CD40-CD40 Ligand Interactions Are Critical in T-B Cooperation but Not for Other Anti-viral CD4$^+$ T Cell Functions

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Summary

CD40-CD40 ligand (CD40L) interaction is required for the generation of antibody responses to T-dependent antigens as well as for the development of germinal centers and memory B cells. The role of the CD40-CD40L interaction in the induction of antigen-specific Th cells and in mediating Th cell effector functions other than cognate help for B cells is less well understood. Using CD40- and CD40L-deficient mice together with lymphocytic choriomeningitis virus and vesicular stomatitis virus as viral model antigens, this study corroborates earlier findings that no Ig isotype switching of virus-specific antibodies was measurable upon infection of CD40- or CD40L-deficient mice. In contrast, in vivo induction of virus-specific CD4$^+$ T cells measured by proliferation and cytokine secretion of primed virus-specific Th cells in vitro was not crucially dependent on the CD40-CD40L interaction. In addition, virus-specific Th cells primed in a CD40-deficient environment, adoptively transferred into CD40-competent recipients, were able to mediate Ig isotype switch. Th-mediated effector functions distinct from and in addition to T-B collaboration were analyzed in CD40- and CD40L-deficient and normal mice: (*a*) local inflammatory reactions upon LCMV infection mediated by LCMV-specific Th cells were not dependent on a functional CD40-CD40L interaction, (*b*) cytokine-mediated protection by CD4$^+$ T cells primed by vesicular stomatitis virus against a challenge infection with recombinant vaccinia virus expressing the glycoprotein of vesicular stomatitis virus was found to be equivalent in CD40L-deficient and normal mice.

Thus, CD40-CD40L interaction plays a crucial role in T-B interactions for Th-dependent activation of B cells but not, or to a much lesser extent, in T cell activation, antigen-specific Th cell responses in vitro, and for interleukin-mediated Th cell effector functions in vivo.

It has been established both in vitro and in vivo that the interaction between CD40 on B cells and its ligand CD40L, which is expressed on activated Th cells, is required for Ig isotype switching, Ig-production and the formation of germinal centers (1–8). In vivo, administration of anti-CD40L antibody or soluble CD40L (9, 10) or CD40-Ig fusion protein (1) as well as studies in CD40- or CD40L-deficient mice (11–13) have shown that the generation of primary and secondary humoral immune responses and the formation of germinal centers to a variety of thymus-dependent antigens were abrogated. Furthermore, patients with hyper-IgM (HIGM)[1] syndrome, a genetic disorder due to mutations in the CD40L gene, exhibit an inability to respond to thymus-dependent antigens and have secondary lymphoid organs which are devoid of germinal centers. Nevertheless, patients suffering from HIGM

[1] *Abbreviations used in this paper:* DNP, dinitrophenol; HEL, hen egg lysozyme; HIGM, hyper-IgM syndrome; KLH, keyhole limpet hemocyanine; LCMV, lymphocytic choriomeningitis virus; LCMV-DNP, DNP covalently coupled to LCMV; OVA, ovalbumin; VaccG_{IND}, recombinant vaccinia virus expressing VSV glycoprotein; VSV, vesicular stomatitis virus.

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have normal T cell numbers and are generally not more susceptible to viral infections than healthy individuals (6, 14–18). This may be due to the presence of IgM antibodies and/or CD40L-independent T cell functions.

Since CD40 is not expressed solely on B cells but also on dendritic cells, follicular dendritic cells, monocytes, hematopoietic progenitor cells, and epithelial cells (19–23) CD40-CD40L interaction might be important for the induction and/or effector phase of Th cells in general. This question was adressed in this study. We confirmed here in two infectious virus disease models that a functional CD40-CD40L interaction is not required for T help-independent anti-viral IgM responses but is strictly required for T-dependent Ig class switching of virus-specific B cells. This study additionally investigated the importance of the CD40-CD40L interaction for the induction of Th cell responses and for Th cell effector functions other than cognate T help for B cells.

Using either CD40- or CD40L-deficient mice, Th cell-mediated antiviral immune responses upon infection with lymphocytic choriomeningitis virus (LCMV) or vesicular stomatitis virus (VSV) were investigated. We found that activation and proliferation of virus-specific Th cells did not require a functional CD40-CD40L interaction. Effector functions of Th cells were characterized by (*a*) the analysis of antiviral humoral immune responses, (*b*) Th cell-mediated inflammatory reaction upon LCMV infection, and (*c*) Th cell–mediated antiviral protection against vaccinia virus. Whereas IgM to IgG switch was completely abrogated in both CD40- and CD40L-deficient mice, the two latter effector functions were comparable in CD40-deficient, CD40L-deficient, and normal mice, demonstrating a dichotomy in the role of CD40-CD40L interaction in Th cell effector functions.

Materials and Methods

Mice. Inbred C57BL/6 (H-2^b) mice were obtained from the breeding colony of the Institut für Zuchthygiene (Tierspital Zürich, Switzerland). The generation of mice deficient for CD40, CD40L, or IgM expression has been described previously (11, 12, 24). Mice were bred in a conventional mouse house facility.

Viruses. The LCMV isolate WE was originally provided by Dr. F. Lehmann-Grube, Hamburg, Germany and grown on L929 cells (ATCC CRL 1; American Type Culture Collection [ATCC], Rockville, MD) with a low multiplicity of infection.

