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OX40-Deficient Mice Are Defective in Th Cell Proliferation but Are Competent in Generating B Cell and CTL Responses after Virus Infection

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Summary

OX40, a member of the TNF receptor superfamily, is expressed on activated T cells and implicated in stimulation of T cells and T-dependent humoral responses. We generated *OX40*$^{-/-}$ mice and found that the formation of extrafollicular plasma cells, germinal centers, and antibody responses was independent of OX40. After infection with LCMV and influenza virus, *OX40*$^{-/-}$ mice retain primary and memory cytotoxic T cell responses with normal expansion and decline of specific CTL. In contrast, CD4$^+$ T cell proliferation and the number of IFN-γ-producing CD4$^+$ T cells were reduced in *OX40*$^{-/-}$ mice. Moreover, the number of CD4$^+$ T cells infiltrating the lungs of influenza virus–infected *OX40*$^{-/-}$ mice was reduced. These results define a unique role of OX40 in the generation of optimal CD4$^+$ T cell responses in vivo.

Introduction

Interaction between members of the TNF receptor (TNFR) and TNF ligand (TNFL) superfamily are important for lymphocyte activation, growth, differentiation, and death (Gravestein and Borst, 1998; Watts and DeBenedette, 1999). The family is rapidly growing and includes the receptors NGFR, TNFRI, TNFRII, LTβR, CD27, CD30, CD40, CD95, Rank (TRANCE-R), Trail-Rs, 4–1BB, and OX40. In mice, OX40 is expressed on both activated CD4$^+$ and CD8$^+$ T cells, whereas expression in rats seems to be restricted to CD4$^+$ T cells (Al-Shamkhani et al., 1996). The ligand for OX40 has a broader tissue distribution and has been found on activated T cells (Baum et al., 1994), B cells (Calderhead et al., 1993), dendritic cells (Ohshima et al., 1997; Brocker et al., 1999), and vascular endothelial cells (Imura et al., 1996). After immunization, OX40 expression on T cells is restricted to the inner and outer parts of the splenic PALS (T cell zone) and coincides in time and localization with the expression of OX40L on antigen activated B cells (Stuber and Strober, 1996). The importance of OX40–OX40L interaction for B cell responses has been demonstrated by enhanced proliferation and Ig secretion after cross-linking of OX40L on B cells (Stuber et al., 1995) and by a severely impaired IgG response to a T-dependent antigen after in vivo neutralization of OX40 with polyclonal antibodies (Stuber and Strober, 1996).

Activation of resting T cells requires a signal through the TCR provided by peptide/MHC complexes, as well as an additional costimulatory signal. CD28 ligation on T cells via B7–1 and B7–2 on APCs appears to be the predominant costimulatory interaction required for activation and survival of T cells (Lenschow et al., 1996). However, studies in CD28-deficient mice showed that CD28 is dispensable for some types of immune responses including primary CTL and DTH responses after LCMV infection (Shahinian et al., 1993; Kündig et al., 1996), Th2 responses after infection with the nematode parasite *Heligmosomoides polygyrus* (Gause et al., 1997), and rejection of skin allografts (Kawai et al., 1996). On the other hand, a number of other molecules, mainly members of the Ig (e.g., ICAMs and CD2), integrin (e.g., LFA-1), and TNFR/TNF superfamilies (e.g., CD40/CD40L, CD27/CD70, 4–1BB/4–1BBL, and OX40–OX40L) have also been invoked in promoting T cell activation and differentiation (Watts and DeBenedette, 1999). This suggests that multiple interactions are required to generate optimal T cell responses. Some of these accessory molecules may directly deliver signals into the T cell (e.g., CD28), while others assist T cells by activation of APCs (e.g., CD40).

TNF family members are able to trigger a complex signaling cascade in target cells. Based on the outcome of the signal that is transduced via a unique set of adaptor molecules, these receptors can be divided in two groups. Some are prone to mediate apoptotic death of the cell on which they are expressed (e.g., CD95, DR3, Trail Rs, and TNFR-1), while others rather promote survival (e.g., CD40, TNFR-2, and RANK). The majority of death-mediating receptors have a cytoplasmic death domain (DD) that associates with adaptor molecules such as FADD and TRADD directly, which leads to the activation of caspases and cell death. Receptors that do not exhibit death domains may recruit RIP and TRAF family members, which can result in the nuclear translocation of NF-κB and protection from apoptosis. However, TRAF binding may also lead to the activation of the SAPK1/JNK pathway, which can result in death of some cells. This indicates that the final outcome of triggering a member of the TNFR family may depend in a subtle way on the balance between activation of caspases, SAPK1/JNK, and NF-κB (Baker and Reddy, 1996).

A number of in vitro studies suggested that OX40–OX40L provides a costimulatory signal resulting in enhanced T cell proliferation and cytokine production (Weinberg et al., 1998). OX40 and CD28 have been demonstrated to act in synergy for the activation of naive T

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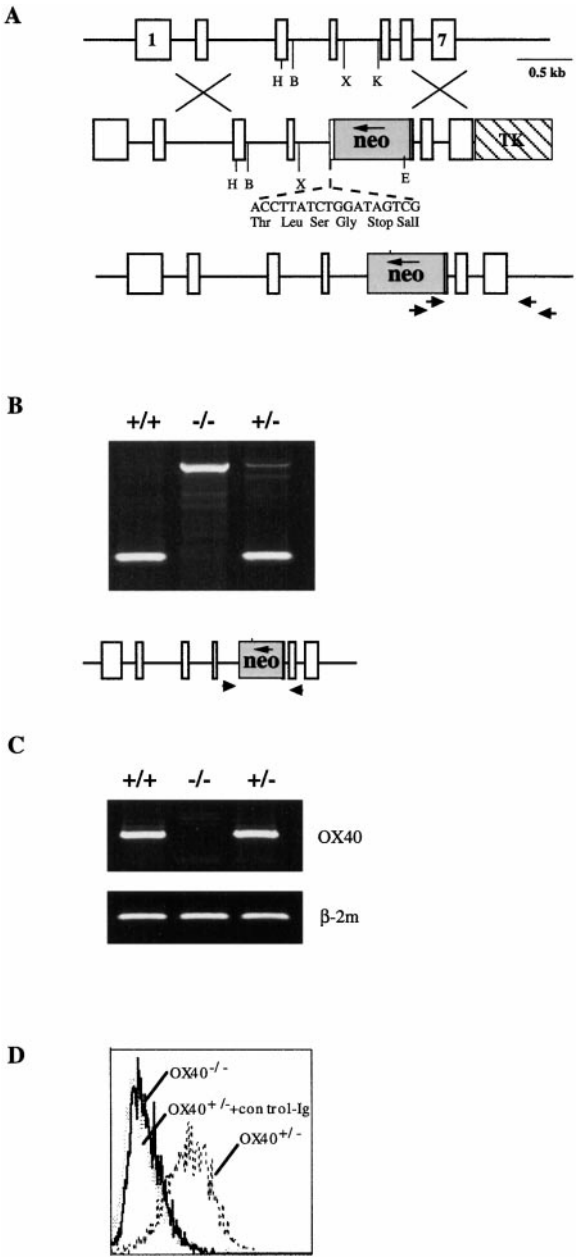

Figure 1. Targeted Disruption of the *OX40* Gene in Mice

(A) Structure of the mouse *OX40* locus (top), the OX40 targeting construct (middle), and the predicted mutated *OX40* gene (bottom). Exons are represented by boxes. The replacement construct contained the *neomycin* (*neo*^{r}) gene replacing part of exon 5 and the *HSV-tk* gene placed 3′ of the homology region allowing positive–negative selection. In exon 5 before the start of the *neo*^{r}, a stop codon was introduced. Large arrow indicates direction of the *neo* gene. Small arrows indicate location of the primers used for nested PCR screening of resistant ES clones.

(B) Tail DNA PCR analysis of wild-type (*OX40*$^{+/+}$), homozygous (*OX40*$^{-/-}$), and heterozygous (*OX40*$^{+/-}$) mutant mice with OX40-specific primers flanking the *neomycin* gene.

(C) RT-PCR analysis of RNA prepared from CD4$^+$ T cells from *OX40*$^{+/+}$, *OX40*$^{-/-}$, and *OX40*$^{+/-}$ mice after stimulation with immobilized anti-CD3 and anti-CD28 for 3 days. cDNA was standardized for the expression of β2-microglobulin before amplification of a fragment from the OX40 transcript using specific primers.

