Stimulation via CD40 can substitute for CD4 T cell function in preventing reactivation of a latent herpesvirus

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Reactivation of latent herpesviruses is a particular problem in immunocompromised individuals, such as AIDS patients, who lack effective CD4 T helper cell function. An important question is whether residual immune defenses can be mobilized to combat such opportunistic infections, in the absence of CD4 T cells. In the present study, we used a mouse model of opportunistic infection to determine whether stimulation via CD40 could substitute for CD4 T cell function in preventing reactivation of a latent herpesvirus. Treatment with an agonistic antibody to CD40 was highly effective in preventing reactivation of latent murine gammaherpesvirus (MHV-68) in the lungs of CD4 T cell-deficient mice. $CD8^+$ T cells were essential for this effect, whereas virus-specific serum antibody was undetectable and IFN-γ production was unchanged. This demonstration that immunostimulation via CD40 can replace CD4 T cell help in controlling latent virus *in vivo* has potential implications for the development of novel therapeutic agents to prevent viral reactivation in immunocompromised patients.

One of the most serious and life-threatening consequences of HIV infection stems from the gradual loss of $CD4^+$ "helper" T cells. These cells play multiple roles in immune defense and can act as effectors, or as helpers, for CD8 cytotoxic T lymphocyte (CTL) and B cell responses. Thus, loss of CD4 T cell function could result in defects in several critical pathways of host immune defense against opportunistic pathogens. In AIDS, CD4 T cell counts are predictive of the types and severity of disease caused by herpesviruses (1). Induction of disease is largely due to reactivation of latent herpesviruses, although new infections may also occur. Although CD8 T cells can clear many viral infections in the absence of CD4 T cells, helper-dependent CD8 T cell responses to viruses have also been demonstrated. Furthermore, in some viral infections, CD4 T cell help is required for long-term but not for acute CD8 T cell responses. For example, whereas lymphocytic choriomeningitis virus-infected mice mount strong primary CTL responses in the absence of CD4 T cell help, long-term or memory CD8 T cell responses are significantly impaired (2–5). The importance of CD4 T cell help in CD8 T cell responses has also been demonstrated in immunosuppressed patients with cytomegalovirus (human herpesvirus-5)-mediated disease. In such patients, it has been shown that the adoptive transfer of cytotoxic CD8 T cell clones to combat cytomegalovirus reactivation is more effective if CD4 T cells are also transferred (6).

Infection of mice with murine gammaherpesvirus-68 (MHV-68) provides a useful small animal model for studying the role of CD4 T cells in the long-term control of persistent viral infections. MHV-68 is a naturally occurring rodent pathogen (7) that is closely related to Epstein–Barr virus (HHV-4) and Kaposi's sarcoma-associated herpesvirus (HHV-8) (8, 9). Intranasal administration of MHV-68 results in acute productive infection of lung alveolar epithelial cells and a latent infection in several cell types, including B lymphocytes, dendritic cells, epithelial cells, and macrophages (10–13). Infectious virus is cleared from the lungs by a T cell-mediated process 10–13 days after infection (14). In normal mice, the lungs remain clear of infectious virus thereafter. MHC Class II $-/-$ mice, which lack functional CD4 T cells, or mice rendered deficient in the latter by antibody treatment, seem initially to control infectious virus (15, 16). However, infectious virus reappears in the lungs 25–35 days after the initial challenge and gradually increases in titer (15, 16). T cell subset depletion experiments in B cell-deficient mice during the latent phase of infection showed that either CD4 or CD8 T cells could prevent reactivation of MHV-68, whereas similar studies in wild-type mice suggested that antibody could also prevent reactivation from latency (12, 15). However, the antibody and CD8 T cell responses do not appear to develop efficiently in the absence of CD4 T cells (15).

A number of recent papers have indicated a central role for CD40-CD40 ligand (CD40L) interactions in mediating CD4 T cell function in the provision of help for CTL (17–19). CD40 is expressed constitutively by B cells, dendritic cells, and macrophages, whereas CD40L is up-regulated on CD4 T cells upon activation. In the present study, we investigated whether treatment with an agonistic antibody to CD40 could substitute for CD4 T cell function in preventing reactivation of a latent herpesvirus. Intriguingly, we found that treatment with the anti-CD40 antibody could reconstitute control of viral latency and prevent reactivation, in the absence of a functional CD4 T cell compartment.