VSV Indiana (Mudd-Summers isolate) seeds, originally obtained from D. Kolakofsky (University of Geneva), were grown on BHK-21 (CRL 8544; ATCC) cells infected at low multiplicity and plaqued on Vero cells.

Vaccinia virus expressing the glycoprotein of VSV was a generous gift of Dr. B. Moss (Laboratory of Viral Diseases, National Institutes of Health, Bethesda, MD) (25). Recombinant viruses were grown at low multiplicity of infection on BSC cells and plaqued on BSC cells.

The recombinant baculovirus expressing the LCMV nucleoprotein has been previously described (26). The recombinant baculovirus was derived from nuclear polyhedrosis virus and was grown at 28°C in *Spodoptera frugiperda* cells in spinner cultures in TC-100 medium. Recombinant proteins were produced as previously described (27).

T Cell Proliferation. Mice were immunized intravenously with 200 PFU LCMV-WE. 14 d later, $CD4^+$ T cells were purified from spleen cell suspensions by MACS-sorting according to the protocol of the supplier (Miltenyi Biotec, Bergisch Gladbach, Germany). 1×10^5 $CD4^+$ T cells were incubated in 96-wells with threefold serial dilutions of either purified, UV-inactivated LCMV (concentrations shown for CD40L−/−, CD40−/−, IgM−/− and C57BL/6 ranged between 0.1 μg/ml and 1 μg/ml), P13 (concentrations shown for CD40L−/−, CD40−/−, IgM−/−, and C57BL/6 ranged between 0.1 μg/ml and 1 μg/ml) or medium only in the presence of 7×10^5 irradiated (2000cGy) C57BL/6 spleen cells for 3 d. Proliferation was assessed by incorporation of [^{3}H]thymidine (25 μCi/well). P13 represents an I-A^b-restricted T cell epitope of the glycoprotein of LCMV which has been described elsewhere (28). We have previously shown that P13 is presented in vivo during a primary immune response against LCMV and that P13-specific $CD4^+$ T cells are induced in vivo (28).

Cytokine Analysis. Supernatants of proliferation assays as described above were analyzed for IL-2 content (24 h after restimulation), IFNγ content (60 h after restimulation), and IL-4 content (60 h after restimulation). IL-2 was determined using the IL-2-dependent cell line CTLL-2. Quantification of viable cells was performed by AlamarBlue™ color reaction (Biosource, International, Camarillo, CA) and measured by fluorescence emission at 590 nm using the CytoFluor™ 2350 (Millipore Corp., Bedford, MA) fluorimeter. IFNγ and IL-4 were assessed by ELISA as described (29, 30).

Adoptive Transfer of Primed Th Cells Followed by Challenge with DNP-modified LCMV. CD40-deficient and CD40-competent mice as well as IgM-deficient mice were immunized with 200 PFU of LCMV-WE into both hind foot pads. 18 d later, single cell suspensions were prepared from spleens. 6×10^7 primed spleen cells or an equivalent number of naive cells were transferred intravenously into naive, sex-matched C57BL/6 recipients. Donor cells were pooled from two to three individuals. A few hours later mice were challenged intravenously with 0.4 μg of purified LCMV to which DNP has been covalently coupled (LCMV-DNP) (31). Mice were bled 9 d later and DNP-specific IgG titers were determined by ELISA. The optimal dose for the challenge immunization with LCMV-DNP was determined by in vivo titration of the LCMV-DNP stock in naive versus LCMV-primed mice. The optimal dose was chosen such that only LCMV-primed mice but not naive mice gave rise to a DNP-specific IgG titer.

In addition, the same adoptive transfer experiment was performed using purified, LCMV-primed $CD4^+$ T cells for transfer. CD40-deficient and heterozygous littermates were immunized with 200 PFU LCMV i.v. 14 d later, $CD4^+$ T cells were purified from spleen cell suspensions by MACS-sorting according to the protocol of the supplier (Miltenyi Biotec) and were used at a purity of at least 95% as checked by FACS analysis. 6×10^6 purified T cells were adoptively transferred into naive C57BL/6 recipients and challenged with 0.4 μg LCMV-DNP. Whole spleen cell transfer from LCMV-primed CD40-deficient mice served as positive control. Mice were bled 10 d later and DNP-specific IgG titers were determined by ELISA.

ELISA. The LCMV nucleoprotein-specific ELISA has been described previously (26). 96-well plates (Petra Plastik, Chur, Switzerland) were incubated with LCMV nucleoprotein (0.01

μg/well) in 0.1 M NaH_2PO_4, pH 9.4, at 4°C. Plates were then preincubated with 2% bovine serum albumin in phosphate-buffered saline for 2 h and washed, and serial dilutions of serum samples (30-fold prediluted) were added to the wells and incubated for 90 min. Plates were washed and incubated with horseradish peroxidase-labeled goat anti-mouse IgG (Sigma Chem. Co., St. Louis, MO). After 90 min, plates were washed and developed with ABTS (5 mg of 2,2'-azino-di-3-ethyl-benzthiazolinsulfonate and 20 μl of H_2O_2 in 50 ml of $NaHCO_3$ [pH 4]). Optical densities were determined at 405 nm.

ELISA-measurement of DNP-specific IgG titers was performed similarly. ELISA plates were coated with 0.25 μg/well of Ovalbumin covalently coupled to DNP (31, 32).