(D) Flow cytometric analysis of OX40 expression of splenocytes from *OX40*$^{+/-}$ and *OX40*$^{-/-}$ mice. Stimulated CD4$^+$ T cells were stained with a rat anti-OX40 mAb or a control Ig followed by a polyclonal goat anti-rat IgG labeled with FITC and anti-CD4 labeled with PE. Shown is fluoresence intensity of OX40 gated on CD4$^+$ T cells. Dead cells were excluded by propidium iodide staining.

cells, whereas OX40 rather than CD28 was required for sustained effector responses (Gramaglia et al., 1998). In addition to this T cell costimulatory function, OX40 also seems to be involved in T cell migration. Specifically, interaction of T cells with OX40 ligand–expressing dendritic cells (DCs) directs them to migrate into B cell follicles (Brocker et al., 1999). Furthermore, in vivo neutralization of OX40 ameliorated the inflammatory diseases EAE and IBD (Weinberg et al., 1996; Higgins et al., 1999), further pointing to a role of OX40 in directing inflammation and T cell migration.

To directly examine the role of OX40 in specific immune responses in vivo, we generated mice deficient for OX40 by targeted gene disruption in embryonic stem cells. Our results demonstrate a nonredundant role of OX40 for the selective augmentation of CD4$^+$ T cell responses, whereas OX40 is dispensable for CTL and humoral responses.

Results

Generation of OX40-Deficient Mice

The *OX40* gene was disrupted by homologous recombination in embryonic stem (ES) cells (E14.1) using the targeting vector illustrated in Figure 1A. Part of exon 5 was replaced with a *neo*^{r} cassette in reverse orientation. Genotypes of progeny from (129Ola × C57BL/6) F_1 matings were determined by PCR with tail DNA and OX40-specific primers flanking the *neo* cassette (Figure 1B). Genotypic frequencies were in the expected Mendelian ratios. The absence of OX40 transcripts was confirmed by RT-PCR with total RNA of purified CD4$^+$ T cells stimulated with anti-CD3 and anti-CD28 for 3 days (Figure 1C). Consistent with this, OX40 protein was undetectable by flow cytomety on the surface of stimulated splenocytes (Figure 1D). To eliminate possible mutations other than OX40 that accumulated in the ES cell line during culture, the mutated *OX40* gene was crossed for two generations to C57BL/6 before heterozygous mice (*OX40*$^{+/-}$) were interbred to obtain *OX40*$^{-/-}$ mice. For all experiments described, we used progeny from *OX40*$^{-/-}$ × *OX40*$^{+/-}$ breedings. *OX40*$^{-/-}$ mice showed normal viability, fertility, growth, anatomy, and lymphocyte subsets in thymus, spleen, lymph nodes, and peyers patches compared with *OX40*$^{+/-}$ littermates (data not shown).

OX40-Deficient Mice Mount Normal Antibody Responses

OX40 has been suggested to be essential for the generation of extrafollicular plasma cells and primary T-dependent antibody responses. To assess the ability of OX40-deficient mice to generate protective antiviral B cell responses, mice were infected with vesicular stomatitis virus (VSV). VSV has been shown to induce an early T cell–independent IgM response followed by a long-lasting, T cell–dependent IgG response (Bachmann and Zinkernagel, 1997), which is essential for the survival of

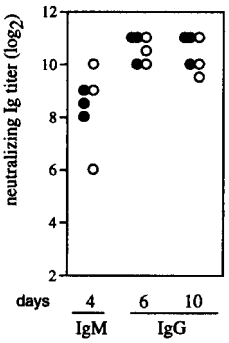

Figure 2. Normal Antibody Response to VSV in *OX40*$^{-/-}$ Mice

Mice were infected intravenously with VSV (Indiana strain, 2×10^6 PFU) and bled at indicated days. Sera were analyzed for neutralizing IgM and IgG antibodies. Neutralizing anti-VSV IgG was absent at day 4 (data not shown). Titers represent 2-fold dilution steps of sera starting with 1:40 dilution. Filled circles (*OX40*$^{+/-}$) and empty circles (*OX40*$^{-/-}$) indicate values from individuals expressed as $\log_2$ of the neutralizing titer.

infected mice (Bachmann et al., 1997). In OX40-deficient mice, both the IgM and the isotype-switched IgG response were comparable to *OX40*$^{+/-}$ control mice (Figure 2). Moreover, all VSV-infected OX40-deficient mice (5/5) survived the infection, indicating that the antibody response was indeed protective.

VSV-specific B cells appear first in the marginal zone and the red pulp with germinal centers developing at around day 6 and persisting for more than 60 days (Bachmann et al., 1996). OX40-deficient mice exhibited normal numbers of VSV-specific plasma cells in the marginal zone and red pulp (Figure 3). Moreover, germinal center formation evaluated by staining germinal center B cells with peanut agglutinin (PNA) and follicular dendritic cells (FDC) with anti-4C11 mAb showed no obvious differences comparing *OX40*$^{-/-}$ and *OX40*$^{+/-}$ controls (Figure 3).

We also immunized mice with haptenated protein (NP-CGG) in alum to study antibody responses against another model antigen. At days 14 after primary and day 7 after secondary immunization, serum levels of NP-specifc IgM, IgG1, and IgG2a and IgG2b antibodies were comparable in OX40-deficient mice and controls (data not shown). Thus, although our data do not exclude a minor contribution of OX40 to B cell responses in other experimental systems, they exclude an essential role for OX40 in such responses.

LCMV-Specific CTL Responses Are Normal in the Absence of OX40

LCMV induces a strong CTL response that peaks around day 8 shortly after peak viral load. Upon elimination of the virus, which occurs usually around day 8–10 after infection, frequencies of specific T cells decline but memory T cells remain detectable for months to years after infection. OX40-deficient and control mice were infected with LCMV (200 pfu), and CTL activity was assessed 8 days later in a ^{51}Cr release assay. Spleen cells from both OX40-deficient and control mice exhibited similar ex vivo lytic activity (Figure 4A). To assess the formation of memory CTLs in the absence of OX40,

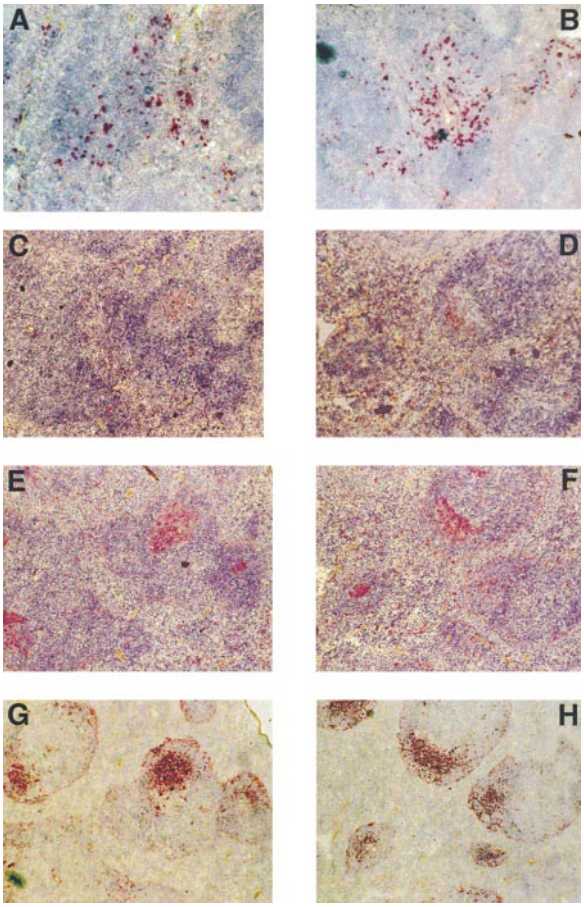

Figure 3. Development of Germinal Centers in *OX40*$^{-/-}$ Mice

Spleen sections from *OX40*$^{+/-}$ (left panel) and *OX40*$^{-/-}$ mice (right panel) infected with VSV 5 days (A and B) or 30 days (C–H) previously were stained for VSV-specific B cells (A, B, E, and F), PNA (C and D), or for follicular dendritic cells with anti-4C11 mAb (G and H). Positive cells are shown in red. Original magnification, 100×.

spleen cells were restimulated 30 (Figure 4B) or 60 days (data not shown) after infection and tested in a ^{51}Cr release assay. No significant difference between the two groups of mice was observed. Limiting dilution analysis revealed similar frequencies of specific CTLs 30 days after infection (Figure 4C). To obtain a more quantitative measurement for the presence of LCMV-specific CTLs, mice were infected with LCMV and splenic CD8$^+$ T cells were stained with LCMV peptide p33-loaded D^b tetramers that specifically stain LCMV-specific CTLs (Figure 4D). Frequencies of tetramer-positive CD8$^+$ T cells were similar 8 days after infection, and specific T cell numbers declined with similar kinetics upon clearance of the virus (Figure 4D and data not shown).

