then be detected in these mice in addition to the wild-type animals (not shown). Altogether, the results from these experiments demonstrate that although LCMV-specific memory CTL recognizing all three H-2^b-restricted LCMV epitopes could be detected in CD40L-deficient mice 2 mo after infection with this virus, the level of memory CTL activity in these animals was much lower than that exhibited by wild-type mice at the same time post-infection.

CTL memory in CD40L-deficient and wild-type mice was also compared in an *in vivo* assay, in which splenocytes were adoptively transferred (without *in vitro* restimulation) from CD40L-deficient or wild-type mice infected 2 mo previously with LCMV into syngeneic wild-type mice persistently infected with LCMV as a result of neonatal inoculation with the virus, and their ability to mediate virus clearance from the recipient animals was assessed by comparing the titres of infectious LCMV in the sera of the recipient animals before and 14 d after the adoptive transfer. The results (Table 4) show that whereas adoptive transfer of memory CTL from wild-type mice infected 2 mo earlier with LCMV into persistently infected recipients led either to virus clearance or to the death of the recipient animal,

mice that received cells from CD40L-deficient donors infected 2 mo earlier with LCMV neither died nor showed a significant decrease in the titres of infectious virus in their serum on day 14 (or days 7, 21, or 28, data not shown) post-transfer. Thus, although memory CTL_p clearly were present in CD40L-deficient mice 2 mo after infection with LCMV (Table 3), they were at too low a frequency to mediate a detectable effect in the *in vivo* virus clearance assay (Table 4).

After infection of wild-type mice with viruses such as LCMV, virus-specific CTL_p reach peak frequencies 7 to 9 d post-infection which decline sharply after virus clearance, but thereafter are maintained at fairly stable levels for the lifetime of the mouse in the absence of viral antigen (57, 58). Although the primary CTL response mounted by virus-infected CD40L-deficient mice was indistinguishable from that made by wild-type animals (Table 1) there was a defect in the induction and/or maintenance of CTL memory in CD40L-deficient mice, as the level of virus-specific memory CTL activity 2 mo after infection with LCMV was much lower in these mice than that in wild-type animals. Although CD8⁺ LCMV-specific primary CTL can be induced in the absence of CD4⁺ T cells, LCMV-specific CD8⁺ memory CTL are not generated/maintained efficiently in CD4-deficient mice (59–61). In the light of our recent observation that expression of CD40L is required for

Table 4. Memory CTL Activity in CD40L-deficient and Wild-type Mice 2 mo after Infection with LCMV, as Assessed by an *In Vivo* Adoptive Transfer Experiment*

Recipient Animal	LCMV titre (PFU/ml) in recipient sera		
	Before adoptive transfer	D14 after adoptive transfer	
Recipients given splenocytes from LCMV-primed wild-type donors	W1	5 × 10 ⁴	Mouse died
	W2	1 × 10 ⁴	<100
	W3	1.4 × 10 ⁴	Mouse died
	W4	4 × 10 ³	Mouse died
	W5	4.8 × 10 ³	Mouse died
	W6	2.2 × 10 ⁴	<100
Recipients given splenocytes from LCMV-primed CD40L-deficient donors	D1	8 × 10 ⁴	1.2 × 10 ⁵
	D2	5 × 10 ⁴	6 × 10 ⁴
	D3	6 × 10 ⁴	3.6 × 10 ⁴
	D4	4 × 10 ⁴	8.5 × 10 ³
	D5	4 × 10 ⁴	8 × 10 ⁴
	D6	2.4 × 10 ⁴	2.8 × 10 ⁴
	D7	5 × 10 ⁴	4.2 × 10 ⁴

*Erythrocyte-depleted splenocyte suspensions from CD40L-deficient and wild-type mice infected 2 mo previously with LCMV were adoptively transferred (4 × 10⁷ cells per recipient) into 2–4-mo-old lightly irradiated (300 rads) syngeneic (C57BL/6) wild-type mice in which a persistent infection with LCMV had been induced by inoculation as neonates with 10³ PFU of LCMV Armstrong ic, to determine whether the donor cells exhibited sufficient LCMV-specific memory CTL activity to mediate virus clearance from the recipient animals. Recipients W1 to W6 received cells from wild-type donors, and recipients D1 to D7 received cells from CD40L-deficient donors. The results shown are the LCMV titres (PFU/ml, measured by plaque assay on Vero cells) in the serum of individual recipient mice just before the adoptive transfer was performed, and on day 14 (D14) after the adoptive transfer. <100 indicates that no infectious virus was detected in a particular serum sample in the plaque assay, the level of sensitivity of which was 100 PFU/ml. Mouse died indicates that the adoptively transferred cells caused the death of the recipient animal before day 14 post-transfer.

in vivo priming of CD4⁺ T cells (15), one factor that may have contributed to the defect in memory CTL activity observed after virus infection of CD40L-deficient mice may have been a deficit in virus-specific CD4⁺ T cell help.

Alternatively or in addition, our results may provide a rationale for the observation that CD40L is expressed (at low levels) on CD8⁺ T cells after activation with anti-CD3 (1, 6) or PMA/ionomycin (3, 4, 7). It is tempting to speculate that CD40L expression may be induced on antigen-specific CD8⁺ T cells activated after virus infection in vivo, and that a signal delivered via this molecule (possibly after interaction with CD40 expressed on interdigitating cells in the T cell zones of secondary lymphoid organs [8]) to a subset of these cells (perhaps those expressing the highest levels of CD40L; or cells that had not migrated into the periphery but still remained in the vicinity of CD40-expressing interdigitating cells) may be involved in the process by which these cells are selected to become memory CTL. Further if, as suggested by recent results of Tough et al. (Tough, D.F., P. Borrow, and J. Sprent, manuscript submitted for publication), CD8⁺ T cell memory is maintained in part through bystander proliferation of memory CTL_p in response to type I interferon production each time an unrelated virus infection occurs, a CD40L-mediated interaction may also be involved in the bystander response of memory CD8⁺ T cells, and thus play a critical role in the maintenance of CTL memory to viruses.

Altogether, these studies of the response of CD40L-deficient mice to virus infection have confirmed the key role of CD40L-mediated interactions in thymus-dependent humoral immunity. In the absence of CD40L, although some virus-specific IgM and IgG2a production was stimulated by a thymus-independent mechanism, only a low-titre antiviral antibody response that decreased over time was achieved, illustrating the necessity for CD40L-mediated interactions for CD4⁺ T cell-assisted B cell activation and isotype switching, germinal center formation, production of long-lived ASC and the establishment of B cell memory to occur. In addition, they have revealed that although activation of the primary virus-specific CD8⁺ CTL response is not dependent on CD40L-mediated interactions, such interactions are involved in the establishment and/or maintenance of CTL memory after virus infection. This observation may provide a possible rationale for the expression of CD40L on activated CD8⁺ T cells, and illustrates that CD40L-mediated interactions play a more ubiquitous role in the immune system than was previously apparent. A better understanding of the involvement of CD40L-mediated interactions in antiviral immunity, particularly in the maintenance of immune memory following virus infection, will facilitate the future design of vaccination strategies for viral infections, and may also have important implications for the prevention of immunopathological responses associated with virus infections.

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