VSV-specific Serum Neutralization Test. Neutralizing titers of sera were determined as described (33). Sera were prediluted 40-fold in supplemented MEM and heat-inactivated for 30 min at 56°C. Serial twofold dilutions were mixed with equal volumes of virus diluted to contain 500 PFU/ml. The mixture was incubated for 90 min at 37°C in an atmosphere containing 5% CO_2. 100 μl of the serum-virus mixture were transferred onto Vero cell monolayers in 96-well plates and incubated for 1 h at 37°C. The monolayers were then overlaid with 100 μl DMEM containing 1% methyl cellulose. After incubation for 24 h at 37°C the overlay was removed and the monolayer was fixed and stained with 0.5% crystal violet. The highest dilution of the serum that reduced the number of plaques by 50% was taken as the neutralizing titer. To determine IgG titers, undiluted serum was first pretreated with an equal volume of 0.1 M 2-ME in saline (34).

Assessment of Foot Pad Swelling. Mice were inoculated with 30 PFU of LCMV-WE into both hind foot pads and foot pad swelling was assessed daily with a spring-loaded caliper (35). In vivo anti-CD4 treatment was performed at day 6 and 8 after inoculation using the monoclonal antibody YTS 191.1 (36).

Protection of Mice from Replication of Recombinant Vaccinia Virus. Mice were immunized with 2×10^6 PFU VSV and challenged i.p. 8 d later with 5×10^6 PFU recombinant vaccinia virus expressing VSV-glycoprotein ($VaccG_{IND}$). Vaccinia titers in ovaries were determined 5 d later as described previously (37). Titers are shown as log10 PFU per animal.

Results

Induction of Virus-specific Th Cells. In some experimental systems B cells have been shown to be crucial for efficient T helper cell induction (38–44). In addition, CD40 is not solely expressed on B cells but also on dendritic cells and follicular dendritic cells (6). We addressed therefore the question whether CD40-CD40L interaction or the presence of B cells serving as antigen presenting cells is required for the induction of virus-specific Th cells in vivo. We compared the induction of LCMV-specific Th cells in vivo in CD40-deficient, CD40L-deficient, normal C57BL/6, and IgM-deficient mice. Since it can be expected that B cells do not serve efficiently as APCs in the absence of proper activation and proliferation induced by the CD40-CD40L interaction, IgM-deficient mice that do not have peripheral B cells (24) were included in this comparison. This made possible the distinction between Th cell induction without the CD40-CD40L interaction and Th cell induction without B cells serving as antigen-presenting cells.

Induction of LCMV- or peptide P13-specific Th cells in vivo was comparable in CD40L-, CD40,- and B cell-deficient mice and in normal C57BL/6 mice as defined by specific proliferation in response to both antigens (Fig. 1). These results demonstrate that LCMV-specific Th cells are induced in vivo independent of a functional CD40-CD40L interaction. Since induction of LCMV-specific Th cells in CD40-deficient as well as in CD40L-deficient mice and B cell-deficient mice was similar, Th cells can apparently be stimulated in vivo without crucial involvement of B cells or of a functional CD40-CD40L-interaction after LCMV infection.

In addition, cytokine secretion patterns were analyzed in the supernatants recovered from the proliferation assays performed with LCMV-primed $CD4^+$ T cells from CD40-deficient, CD40L-deficient, or control C57BL/6 mice (Fig. 2). IL-2 and IFNγ were secreted in comparable amounts by $CD4^+$ T cells originating from CD40-deficient, CD40L-deficient, or control mice whereas no IL-4 could be detected in the same supernatants (Fig. 2). Thus, the Th1 cytokine secretion pattern which is normally ob-

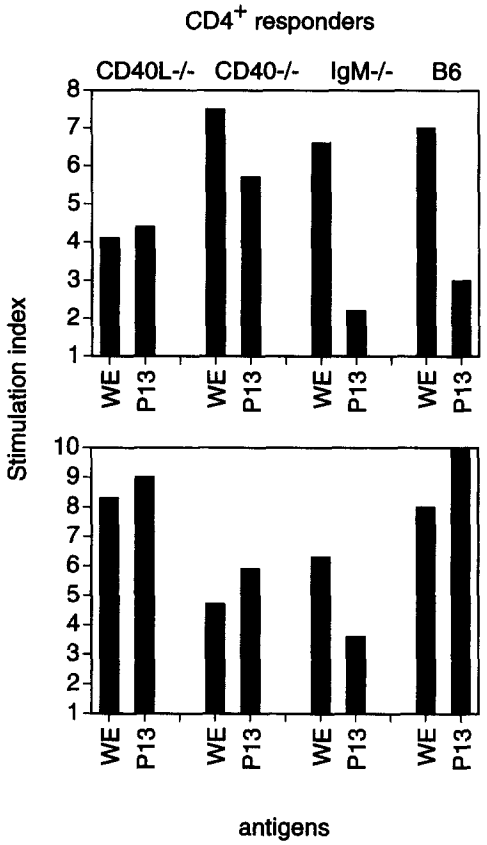

Figure 1. LCMV-specific proliferation of purified Th cells. CD40L-deficient, CD40-deficient, IgM-deficient, and C57BL/6 mice were immunized with LCMV and 14 d later LCMV-specific proliferation of purified $CD4^+$ T cells was determined using either UV-inactivated LCMV (*WE*) or peptide 13 (*P13*) as LCMV-specific antigens (concentrations of stimulating antigens are described in Materials and Methods). P13 is a LCMV-GP-derived peptide recognized by $CD4^+$ T cells. Stimulation indices were calculated in relation to proliferation in medium control. Background cpm counts in medium control were always ~2,000 cpm. Two of four equivalent experiments are shown.