Impaired LCMV-Specific CD4$^+$ T Cell Responses in the Absence of OX40

To assess the role of OX40 in the generation of LCMV-specific Th cell responses, mice were immunized with LCMV (200 pfu), and 15 days later, spleen cells were isolated and CD4$^+$ T cells subsequently purified using magnetic beads. CD4$^+$ T cells were stimulated in vitro

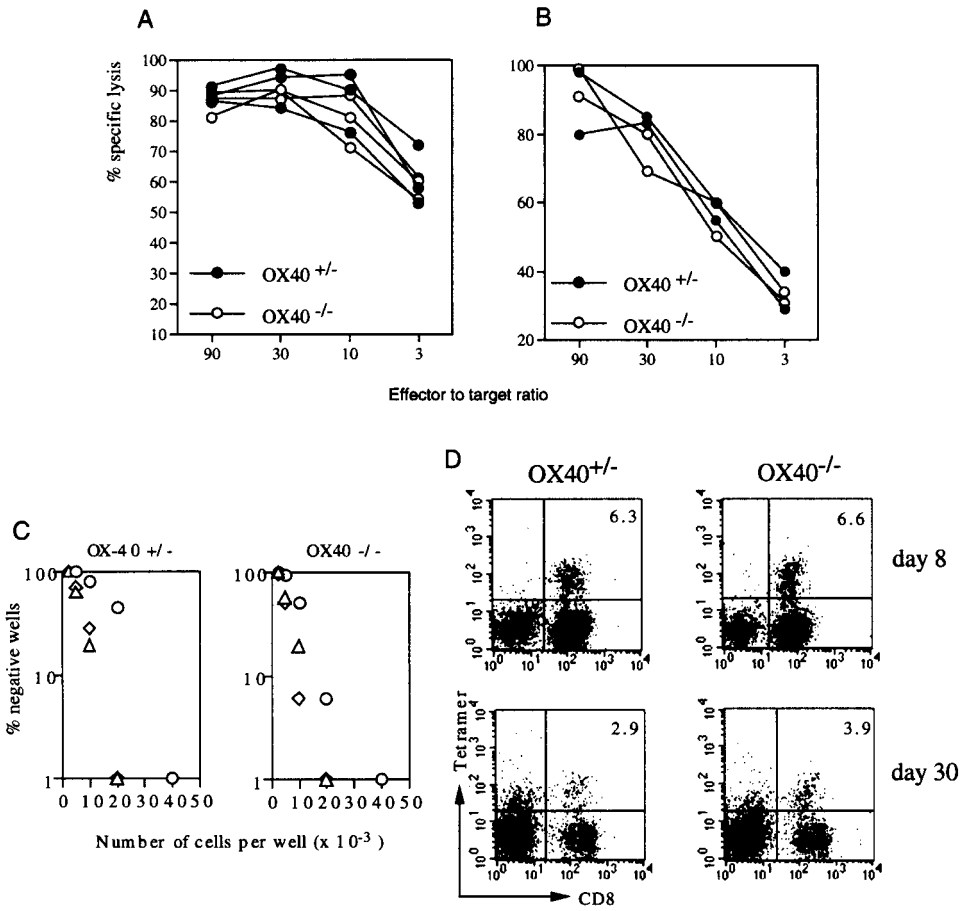

Figure 4. Unaltered Primary and Secondary CTL Responses to LCMV in *OX40*$^{-/-}$ Mice

(A) Mice were infected with 200 PFU of LCMV (WE strain), and 8 days later primary ex vivo CTL lytic activity was measured using EL4 cells pulsed with the specific peptide p33 in a ^{51}Cr release assay as described in the Experimental Procedures. Unspecific killing of all samples was below 7% (data not shown).

(B) A memory CTL response was assayed 30 days after infection by culturing splenocytes from infected mice together with irradiated splenocytes (H2^{b}) and peptide p33 before measurement of lytic activity as described above. Unspecific killing was below 10% (data not shown).

(C) Serial dilutions of spleen cells were restimulated with peptide-pulsed splenocytes in the presence of IL-2 in 96-well plates. Cells were resuspended 5 days later, and lytic activity was assessed by adding peptide-pulsed ^{51}Cr-loaded EL-4 cells. Wells were scored positive if lytic activity was two standard deviations above background. Average frequencies of specific CTLs were 1:17000 for *OX40*$^{+/-}$ mice and 1:13000 for *OX40*$^{-/-}$ mice.

(D) Mice were infected with 200 PFU of LCMV (WE strain), and the frequency of LCMV-specific CD8^{+} T cells was measured by flow cytometry at day 8, the peak of the effector response, and at day 30, about 2.5 weeks after elimination of the virus. Splenocytes were stained with p33-loaded tetramers labeled with PE and anti-CD8 labeled with APC.

with LCMV-infected splenic APCs for 4 days before measurement of T cell proliferation by [^{3}H]thymidine incorporation and IFN-γ levels in supernatants. In the absence of OX40, both the proliferation (Figure 5A) and production of IFN-γ (Figure 5B) were severely impaired. As a more direct readout for the numbers of activated T cells present after viral infection, splenocytes were stimulated for 4 hr using PMA/ionomycin and CD4^{+} and CD8^{+} T cells were stained intracellularly for the production of IFN-γ. As compared to controls, in *OX40*$^{-/-}$ mice the frequency of IFN-γ-producing CD8^{+} T cells was not significantly different, whereas the frequency of IFN-γ-producing CD4^{+} T cells was strongly reduced (Figure 5C). To investigate whether the impaired CD4^{+} T cell proliferation and differentiation had any consequence for the humoral response, we measured LCMV-specific serum antibody levels at day 12 after infection. As shown in Figure 6, *OX40*$^{-/-}$ mice mounted an efficient specific IgG1, IgG2a, and IgG2b antibody response to LCMV.

OX40 Is Critical for Efficient Induction of Influenza Virus–Specific Th Cell Responses

To assess the role of OX40 in the generation of specific Th cell responses to infection with a virus, which causes a more local infection mainly restricted to a mucosal site, *OX40*$^{-/-}$ and control mice were infected intranasally with influeza virus PR8. Lung-infiltrating cells were isolated by bronchoalveolar lavage (BAL) 10 days later and used to directly measure the presence and function of specific cytotoxic T cells in a ^{51}Cr release assay. As

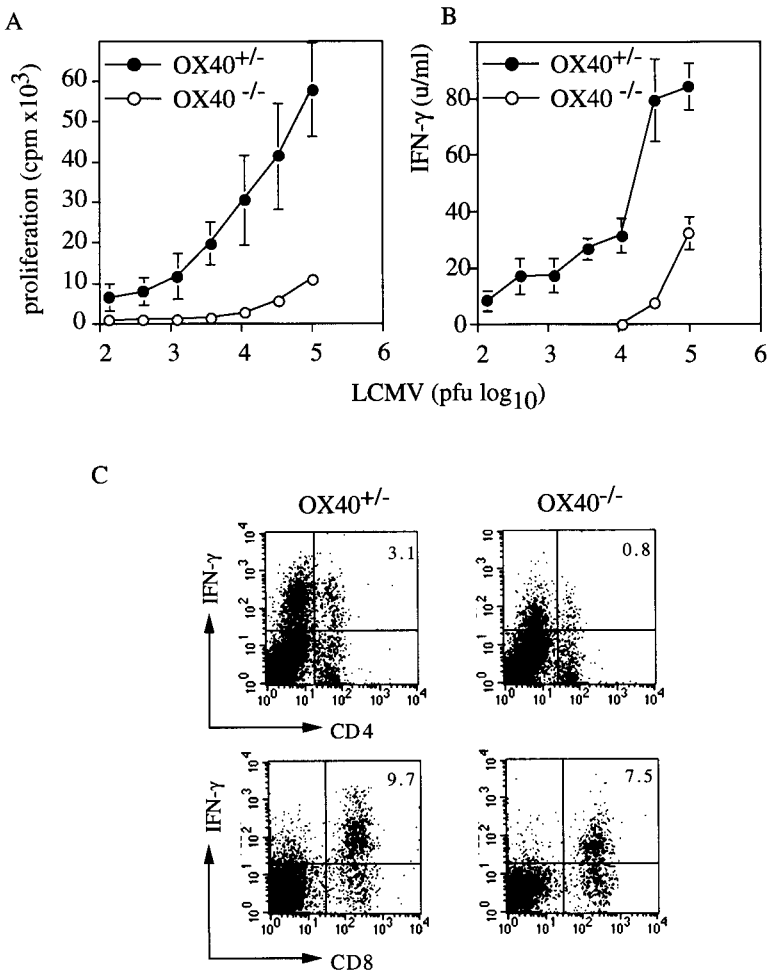

Figure 5. OX40 Is Required for Optimal Responses of CD4+ T Cells to LCMV Infection

Mice were infected with LCMV as described above 17 days earlier. Splenic CD4+ T cells were purified using magnetic beads and stimulated with irradiated splenocytes from uninfected C57BL/6 mice together with graded doses of virus before measurement of (A) proliferation by [^{3}H]thymidine incorporation and (B) IFN-γ levels in the supernatants by ELISA. (C) Spleen cell suspensions from LCMV-infected mice (day 15) were stimulated in vitro using PMA and ionomycin for 4 hr and production of IFN-γ was assessed by intracellular cytokine staining on CD4+ (upper panel) and CD8+ T cells (lower panel). Percentages of IFN-γ-producing CD4+ and CD8+ T cells are indicated in the figure.

shown in Figure 7A, a potent CTL activity against influenza virus was detected in *OX40*$^{+/-}$ mice, which was comparable in *OX40*$^{-/-}$ mice. Interestingly, the total number of BAL cells was reduced in *OX40*$^{-/-}$ mice (Table 1). Flow cytometry analysis showed that there was a specific reduction in infiltrating CD4+ T cells but not CD8+ T cells (Table 1). Moreover, splenic CD4+ T cells purified from *OX40*$^{-/-}$ mice at day 7 after infection showed reduced proliferation upon in vitro restimulation with viral antigen (Figure 7B) and also secreted reduced amounts of IFN-γ (Figure 7C). No detectable amounts of IL-4 were secreted by the Th cells, indicating that the absence of OX40 did not result in immune deviation (data not shown). In agreement with results obtained after LCMV and VSV infection, anti–influenza virus serum antibody levels were unaltered in *OX40*$^{-/-}$ mice compared to *OX40*$^{+/-}$ mice (Figure 7D).

Discussion

This report describes the generation and immunological characterization of mice deficient for the expression of OX40. Our results demonstrate that OX40 plays an important role in the stimulation of antiviral CD4+ T cell responses, while antiviral CTL responses were normal. Contrary to expectations, *OX40*$^{-/-}$ mice mounted normal humoral responses.

The defective CD4+ T cell responses to LCMV and influenza virus in *OX40*$^{-/-}$ mice adds to the accumulating in vitro data suggesting a role for OX40 in costimulation of CD4+ T cells (Weinberg et al., 1998). In the present study, we found strongly reduced proliferation of antiviral CD4+ T cells and Th1 differentiation in OX40-deficient

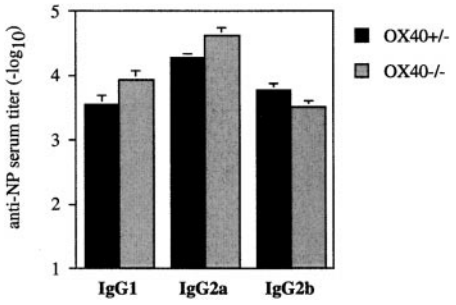

Figure 6. OX40 Is Dispensable for Antibody Responses to LCMV

Mice were intravenously infected with 200 pfu LCMV-WE and bled at day 12 to measure anti-LCMV NP serum IgG1, IgG2a, and IgG2b antibody levels by ELISA. Shown are averages ± SEM of the end point titers from groups of mice (n = 4/group).

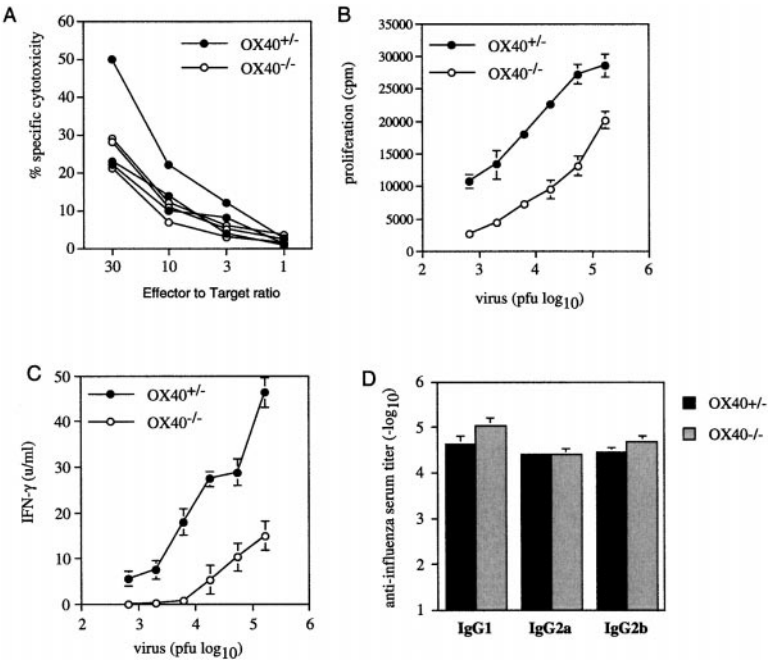

Figure 7. OX40 Is Required for Optimal Responses of CD4+ T Cells to Influenza Virus Infection

Mice (n = 3/group) were infected intranasally with influenza virus (500 pfu, strain PR8).

(A) The bronchoalveolar lavage was harvested 10 days later and used directly to determine primary ex vivo cytolytic activity using EL4 cells pulsed with the specific peptide NP68 in a ^{51}Cr release assay as described in the Experimental Procedures. Shown are results of individual mice. Unspecific killing of all samples was below 4% (data not shown).

(B and C) At day 7 after after infection, splenic CD4+ T cells were purified using magnetic beads as described by the manufacturer and stimulated with irradiated splenocytes from uninfected C57BL/6 mice together with graded doses of UV light–inactivated influenza virus before measurement of (B) proliferation by [^{3}H]thymidine incorporation and (C) IFN-γ levels in the supernatants by ELISA. Shown are averages ± SEM of groups of mice (n = 3/group).

(D) Mice were bled at day 10 after infection to measure anti-influenza virus serum IgG1, IgG2a, and IgG2b antibody levels by ELISA. Shown are averages ± SEM of the end point titers from groups of mice (n = 4/group).

mice. Additionally, the frequency of IFN-γ-producing CD4+ T cells (Th1) after LCMV infection was strongly reduced in *OX40*$^{-/-}$ mice compared to *OX40*$^{+/-}$ mice. It has been proposed that OX40 stimulation is mainly required to promote Th2 differentiation (Flynn et al., 1998; Ohshima et al., 1998). However, our results indicate that Th1 responses to viruses were affected in the absence of OX40. While Th2 responses remain to be investigated in *OX40*$^{-/-}$ mice, we think that OX40 signaling is required for activation or maintenance rather than for inducing differential polarization of CD4+ T cells.

OX40L is expressed by professional APCs as well as by vascular endothelial cells (Imura et al., 1996). It is therefore possible that the reduced number of CD4+ T cells in the BAL of infuenza virus–infected *OX40*$^{-/-}$ mice resulted from impaired Th cell priming in central lymphoid organs or impaired extravasation from blood vessels. While we have clear data indicating reduced Th cell priming in spleens of influenza virus–infected mice, we have at present time no evidence for or against a role for OX40 in directing extravasation from blood vessels.

Originally, OX40 was identified in the rat and shown to be expressed only on activated CD4+ T cells (Paterson et al., 1987). Possibly because of this reason, previous studies have not yet addressed a role of OX40 in the generation of CTL responses. However, more recent reports clearly showed that both in mouse and man, in contrast to rats, OX40 is also expressed on CD8+ T cells (Al-Shamkhani et al., 1996). Thus, it was important to define the role of OX40 in the generation of CTL responses using the *OX40*$^{-/-}$ mice. Our analysis, including the measurement of both a primary in vivo and secondary in vitro CTL response by classical ^{51}Cr release and the determination of the frequency of virus-specific CD8+ T cells by limiting dilution analysis and latest tetramer-staining technology (Altman et al., 1996), clearly rules out a role of OX40 for CTL responses to LCMV, in contrast to its requirement for T helper cell responses. Since CTL responses to LCMV do not require CD4+ T cell help, it will be interesting to look at CTL responses in *OX40*$^{-/-}$ mice that are known to be more dependent on T cell help such as antitumor or allograft CTL responses.