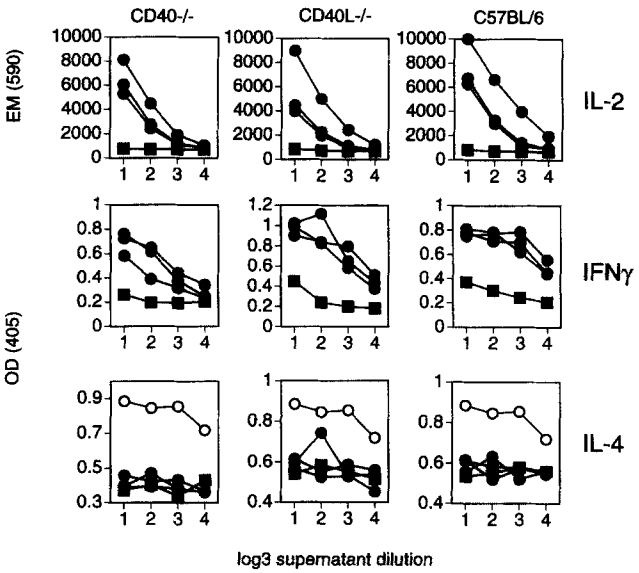

Figure 2. LCMV-specific cytokine secretion. In vivo LCMV-primed CD40-, CD40L-deficient or control $CD4^+$ T cells were restimulated in vitro with the LCMV-glycoprotein-derived $I\text{-}A^b$-binding peptide P13 (*circles*) or without antigen (medium control (*squares*). IL-2 content in the supernatants (5,000 EM U correspond to 30 U IL-2/ml) was analyzed 24 h after restimulation (*top*) whereas IFNγ (OD = 0.5 corresponds to 500 U IFNγ/ml) and IL-4 levels (OD = 0.5 corresponds to 25 U IL-4/ml) were assessed 60 h after restimulation (*middle* and *bottom*). Recombinant IL-4 (*open circles*) served as positive control in the IL-4 ELISA. Each line represents a separate proliferation assay from an individual mouse. One of three equivalent experiments is shown.

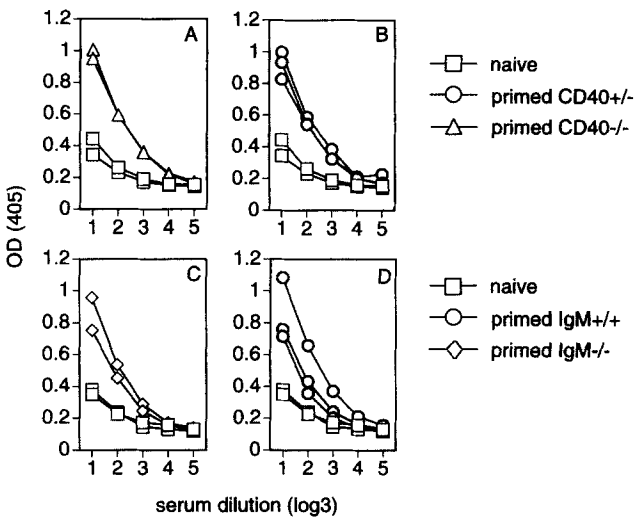

Figure 3. Cognate help to CD40-expressing B cells after adoptive transfer of LCMV-primed Th cells. CD40-deficient mice (*triangles*) and heterozygous littermates (*circles*) as well as IgM-deficient mice (*diamonds*) and control mice (*circles*) were immunized with LCMV. 20 d after infection spleen cells were adoptively transferred into CD40 competent naive recipients and the recipients as well as naive CD40 competent control animals (*squares*) were challenged with LCMV-DNP. After 9 d, DNP-specific IgG titers were determined by ELISA from 30-fold prediluted sera. Each line represents one individual mouse. One of three similar experiments is shown. Transfer of naive $CD4^+$ cells from $CD40^{-/-}$ donors did not result in the generation of significant DNP-specific IgG antibodies (data not shown).

served for LCMV-specific Th cells is not influenced by the absence of functional CD40-CD40L interactions.

In a second experimental approach, induction of LCMV-specific Th cells was assessed in CD40-deficient mice and in heterozygous littermates (both being competent for CD40L on Th cells) by measuring T helper cell function upon transfer of primed Th cells into CD40-competent naive recipients. Thus, CD40L competent cells were primed in a CD40-deficient environment. Adoptive transfer of CD40L-positive, LCMV-primed Th cells into naive recipients with normal $CD40^+$ B cells restores functional T-B cooperation. The conditions for challenging the recipient mice were chosen such that only Th cells already primed in the CD40-deficient donors were able to induce a significant isotype switch in the recipient. To assess the importance of B cells for the induction of Th cells, B cell-deficient mice were included in the experiment. CD40-deficient, B cell-deficient and control mice were infected with LCMV and 18 d after infection spleen cells were transferred into naive recipients. This was followed by a challenge immunization with LCMV-DNP. DNP-specific IgG antibodies were only significantly induced after challenge if LCMV-specific Th cells had been induced in the donor animals before transfer (Fig. 3). Since B cells in the recipients express CD40, equivalent DNP-specific IgG titers in recipients that received primed Th cells from immunized CD40-deficient mice or from heterozygous littermates revealed that induction of LCMV-specific Th cells was not dependent on a functional CD40-CD40L interaction in vivo (Fig. 3, *A* and *B*). Similarly, equivalent IgG titers in recipients that received Th cells from LCMV-immunized B cell-deficient mice or B cell-competent C57BL/6 mice indicated that induction of LCMV-specific Th cells was not dependent on the presence of B cells serving as antigen presenting cells (Fig. 3, *C* and *D*).