It is interesting to compare the phenotype of *OX40*$^{-/-}$ mice with mice deficient for other accessory and costimulatory molecules. The prototype of a T cell costimulatory molecule is CD28. After LCMV infection, CD28$^{-/-}$ mice show severly reduced CD4+ T cell proliferation (M. K., A. Oxenius, and M. F. B., unpublished data), while CTL responses remain relatively intact (Shahinian et al., 1993; Kündig et al., 1996). In this sense, *OX40*$^{-/-}$ mice and CD28$^{-/-}$ mice behave quite similarly. This may suggest that CD28 and OX40 signaling acts sequentially

Table 1. Reduced CD4+ T Cells in the Lung of Influenza Virus–Infected OX40$^{-/-}$ Mice

Mice	BAL Cells Total Number ± SEM (× 10^4)	CD4+ Percent	CD8+ Percent	CD4+ T Cells Total Number ± SEM (× 10^4)	CD8+ T Cells Total Number ± SEM (× 10^4)
OX40$^{+/-}$	11.83 ± 3.6	17.7 ± 2.1	73.3 ± 1.6	2.68 ± 0.48	8.6 ± 2.4
OX40$^{-/-}$	7.75 ± 2.5	11.5 ± 2.5	79.5 ± 1.7	0.99 ± 0.49	6.2 ± 1.83

BAL was harvested at day 10 after influenza virus infection and analyzed by flow cytometry after staining with CD4 and CD8 mAbs and gating on live lymphocytes. Shown are average values ± SEM of four mice per group.

(e.g., CD28 ligation induces OX40 expression) or that CD28 and OX40 act independently with overlapping functions. We are currently investigating these possibilities. A previous report suggested that CD28 and OX40 synergize in the induction of CD4$^+$ T cell proliferation and IL-2 production but that OX40 ligation, rather than CD28 ligation, is responsible for sustained CD4$^+$ effector responses (Gramaglia et al., 1998). This is compatible with the idea that OX40 enhances CD4$^+$ T cell responses by providing survival signals mediated by the TRAF-2/NF-κB pathway (Kawamata et al., 1998), whereas CD28 augments TCR-mediated signals intracellularly and is therefore required early to promote T cell activation (Tuosto and Acuto, 1998). Thus, while our results clearly show that CD28 and OX40 are not completely redundant for CD4$^+$ T cell stimulation, this possibility remains for CTL responses. More recently, it was shown that the CTL response to LCMV was normal in *4–1BBL*$^{-/-}$ mice and in *CD28*$^{-/-}$ mice but was reduced (though not absent) in *CD28/4–1BBL* double-deficient mice (DeBenedette et al., 1999). 4–1BB is another member of the TNFR familiy expressed on both activated CD4$^+$ and CD8$^+$ T cells (Vinay and Kwon, 1998). Like OX40, 4–1BB has been implicated in T cell costimulation (Vinay and Kwon, 1998). While it remains to be investigated whether *CD28/OX40* double-deficient mice show pronounced defects in CTL responses, it appears possible that 4–1BB and OX40 signals are functional correlates for the induction of CD8$^+$ and CD4$^+$ T cells, respectively. Thus, CD8$^+$ rather than CD4$^+$ T cell responses appeared reduced in *4–1BB*$^{-/-}$ mice (DeBenedette et al., 1999), and 4–1BB cross-linking with agonistic antibodies preferentially stimulates proliferation and cell survival of CD8$^+$ T cells over CD4$^+$ T cells (Shuford et al., 1997; Takahashi et al., 1999). In contrast, as shown here, CD4$^+$ but not CD8$^+$ T cell responses are reduced in *OX40*$^{-/-}$ mice. Considering that the primary mediators of viral clearance in influenza virus infection are the CD8$^+$ effectors and that the CD4$^+$ T cell effector mechanism involving cytokine secretion is relatively inefficient and may be limited to promote humoral immunity (Doherty et al., 1997), it was not surprising that *OX40*$^{-/-}$ efficiently cleared the virus by day 11 after infection without any delay compared to *OX40*$^{+/-}$ mice (data not shown).

Unlike 4–1BB, CD27, and OX40, which are expressed on activated T cells, other members of the TNFR family, such as CD40 and RANK (TRANCE-R), are expressed on and deliver signals into the APC. CD40 and RANK (TRANCE-R) triggering activates dendritic cells to produce IL-12, to upregulate costimulatory ligands (e.g., CD80, CD86) and MHC class II peptide complexes, and to prolong DC survial (Anderson et al., 1997; Wong et al., 1997; Grewal and Flavell, 1998; Bachmann et al., 1999a). Together, this allows for optimal T cell stimulation. In addition, CD40 triggering may promote T cell stimulation also by regulating OX40–OX40L interaction. However, we found that CD40-deficient and CD40-competent DCs loaded with specific peptide comparably stimulated OX40 expression on CD4$^+$ T cells, but at present, we cannot exclude reduced OX40L expression on DC in the absence of CD40 (C. R., M. F. B., and M. K., unpublished data). Comparing *CD40*$^{-/-}$ mice and *OX40*$^{-/-}$ mice, there are some similarities but also some notable differences. Primary CTL responses to LCMV are unaltered in both mutant strains (Figure 4A; Borrow et al., 1996; Whitmire et al., 1996), whereas memory CTL responses are significantly impaired in *CD40*$^{-/-}$ mice (Borrow et al., 1996) but remained intact in *OX40*$^{-/-}$ mice (Figure 4B). Although *CD40*−/− mice fail to mount a proper Th1 response to a number of pathogens (Foy et al., 1996; Grewal and Flavell, 1998), CD4$^+$ T cell responses against some viruses including LCMV are normal (Oxenius et al., 1996). In contrast, as discussed above, antiviral CD4$^+$ T cell responses to LCMV are reduced in the absence of OX40. In this regard, *OX40*$^{-/-}$ mice have a more severe defect than *CD40*$^{-/-}$ mice.

We did not observe any effect of OX40 deficiency on primary T-dependent antibody responses to VSV, LCMV, and influenza virus infection. Additionally, primary and secondary antibody responses after immunization with NP-CGG resulted in NP-specific IgM, IgG1, IgG2a, and IgG2b serum levels that did not differ comparing *OX40*$^{-/-}$ mice and *OX40*$^{+/-}$ control littermates (data not shown). Thus, OX40 played no critical role for the secretion of isotype-switched antibodies and plasma cell formation. Additionally, the formation of germinal centers in response to VSV was largely normal in the absence of OX40. In this regard, the role of OX40 clearly differs from the role of CD40, since CD40 is absolutely essential for T/B collaboration (Foy et al., 1996).

Previously, it has been suggested that OX40–OX40L interaction is essential for primary IgG responses. Treatment of TNP-KLH-immunized mice with anti-OX40 antibodies resulted in a reduced number of extrafollicular plasma cells in the PALS and abrogated IgG responses. Since germinal center formation was unaltered, it has been argued that activated CD4$^+$ T cells were not depleted by anti-OX40 treatment (Stuber and Strober, 1996). However, it remains possible that only a subset of activated CD4$^+$ T cells express OX40, which are located mainly in PALS but not in the B cell follicle (Stuber and Strober, 1996), and which were, in fact, depleted. On the other hand, there is strong evidence that OX40 ligation induces CD4$^+$ T cell migration to B cell follicles, where CD4$^+$ T cells accumulate after immunization in transgenic mice expressing OX40L on DC (Brocker et al., 1999). Thus, the injected anti-OX40 antibodies in the earlier experiments may have directed the Th cells to migrate into the B cell follicles, inhibiting the Th cell–dependent formation of extrafollicular plasma cells.

Considering impaired CD4$^+$ T cell proliferation and IFN-γ production, it may appear somewhat surprising that *OX40*$^{-/-}$ mice mounted normal antiviral IgG responses. However, during primary neutralizing antiviral B cell responses, the frequency of specific B cells is usually more limiting than the frequency of specific Th cells (Charan et al., 1986), indicating that reduced Th cell responses may still suffice to trigger largely normal B cell responses. In addition, it may be possible that the population of Th cells proliferating and secreting IFN-γ in vitro may not be identical to the population of Th cells assisting B cells to secrete antibodies. Thus, a reduced CD4$^+$ proliferation and differentiation may not necessarily be paralleled by a reduced B cell help. Nevertheless, it is conceivable that different doses of antigen, different adjuvants, or different immunization regimens will reveal more subtle effects of OX40 on B cell responses.

Taken together, our results demonstrate that the function of OX40 is limited to $CD4^+$ T cell activation, which makes it an ideal target with a great therapeutic potential for human diseases including multiple sclerosis, inflammatory bowel disease, rheumatoid arthritis, and graft versus host disease, where the $CD4^+$ T cell subset has been suggested to be central for pathogenesis (Weinberg et al., 1996; Higgins et al., 1999).

Experimental Procedures

Generation of OX40-Deficient Mice

Genomic DNA from ES cells (E14.1) was used to PCR amplify a 2.2 kb fragment from the *OX40* locus extending from exon 1 to the beginning of exon 5. Primers (OX40for: CCAAAGCACTTCTTAGCT TATCATG; OX40rev: GTCGACTATCCAGATAAGGTACA) contained restrictions sites for NotI and SalI at their 5′ ends. This fragment was used as the long arm of homology for the target vector and cloned upstream of the *neomycin* cassette, which was derived from the plasmid pMC1NeoPolyA (Stratagene). In exon 5, a mutation was introduced at amino acid (aa) 150 to generate a stop codon (K: → stop). The short arm of homology was generated by amplification of a 600 bp fragment with primers (OX40for: CCCGCCACCCAGC CAGTG; OX40rev: ATATATCTCGAGGCACCTAGAACGGTGTGGAG GGTA) containing a XhoI site at their 5′ ends and cloned downstream of the *neo* cassette. The replacement target vector contained the *HSV-tk* gene to allow positive–negative selection. The *OX40* gene in embryonic stem cells (ES) E14.1 (129 Ola) was targeted as previously described (Kopf, 1996). Briefly, ES cells were electroporated before selection with G418 (400 μg/ml) and Gancyclovir (2 μM) and screened by nested PCR with oligonucleotides specific for *neomycin* (neo.p1for: GCCGATTGTCTGTTGTGCCC; neo.p2rev: GGA GAACCTGCGTGCAATCC) and OX40 (p1rev: TAGCATGTTTATTAG GAGCACC; p2rev: CCAGGCAGGTAGTATGCATAGC) located external to the short arm of the target vector. Chimeric mice were generated by standard protocols, and tail DNA from F1 offspring and subsequent generations was analyzed by PCR for the disrupted OX40 allele. OX40 mutant mice were backcrossed for three generations to C57BL/6 before heterozygous mice were used for interbreeding to obtain homozygous OX40-deficient mice. PCR genotyping with OX40 specfic primers (for: AAGATGGCTCAGTGCAA CTTCC; rev: CAAGGTGGGTGGAGAGGGCAA) upstream and downstream of the *neomycin* gene was performed as described (Kopf, 1996) and resulted in a 225 bp and a 1375 bp fragment from the OX40 wild-type and mutant allele, respectively. The offspring of $OX40^{-/-} \times OX40^{+/-}$ breedings were used for all experiments described.

In Vitro Stimulation of T Cells and OX40 Expression Analysis

Spleens and lymph nodes of mice were teased to single-cell suspensions and depleted of $CD8^+$ (mAb 53–6.72) and MHC class II^+ cells (mAb M5/114) by negative selection using magnetic beads (Dynal, Oslo, Norway) according to the manufacturer's instructions. The resulting $CD4^+$ T cell population was plated (2×10^5 cells/0.2 ml) in flat-bottomed microtiter plates (Costar, Cambridge, MA) precoated with anti-CD3 and anti-CD28 mAbs (5 μg/ml each) for 72 hr. Stimulated cells were used for analyis of OX40 expression by flow cytometry and by RT-PCR. For FACS analysis, cells were stained with a rat anti-OX40 mAb (Serotec, Oxford, UK) and a secondary goat anti-rat mAb labeled with FITC. Total cellular RNA was prepared of originally 2×10^6 plated $CD4^+$ T cells, and RNA samples were reverse transcribed using random hexamers for priming and Superscript-RT (GIBCO-BRL) (Kopf et al., 1996). cDNA samples were diluted 1:4 and standardized for the expresssion of β2-microglobulin essentially as described (Kopf et al., 1996). OX40 transcripts were amplified by semiquantitative PCR using specific primers (for: ACAAGTGCTGTC GTGAGTGC; rev: CAAGGTGGGTGGAGAGGGCAA) and standardized cDNA aliquots.

Pathogens

The LCMV isolate WE was originally provided by Dr. P. S. Ohashi (University of Toronto, Canada) and grown on L cells at a low multiplicity of infection. LCMV (200 pfu) was injected intravenously per mouse.

Influenza virus PR8 (A/Puerto Rico8/34, H1N1 subtype) and the MDCK cell line (Madien-Darby canine kidney cells) were originally provided by Dr. J. Pavlovic (University of Zürich, Switzerland). Infectious stocks of influenza virus PR8 were grown for 3 days at 37°C in the allantoic cavity of day 10 fertilized eggs (Gipf, Oberfrick, Switzerland) and purified as described (Bachmann et al., 1999b). Viral titers were determined on MDCK cells as described (Bachmann et al., 1999b). Mice were infected intranasally with 10^4 pfu of live influenza virus.

VSV serotype Indiana was originally obtained from Dr. P. S. Ohashi and grown at low multiplicity of infection in BHK cells.

Serum Neutralization Test after VSV Infection

Serum was collected from mice at specific time points after VSV infection. The sera were prediluted 40-fold in minimal essential medium containing 5% FCS, then heat-inactivated for 30 min at 56°C. Serial 2-fold dilutions were mixed with equal volumes of VSV diluted to contain 500 pfu/ml. The mixture was incubated for 90 min at 37°C in an atmosphere with 5% CO_2. The serum–virus mixture (100 μl) was transferred onto Vero cell monolayers in 96-well plates and incubated for 1 hr at 37°C. The monolayers were then overlaid with 100 ml of DMEM containing 1% methyl cellulose. After incubation for 24 hr at 37°C, the overlay was flicked off and the monolayer was fixed and stained with 0.5% crystal violet. The highest dilution of serum that reduced the number of plaques by 50% was taken as titer. Due to the addition of an equal volume of virus, the titer of serum was considered to be one step higher. To determine IgG titers, undiluted serum was pretreated with an equal volume of 0.1 mM-2-mercaptoethanol in saline.

Detection of Antibodies by ELISA

Antibodies against the NP of LCMV and influenza virus were detected by an ELISA. Recombinant baculovirus-expressing NP (Battegay et al., 1993), a kind gift from Rolf Zinkernagel (University of Zürich), was coated onto a 96-well plate (Nunc maxisorp) in a concentration of 200 ng/well in 50 μl PBS overnight at 4°C. UV-inactivated influenza virus was coated with 1000 PFU/well. Subsequent steps were done at room temperature and followed by three washing procedures with PBS. Coated plates were blocked with 1% BSA for 1–2 hr, and then sera serially diluted in PBS containing 0.1% BSA were added for 3–5 hr. Alkaline phospatase–labeled goat anti-mouse antibodies to IgG1, IgG2a, or IgG2b (Southern Biotechnology Associates) were added for 1–2 hr followed by the addition of p-nitrophenyl phosphate (Sigma) as substrate before reading the OD at 405 nm. Positive titers were defined as three standard deviations above mean values of negative controls.

Histology

Histology was perfomed essentially as described (Bachmann et al., 1996). In brief, organs were snap-frozen in liquid nitrogen, and tissue sections of 5 μm thickness were cut in a cryostate. PNA staining was performed using PNA and rabbit anti-PNA antibodies. For FDC staining, the rat 4C11 antibody was used (Gray et al., 1991). Sections were then incubated with alkaline phosphatase–labeled antibodies to rabbit or rat immunoglobulins and developed using naphtol AS-BI phosphate and New Fuchsin. VSV-specific B cells were visualized using VSV particles followed by rabbit-anti-VSV antibodies. Sections were then developed as described above. All sections were counterstained using hemalum.

Proliferation and IFN-γ Production of LCMV- and Influenza Virus–Specific $CD4^+$ T Cells

Mice were immunized intravenously with LCMV-WE (200 pfu), and 15 days later, $CD4^+$ T cells were purified from spleen cell suspensions by magnetic separation (MACS; Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. Purity was greater than 90%. $CD4^+$ T cells (1×10^5) were incubated in 96 wells with 3-fold serial dilutions of live LCMV (highest concentration, MOI = 0.3) or medium only in the presence of 3×10^5 irradiated (2000 cGy) C57BL/6 spleen cells for 3 days. The supernatants (100 μl) were removed for measurement of IFN-γ by ELISA with specific mAbs (Pharmingen, San Diego, CA). Proliferation was

assessed by incorporation of [^{3}H]thymidine (1 μCi/well). For influenza virus–specific CD4$^+$ T cell proliferation, spleen cells were isolated 7 days after infection. Proliferation assays were performed as for LCMV, with the exception that 2×10^5 CD4$^+$ T cells were stimulated with UV light–inactivated influenza virus (5×10^4 pfu/ml before inactivation).