To confirm that the transferred $CD4^+$ T cells were alone responsible for the enhanced DNP-specific IgG titers, purified $CD4^+$ T cells from LCMV-primed CD40-deficient mice or heterozygous littermates were transferred into naive C57BL/6 recipients which were challenged by injection of LCMV-DNP. DNP-specific IgG titers were determined 10 d later (Fig. 4, *A* and *B*). In addition, transfer of unseparated spleen cells from LCMV-primed CD40-deficient donors into naive C57BL/6 recipients served as positive control (Fig. 4 *C*), whereas unmanipulated, unprimed C57BL/6 mice served as negative control. Both $CD4^+$ T cells originating from either LCMV-primed CD40-deficient mice or heterozygous littermates were able to comparably provide help for CD40-competent B cells (Fig. 4, *A* and *B*), albeit whole spleen cell transfers resulted in a more pronounced enhancement of DNP-specific IgG titers as compared to purified $CD4^+$ T cells.

Thus, three independent assays showed that the induction of LCMV-specific Th cell responses was not limited by CD40-CD40L interactions and was independent of B cells acting as critical APCs.

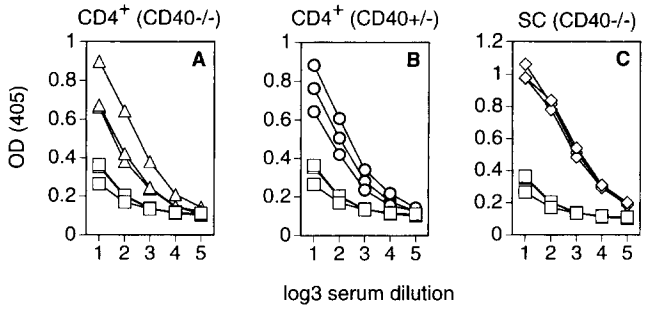

Figure 4. Cognate help to CD40-expressing B cells after adoptive transfer of purified, LCMV-primed $CD4^+$ T cells. CD40-deficient mice (*triangles*) and heterozygous littermates (*circles*) were immunized with 200 PFU LCMV i.v. 14 d later $CD4^+$ T cells were purified and adoptively transferred into CD40-competent, naive recipients (*A* and *B*). In addition, spleen cells from LCMV-primed CD40-deficient donors (*diamonds*) were transferred into CD40-competent naive recipients (*C*). All recipients as well as naive control animals (*squares*) were challenged with LCMV-DNP and 10 d later DNP-specific IgG titers were determined in 90-fold prediluted sera. Each line represents an individual recipient. One of two similar experiments is shown.

Th Cell Effector Function: Cognate Help to B Cells. It has already been demonstrared in detail that T-dependent IgG humoral immune responses are strictly dependent on a functional CD40-CD40L interaction and that a missing ligand in this complex totally abrogates germinal center formation as well as immunoglobulin isotype switch and generation of memory B cells (1–10). To ensure that Ig responses to LCMV as a model viral antigen require CD40-CD40L engagement, we infected CD40-, CD40L-deficient, or C57BL/6 mice with LCMV and then measured antiviral primary antibody responses. VSV was used as a second viral model antigen.

Infection of normal mice with LCMV leads to a pronounced IgG response specific for the nucleoprotein of LCMV (LCMV-NP) (Fig. 5). In contrast, no LCMV-NP-specific IgG titers were obtained using CD40L- or CD40-deficient mice (Fig. 5). Thus, CD40-CD40L interaction is crucial for isotype class switching in response to LCMV-infection.

In addition, we analyzed virus neutralizing antibody responses in CD40L-deficient and normal mice after infection with VSV. VSV infection of mice normally induces a type I T-independent neutralizing IgM-response followed by a T-dependent neutralizing IgG response (45–47). As expected control C57BL/6 mice mounted high neutralizing IgM and IgG responses (Fig. 6). In contrast, CD40L-deficient mice mounted a neutralizing T-independent IgM response but no VSV-specific IgG was detected upon VSV infection (Fig. 6). The same results were obtained using CD40-deficient mice (data not shown). These data corroborate previous findings in nonviral systems, demonstrating that cognate T-B cooperation leading to Ig class switching is critically dependent on functional CD40-CD40L interactions.