^{51}Cr Release Assays

Splenocytes from LCMV-WE-infected mice were used for measurement of primary and secondary CTL responses in a conventional ^{51}Cr release assay essentially as described (Bachmann, 1997). Briefly, for primary responses, at day 8 after infection, EL-4 target cells were pulsed with peptide p33 (KAVYNFATM, aa 33–42 derived from the LCMV glycoprotein) at a concentration of 10^{-7} M for 90 min at 37°C in the presence of [^{51}Cr]sodium chromate in IMDM supplemented with 10% FCS. Ex vivo isolated spleen cell suspensions from infected mice were serially diluted and mixed with peptide-pulsed target cells. For secondary responses, splenocytes were isolated at day 30 after infection and cultured for 5 days at a density of 4×10^6 together with peptide p33-pulsed irradiated (25 Cy) C57BL/6 spleen cells (10^6 cells) in 2 ml of IMDM supplemented with 10% FCS. Restimulated spleen cells were resuspended in 0.5 ml of medium per culture well and serial 3-fold dilutions of effectors were performed (referred to as dilution of standard culture) and measured in a conventional ^{51}Cr release assay, using GP33-labeled EL-4 cells as targets. Limiting dilution analysis was performed as described (Bachmann, 1997).

Lung lavage cells from day 10 influenza virus–infected mice were tested directly for the presence of specific cytotoxic T cells. EL-4 target cells were pulsed with peptide NP68 (ASNENMDAM, aa 366–374, derived from the influenza virus nucleoprotein of the 1968 strain A/HK/8/68) at a concentration of 20 ng/ml in the presence of ^{51}Cr as described above. Lung lavage cells were serially diluted and mixed with peptide-pulsed target cells and levels of cytotoxicity measured in a 6 hr ^{51}Cr release assay.

Generation and Use of Tetramers

Soluble, biotinylated class I monomers, comprising the murine H2-D^b molecule, human β2M, and LCMV peptide p33, were generated as described previously (Gallimore et al., 1998). Tetrameric complexes were subsequently generated by stepwise addition of phycoerythrin-labeled extravidin (Sigma) to the biotinylated monomers at a 1:4 molar ratio. Single-cell suspensions were prepared from spleens and incubated with PE-conjugated tetrameters at 37°C for 15 min. APC-conjugated anti-CD8 mAb (Pharmingen, San Diego, CA) was added subsequently and kept on ice for 15 min. Cells were washed and resuspended in PBS/2% FCS/0.5 mM EDTA (FACS buffer) and analyzed by flow cytometry (FACSCAN; Beckton Dickinson, Mountain View, CA) using Cellquest software.

Intracellular Cytokine Analysis

Staining for intracellular cytokines was performed using a modification of standard techniques. Briefly, cells were stimulated with PMA (10^{-7} M) and ionomycin (1 μg/ml) for 4 hr. Brefeldin A (10 μg/ml) was added to cultures 2 hr prior to harvesting. Cells were washed once with PBS and fixed with 2% paraformaldehyde for 30 min at room temperature. Subsequently, cells were stained with PE-labeled anti-CD4 mAb or anti-CD8 mAb, washed, and stained for another 30 min with FITC-labeled anti-IFN-γ in a 1% BSA/PBS solution containing 0.5% saponine for permeabilization. After staining, cells were washed and resuspended in 1% BSA/PBS solution and analyzed by flow cytometry.

Bronchoalveolar Lavage

At day 10 after influenza virus infection, BAL was performed by injecting 0.3 ml of PBS into the lungs via the trachea and immediate withdrawal of the fluid. This procedure was repeated a total of three times. Isolated cells were washed, counted, and used for CD4 and CD8 expression analysis by flow cytometry or for a ^{51}Cr release assay as described above.

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References

Al-Shamkhani, A., Birkeland, M.L., Puklavec, M., Brown, M.H., James, W., and Barclay, A.N. (1996). OX40 is differentially expressed on activated rat and mouse T cells and is the sole receptor for the OX40 ligand. Eur. J. Immunol. *26*, 1695–1699.

Altman, J.D., Moss, P.A.H., Goulder, P.J.R., Barouch, D.H., McHeyzer-Williams, M.G., Bell, J.I., McMichael, A.J., and Davis, M.M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. Science *274*, 94–96. Erratum: Science *280(5371)*, 1998.

Anderson, D.M., Maraskovsky, E., Billingsley, W.L., Dougall, W.C., Tometsko, M.E., Roux, E.R., Teepe, M.C., DuBose, R.F., Cosman, D., and Galibert, L. (1997). A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature *390*, 175–179.

Bachmann, M.F. (1997). Evaluation of lymphocytic choriomeningitis virus–specific cytotoxic T cell responses. In Immunology Methods Manual, I. Lefkovits, ed. (New York: Academic Press), pp. 1921–1933.

Bachmann, M.F., and Zinkernagel, R.M. (1997). Neutralizing antiviral B cell responses. Annu. Rev. Immunol. *15*, 235–270.

Bachmann, M.F., Odermatt, B., Hengartner, H., and Zinkernagel, R.M. (1996). Induction of long-lived germinal centers associated with persisting antigen after viral infection. J. Exp. Med. *183*, 2259–2269.

Bachmann, M.F., Kalinke, U., Althage, A., Freer, G., Burkhart, C., Roost, H.-P., Aguet, M., Hengartner, H., and Zinkernagel, R.M. (1997). The roles of antibody concentration and avidity in anti-viral protection. Science *276*, 2024–2027.

Bachmann, M.F., Wong, B.R., Josien, R., Steinman, R.M., Oxenius, A., and Choi, Y. (1999a). TRANCE, a TNF family member critical for CD40-independent T helper cell activation. J. Exp. Med. *189*, 1025–1031.

Bachmann, M.F., Ecabert, B., and Kopf, M. (1999b). Influenza virus: a novel method to assess viral and neutralizing antibody titers in vitro. J. Immunol. Methods *225*, 105–111.

Baker, S.J., and Reddy, E.P. (1996). Transducers of life and death: TNF receptor superfamily and associated proteins. Oncogene *12*, 1–9.

Battegay, M., Moskophidis, D., Waldner, H., Brundler, M.A., Fung-Leung, W.P., Mak, T.W., Hengartner, H., and Zinkernagel, R.M. (1993). Impairment and delay of neutralizing antiviral antibody responses by virus-specific cytotoxic T cells. J. Immunol. *151*, 5408–5415. Erratum: J. Immunol. *151(5)*, 1994.

Baum, P.R., Gayle, R.B., 3rd, Ramsdell, F., Srinivasan, S., Sorensen, R.A., Watson, M.L., Seldin, M.F., Baker, E., Sutherland, G.R., Clifford, K.N., et al. (1994). Molecular characterization of murine and human OX40/OX40 ligand systems: identification of a human OX40 ligand as the HTLV-1-regulated protein gp34. EMBO J. *13*, 3992–4001.

Borrow, P., Tishon, A., Lee, S., Xu, J., Grewal, I.S., Oldstone, M.B., and Flavell, R.A. (1996). CD40L-deficient mice show deficits in antiviral immunity and have an impaired memory CD8$^+$ CTL response. J. Exp. Med. *183*, 2129–2142.

Brocker, T., Gulbranson-Judge, A., Flynn, S., Riedinger, M., Raykundalia, C., and Lane, P. (1999). CD4 T cell traffic control: in vivo evidence that ligation of OX40 on CD4 T cells by OX40-ligand expressed on dendritic cells leads to the accumulation of CD4 T cells in B follicles. Eur. J. Immunol. *29*, 1610–1616.

Calderhead, D.M., Buhlmann, J.E., van den Eertwegh, A.J., Claassen, E., Noelle, R.J., and Fell, H.P. (1993). Cloning of mouse Ox40:

a T cell activation marker that may mediate T-B cell interactions. J. Immunol. *151*, 5261–5271.

Charan, S., Hengartner, H., and Zinkernagel, R.M. (1986). Primary antibody responses to a well-defined and unique hapten are not enhanced by preimmunization with carrier: analysis in a viral model. Proc. Natl. Acad. Sci. USA *83*, 2604–2608.

DeBenedette, M.A., Wen, T., Bachmann, M.F., Ohashi, P., Barber, B.H., Stocking, K.L., Peschon, J., and Watts, T.H. (1999). Analysis of 4–1BB ligand-deficient mice and of mice lacking both 4–1BB ligand and CD28 reveals a role for 4–1BB ligand in skin allograft rejection and the cytotoxic T cell response to influenza virus. J. Immunol. *163*, 4833–4841.

Doherty, P.C., Topham, D.J., Tripp, R.A., Cardin, R.D., Brooks, J.W., and Stevenson, P.G. (1997). Effector $CD4^+$ and $CD8^+$ T-cell mechanisms in the control of respiratory virus infections. Immunol. Rev. *159*, 105–117.

Flynn, S., Toellner, K.M., Raykundalia, C., Goodall, M., and Lane, P. (1998). CD4 T cell cytokine differentiation: the B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and upregulates expression of the chemokine receptor, Blr-1. J. Exp. Med. *188*, 297–304.

Foy, T.M., Aruffo, A., Bajorah, J., Buhlmann, J.E., and Noelle, J.N. (1996). Immune regulation by CD40 and its ligand. Annu. Rev. Immunol. *14*, 591–617.

Gallimore, A., Glithero, A., Godkin, A., Tissot, A.C., Pluckthun, A., Elliott, T., Hengartner, H., and Zinkernagel, R. (1998). Induction and exhaustion of lymphocytic choriomeningitis virus–specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I–peptide complexes. J. Exp. Med. *187*, 1383–1393.

Gause, W.C., Chen, S.J., Greenwald, R.J., Halvorson, M.J., Lu, P., Zhou, X.D., Morris, S.C., Lee, K.P., June, C.H., Finkelman, F.D., et al. (1997). CD28 dependence of T cell differentiation to IL-4 production varies with the particular type 2 immune response. J. Immunol. *158*, 4082–4087.

Gramaglia, I., Weinberg, A.D., Lemon, M., and Croft, M. (1998). Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. J. Immunol. *161*, 6510–6517.

Gravestein, L.A., and Borst, J. (1998). Tumor necrosis factor receptor family members in the immune system. Semin. Immunol. *10*, 423–434.

Gray, D., Kosco, M., and Stockinger, B. (1991). Novel pathways of antigen presentation for the maintenance of memory. Int. Immunol. *3*, 141–148.

Grewal, I.S., and Flavell, R.A. (1998). CD40 and CD154 in cell-mediated immunity. Annu. Rev. Immunol. *16*, 111–136.

Higgins, L.M., McDonald, S.A., Whittle, N., Crockett, N., Shields, J.G., and MacDonald, T.T. (1999). Regulation of T cell activation in vitro and in vivo by targeting the OX40–OX40 ligand interaction: amelioration of ongoing inflammatory bowel disease with an OX40–IgG fusion protein, but not with an OX40 ligand–IgG fusion protein. J. Immunol. *162*, 486–493.

Imura, A., Hori, T., Imada, K., Ishikawa, T., Tanaka, Y., Maeda, M., Imamura, S., and Uchiyama, T. (1996). The human OX40/gp34 system directly mediates adhesion of activated T cells to vascular endothelial cells. J. Exp. Med. *183*, 2185–2195.

Kawai, K., Shahinian, A., Mak, T.W., and Ohashi, P.S. (1996). Skin allograft rejection in CD28-deficient mice. Transplantation *61*, 352–355.

Kawamata, S., Hori, T., Imura, A., Takaori-Kondo, A., and Uchiyama, T. (1998). Activation of OX40 signal transduction pathways leads to tumor necrosis factor receptor–associated factor (TRAF) 2– and TRAF5-mediated NF-κB activation. J. Biol. Chem. *273*, 5808–5814.

Kopf, M. (1996). Protocols for gene targeting. In Immunology Methods Manual: The Comprehensive Sourcebook of Techniques, I. Lefkovits, ed. (San Diego, CA: Academic Press), pp. 199–216.

Kopf, M., Brombacher, F., Kohler, G., Kienzle, G., Widmann, K.H., Lefrang, K., Humborg, C., Ledermann, B., and Solbach, W. (1996). IL-4-deficient Balb/c mice resist infection with Leishmania major. J. Exp. Med. *184*, 1127–1136.

Kündig, T.M., Shahinian, A., Kawai, K., Mittrücker, H.-W., Sebzda, E., Bachmann, M.F., Mak, T.W., and Ohashi, P.S. (1996). Duration of TCR stimulation determines costimulatory requirement of T cells. Immunity *5*, 41–52.

Lenschow, D.J., Walunas, T.L., and Bluestone, J.A. (1996). CD28/B7 system of T cell costimulation. Annu. Rev. Immunol. *14*, 233–258.

Ohshima, Y., Tanaka, Y., Tozawa, H., Takahashi, Y., Maliszewski, C., and Delespesse, G. (1997). Expression and function of OX40 ligand on human dendritic cells. J. Immunol. *159*, 3838–3848.

Ohshima, Y., Yang, L.P., Uchiyama, T., Tanaka, Y., Baum, P., Sergerie, M., Hermann, P., and Delespesse, G. (1998). OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human CD4(+) T cells into high IL-4-producing effectors. Blood *92*, 3338–3345.

Oxenius, A., Campbell, K.A., Maliszewski, C.R., Kishimoto, T., Kikutani, H., Hengartner, H., Zinkernagel, R.M., and Bachmann, M.F. (1996). CD40-CD40 ligand interactions are critical in T-B cooperation but not for other anti-viral $CD4^+$ T cell functions. J. Exp. Med. *183*, 2209–2218.

Paterson, D.J., Jefferies, W.A., Green, J.R., Brandon, M.R., Corthesy, P., Puklavec, M., and Williams, A.F. (1987). Antigens of activated rat T lymphocytes including a molecule of 50,000 Mr detected only on CD4 positive T blasts. Mol. Immunol. *24*, 1281–1290.

Shahinian, A., Pfeffer, K., Lee, K.P., Kündig, T.M., Ohashi, P.S., Thompson, C.B., and Mak, T.W. (1993). Differential T cell costimulatory requirements in CD28-deficient mice. Science *261*, 609–612.

Shuford, W.W., Klussman, K., Tritchler, D.D., Loo, D.T., Chalupny, J., Siadak, A.W., Brown, T.J., Emswiler, J., Raecho, H., Larsen, C.P., et al. (1997). 4–1BB costimulatory signals preferentially induce $CD8^+$ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses. J. Exp. Med. *186*, 47–55.

Stuber, E., and Strober, W. (1996). The T cell–B cell interaction via OX40–OX40L is necessary for the T cell–dependent humoral immune response. J. Exp. Med. *183*, 979–989.

Stuber, E., Neurath, M., Calderhead, D., Fell, H.P., and Strober, W. (1995). Cross-linking of OX40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic cells. Immunity *2*, 507–521.

Takahashi, C., Mittler, R.S., and Vella, A.T. (1999). Cutting edge: 4–1BB is a bona fide CD8 T cell survival signal. J. Immunol. *162*, 5037–5040.

Tuosto, L., and Acuto, O. (1998). CD28 affects the earliest signaling events generated by TCR engagement. Eur. J. Immunol. *28*, 2131–2142.

Vinay, D.S., and Kwon, B.S. (1998). Role of 4–1BB in immune responses. Semin. Immunol. *10*, 481–489.

Watts, T.H., and DeBenedette, M.A. (1999). T cell co-stimulatory molecules other than CD28. Curr. Opin. Immunol. *11*, 286–293.

Weinberg, A.D., Bourdette, D.N., Sullivan, T.J., Lemon, M., Wallin, J.J., Maziarz, R., Davey, M., Palida, F., Godfrey, W., Engleman, E., et al. (1996). Selective depletion of myelin-reactive T cells with the anti-OX-40 antibody ameliorates autoimmune encephalomyelitis. Nat. Med. *2*, 183–189.

Weinberg, A.D., Vella, A.T., and Croft, M. (1998). OX-40: life beyond the effector T cell stage. Semin. Immunol. *10*, 471–480.

Whitmire, J.K., Slifka, M.K., Grewal, I.S., Flavell, R.A., and Ahmed, R. (1996). CD40 ligand-deficient mice generate a normal primary cytotoxic T-lymphocyte response but a defective humoral response to a viral infection. J. Virol. *70*, 8375–8381.

Wong, B.R., Josien, R., Lee, S.Y., Sauter, B., Li, H.L., Steinman, R.M., and Choi, Y. (1997). TRANCE (tumor necrosis factor [TNF]–related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. J. Exp. Med. *186*, 2075–2080.