Th Cell Effector Function: Inflammatory Reaction. Infection of normal mice with LCMV in the hind footpads induces a local inflammatory reaction consisting of two sequential swelling phases. A pronounced swelling reaction mediated by $CD8^+$ T cells is observed 7–10 d after infection; this is followed by a $CD4^+$ T cell–mediated swelling phase declining around day 14–16 after infection (46, 48). The second phase of the swelling reaction exhibits no sharp peak but has the shape of a shoulder. We analyzed whether or not the second swelling phase mediated by LCMV-specific $CD4^+$ T cells was dependent on CD40-CD40L interactions. CD40-deficient mice as well as heterozygous littermates were infected with LCMV in both hind footpads and the subsequent swelling reaction was monitored daily (Fig. 7 *A*). In fact, there was a tendency noted that CD40-defi-

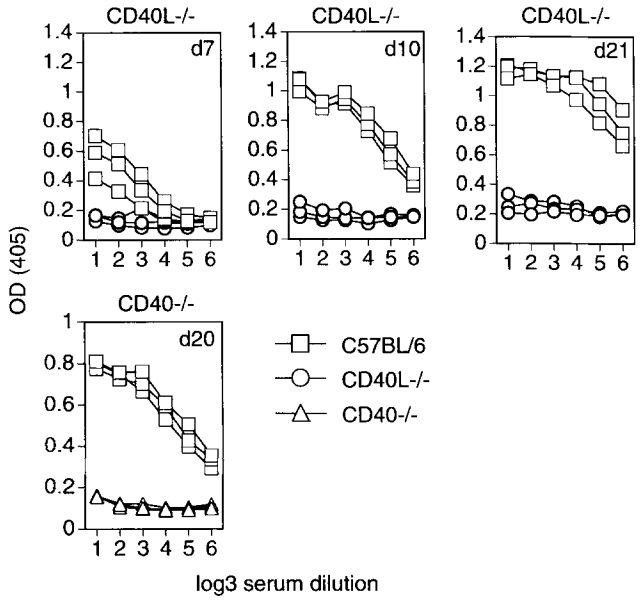

Figure 5. LCMV-NP specific IgG antibody response. CD40L-deficient (*circles*), CD40-deficient (*triangles*) and normal C56BL/6 (*squares*) mice were immunized with LCMV. LCMV-NP-specific IgG titers were determined 7, 10, and 21 d after infection for CD40L-deficient and normal C56BL/6 mice. LCMV-NP specific IgG titers in CD40-deficient and normal C57BL/6 mice were assessed 20 d after LCMV infection in 30-fold prediluted sera. Each line represents one individual mouse. Variations were smaller than two dilution steps. One of three comparable experiments is shown.

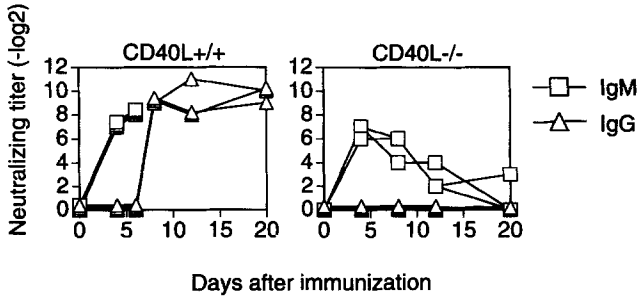

Figure 6. VSV neutralizing antibody response. CD40L-deficient and C57BL/6 mice were immunized with VSV. VSV-neutralizing IgM titers (*squares*) and VSV-neutralizing IgG titers (*triangles*) were determined from 40-fold prediluted sera 4, 6, 8, 12, and 20 d after infection. Each line represents one individual mouse. One of three comparable experiments is shown.

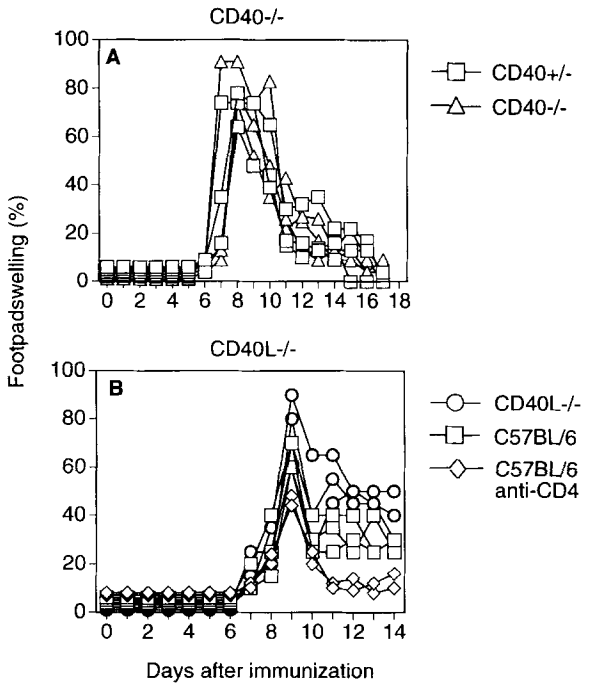

Figure 7. LCMV-specific foot pad swelling reaction. In *A* CD40-deficient mice (*triangles*) and heterozygous littermates (squares) were immunized into the footpads with 200 PFU of LCMV and the local LCMV-specific foot pad swelling reaction was daily monitored. In *B* LCMV-specific foot pad swelling reaction was similarly analyzed in CD40L-deficient (*circles*) and normal C57BL/6 mice (*squares*) as well as in anti-CD4 treated C57BL/6 mice (*diamonds*). Each line represents one individual mouse.

cient mice exhibited a slightly increased maximal early CD8-dependent peak response and that subsequently the CD4-dependent shoulder tended to be slightly higher. Thus, neither the $CD8^+$ T cell–mediated swelling phase nor in the $CD4^+$ T cell–mediated swelling phase was dependent upon a functional CD40 molecule. Compatible results were obtained with CD40L-deficient mice (Fig. 7 *B*). This indicates comparable abilities to mount LCMV-specific inflammatory reactions in the presence or absence of CD40-CD40L interaction. To confirm that the second phase of the swelling reaction was actually $CD4^+$ T cell mediated, C57BL/6 mice were treated with a $CD4^+$ T cell-depleting monoclonal antibody at day 6 and 8 after inoculation with LCMV. Foot pad swelling was less marked and the subsequent decline was much more rapid compared to untreated controls (Fig. 7 *B*).

Th Cell Effector Function: Antiviral Protection. A third Th cell effector function was analyzed in CD40L-deficient and normal mice. VSV primed C57BL/6 mice have been shown to rapidly control replication of a recombinant vaccinia virus expressing the glycoprotein of VSV ($VaccG_{IND}$). This antiviral protective mechanism is virtually exclusively mediated by IFNγ and TNFα which are secreted by VSV glycoprotein-specific $CD4^+$ T cells (37, 49). Neither $CD8^+$ T cells nor VSV-neutralizing antibodies have been shown to be protective in this infection. Because G_{IND} is not expressed in the $VaccG_{IND}$ envelope, VSV-specific antibodies cannot bind or neutralize $VaccG_{IND}$ recombinant virus. CD40L-deficient and normal mice were infected with VSV and 8 d later the VSV primed mice as well as naive control animals were challenged intraperitoneally by infection with $VaccG_{IND}$. Vaccinia titers in the ovaries were determined 5 d after challenge (Fig. 8). As with the foot pad swelling results (Fig. 7 *B*), we did not observe a reduced $CD4^+$ T cell mediated antiviral effector function in CD40L-deficient as compared to CD40L-competent mice.

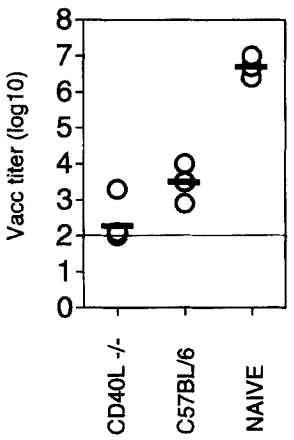

Figure 8. $CD4^+$ T cell dependent protection against challenge infection with recombinant vaccinia virus. CD40L-deficient and normal C57/BL6 mice were immunized with 2×10^6 PFU of VSV and 8 d later these primed mice as well as naive control mice were challenged i.p. with 5×10^6 PFU $VaccG_{IND}$. Vaccinia titers in ovaries were determined 5 d after challenge infection. One of two experiment is shown.

Discussion

Many reports have focussed on the key importance of the CD40-CD40L interaction for the generation of humoral immune responses (1–6). This was confirmed here for antiviral IgG immune responses. In contrast to most previous reports, this study examined in addition the role of CD40-CD40L interaction on Th cell activation, proliferation and effector functions. Surprisingly, neither Th cell induction nor Th cell effector functions other than cognate help for B cells were compromised by the lack of the CD40- or the CD40L molecules.

CD40 is expressed on different APCs such as dendritic cells and B cells and macrophages (19–23). In addition, it has been shown that CD40-CD40L interaction induces upregulation of MHC class II, B7 and ICAM-1 on dendritic cell lines which potentiate the stimulatory capacity of the APC (50). The CD40-CD40L interaction-dependent upregulation of these and maybe other accessory molecules on the surface of the APC signals then back to the T cell, a process which may be supported by interleukins in addition. Thus, it could be expected that activation and proliferation of specific Th cells would be impaired in the absence of the CD40-CD40L interaction as in fact has been recently reported (51, 52). However, we report here that antigen-specific Th cells are induced normally in vivo in the absence of a functional CD40-CD40L interaction after two different virus infections; hence the formation of this receptor-ligand pair is apparently not critical for the activation of naive virus-specific Th cells. Since B cell activation and proliferation seems to depend critically upon the presence of cognate T-help (5, 7, 53, 54), absence of CD40-CD40L interaction will interfere with proliferation of spe-

cific B cells and therefore reduce class II associated antigen presentation by specific B cells. Because it has been reported that antigen presentation by B cells is important for the normal induction of T-helper cells in several systems (38–44), our results suggest that either B cell proliferation and activation is impaired to a smaller degree than expected in the absence of CD40-CD40L interaction, or, alternatively and more likely, that the presence of specific B cells plays only a minor if any role for the induction of T-helper cells with the infectious virus-derived antigens studied here (55). This latter possibility is supported by the fact, that Th cells primed in the absence of B cells proliferated normally and could mediate isotype switch after adoptive transfer (Fig. 3, *C* and *D*). These findings are in contrast to recently presented data (51, 52). Grewal et al. (51) showed a significant impairment of KLH- or HEL-specific T cell priming in mice lacking CD40 ligand. This impairment was evidenced by strongly reduced recall proliferation responses and by a failure of adoptively transferred CD40L-deficient T cells to expand in vivo after antigenic challenge. In contrast to these findings, van Essen et al. observed normal KLH-specific Th cell induction in CD40-deficient mice, but described these KLH-primed T cells as being qualitatively different from T cells primed in a CD40-competent environment; they were apparently not able to provide help to B cells. These differences concerning the importance of a functional CD40-CD40L interaction for the induction and effector functions of T cells between the virus models used in this study and the soluble KLH antigen system used by Grewal et al. (51) and by van Essen et al. (52) probably reflect different critical in vivo mechanisms governing both the induction and effector functions of T cells. There are at least two possible explanations that might account for these differences: (*a*) The efficient activation of T cells after a viral infection such as with LCMV might over-ride some of the more subtle requirements for responses to soluble protein antigens such as KLH. In addition, the failure of CD40-deficient mice to resolve a Leishmania infection (H. Kikutani, personal communication), despite exhibiting a Leishmania-resistant genetic background, could be due to the lack of CD40 signals to macrophages or dendritic cells which then could lead to a deficit in IL-12 production and therefore to an impaired Th1 development. Additional evidence for a requirement of a functional CD40-CD40L interaction for IL-12 induction in vivo and hence the induction of a Th1 response has recently been presented by Stüber et al. (56). Thus, T helper cells may influence their responses via CD40L indirectly by activating their APCs. In a viral infection the direct secretion of IFN and possibly other interleukins by T cells may render this pathway less critical. (*b*) Alternatively, B cells may be of key importance as APCs for T cell induction in a soluble antigen system in contrast to virus model infections. There still exists an unresolved controversy on the issue whether B cells play a central role in the initiation of T cell immune responses. Even studies using the same experimental antigen such as KLH as soluble antigen and B cell-deficient mice revealed different results: Epstein et al. (57) reported successful T cell priming in B cell-deficient mice using KLH as antigen whereas Liu et al. (44) failed to obtain T cell priming in B cell-deficient mice using either KLH or OVA as soluble protein antigens. Constant et al. (58) demonstrated that mice lacking B cells were impaired in their priming of T cells to protein but not to peptide antigens. This dominant role of B cells as APCs found in several experimental systems using soluble antigens is possibly abrogated if the CD40 signaling pathway is not functional. We deliberately chose an experimental system where T cell induction is not dependent on B cells functioning as APCs and analyzed the role of CD40-CD40L interaction in T cell priming and Th cell effector functions independently of the presence or absence of B cells.

The effector functions of Th cells are differentially influenced by CD40-CD40L interaction. Thymus-dependent Ig-isotype switched humoral immune responses are completely abrogated in CD40- or CD40L-deficient mice whereas thymus-independent IgM-responses to VSV are comparable to normal mice; the latter apparently do not require a functional interaction between CD40 and its ligand. Although T-helper cells do not induce antibody isotype switching in B cells in the absence of CD40-CD40L interaction, $CD4^+$ T cells could be functionally primed in CD40-deficient mice since upon transfer they were able to provide help for CD40-competent B cells to undergo isotype switch. These findings are in contrast to the recent data of van Essen et al. (52) showing that KLH-specific T cells primed in a CD40-deficient environment are not able to help CD40-competent B cells. Although the experimental setups are not identical, it can be concluded that Th cell priming by virus infection seems to be considerably more efficient in a CD40-deficient environment than is possible for soluble protein antigens. Not only were induction, proliferation and cytokine production of specific T-helper cells normal in the absence of CD40-CD40L interaction but also effector functions of T-helper cells other than mediating Ig isotype switching were largely normal in the absence of CD40 or CD40L. $CD4^+$ T cell mediated inflammatory reaction upon LCMV infection was not impaired in CD40- or CD40L-deficient mice. In addition, Th cell-dependent antiviral protection was not impaired by the lack of CD40-CD40L interaction: activated VSV-specific Th cells in CD40L-deficient mice were able to inhibit the replication of VSV-G recombinant vaccinia virus by a cytokine mediated effector function; this protective Th cell function has been shown to be mediated by release of IFNγ and TNFα by activated $CD4^+$ Th cells (37). It has recently been suggested, that CD40L may exert a direct protective effect against vaccinia virus (59). Our data show that Th cells can efficiently protect against vaccinia virus replication also in the absence of CD40L, suggesting that this parameter is not limiting under the conditions tested in vivo.

In conclusion, these results reveal a dichotomy in the role of the CD40-CD40L interaction in Th cell effector functions: the functional interaction is of critical importance in T-B cell cooperation for the induction of thymus-dependent humoral immune responses but the same inter-

action is not limiting for Th cell mediated effector functions such as virus-induced inflammatory reactions or antiviral protection, which probably depend on interactions between Th cells with macrophages and/or dendritic cells. Our data strongly suggest that CD40-CD40L interaction divides anti-viral Th cell effector functions in two categories: one category consisting of cognate T help mediating B cell activation followed by Ig class switching which is strictly dependent on a functional CD40-CD40L interaction; and a second category, represented in this report by virus-induced, $CD4^+$ T cell-mediated inflammatory reaction as well as antiviral protection is largely independent of a functional CD40-CD40L interaction. Similarly, activation and proliferation of Th cells, albeit not a Th cell effector function, may be placed in this second category. Thus, CD40-CD40L interaction is critically and probably unidirectionally-needed for B cell activation followed by Ig class switch, but in addition is possibly of importance for macrophage/dendritic cell activation especially in the case of nonviral antigens.

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