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Materials and Methods

Mice. C57BL/6 mice that were homozygous for the disruption of the H-IA^b gene (MHC Class II $-/-$; ref. 20) were obtained from Taconic Farms or from breeding colonies at La Jolla Institute for Allergy and Immunology (LIAI). Mice were bred and housed under specific pathogen-free conditions in the vivarium at LIAI. The genotype of the mice was confirmed by determining the percentage of CD4 T cells in splenocyte populations by fluorescence-activated cell sorter (Becton Dickinson) analysis. Age-matched 6- to 15-wk-old female MHC Class II $-/-$ and wild-type mice were used in all experiments.

Viral Infection and Sampling. MHV-68 virus (clone G2.4) was obtained from A. A. Nash, Edinburgh, U.K., and stocks were grown in owl monkey kidney cells (ATCC CRL 1556). Mice were anesthetized with Avertin (2,2,2-tribromoethanol) and infected intranasally with 2×10^4 plaque-forming units (pfu) of the virus in PBS. Virus-infected MHC Class II $-/-$

Abbreviations: CTL, cytotoxic T lymphocyte; MHV-68, murine gammaherpesvirus-68; CD40L, CD40 ligand; pfu, plaque-forming unit; APC, antigen-presenting cell; BAL, bronchoalveolar lavage.

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mice (regardless of whether they had been treated with anti-CD40) appeared to be susceptible to secondary bacterial infections, and, therefore, in the majority of experiments, both MHC Class II −/− and wild-type control mice were housed in sterile caging with sterile food and water. Seven to sixty days after infection, the mice were terminally anesthetized with Avertin. The lungs were removed and homogenized in medium on ice by using a Tissue Tearor homogenizer (Fisher Scientific, Pittsburgh, PA) before virus titration. Single cell suspensions were prepared from the spleen, as previously described (21). Cell viability was determined by trypan blue exclusion.

Treatment with Antibodies to CD40 *in Vivo*. Mice were treated with 100 μg FGK45, a rat monoclonal antibody to mouse CD40 (22), or with either a control whole rat Ig preparation or an isotype control antibody (clone R35-95 from PharMingen). The antibodies were diluted in sterile PBS and injected i.v., 1 and 15 days after infection with MHV-68.

Depletion of CD8 T Cells. Mice were depleted of CD8 T cells by i.p. injection of 100 μg of the monoclonal anti-CD8 antibody, 2.43, on days 18, 19, and 20 postinfection and weekly thereafter.

Virus Titration and Infectious Centers Assay. Titers of replicating virus were determined by plaque assay on NIH 3T3 cells (ATCC CRL1658) as described previously (15). Briefly, dilutions of stock virus or homogenized mouse tissues were adsorbed onto NIH 3T3 monolayers for 1 h at 37°C and overlaid with carboxymethyl cellulose. After 6 days, the carboxymethyl cellulose overlay was removed, and the monolayers were fixed with methanol and stained with Giemsa to facilitate determination of the number of plaques. The detection limit of this assay is 10 pfu/0.1 g lung tissue or 3 pfu/10^7 splenocytes, based on plaques recovered from homogenates of uninfected lung or splenocytes spiked with known amounts of virus.

The frequency of latently infected lymphocytes was determined by using an infectious centers assay. Leukocyte suspensions prepared from lymph nodes or spleen were plated at various cell densities on monolayers of NIH 3T3 cells, incubated overnight, and then overlaid with carboxymethyl cellulose. The cells were cocultured for 5–6 days, after which the overlay was removed and the number of plaques was determined as described above.

Flow Cytometric Analysis. Cells were stained with phycoerythrin- or FITC-conjugated monoclonal antibodies as described previously (23). All antibodies were purchased from PharMingen. Isotype controls were included in each assay.

Cytokine ELISAs. IFN-γ levels in culture supernatants from cells that had been restimulated *in vitro* with virus-infected splenic antigen-presenting cells (APCs) were assayed by sandwich ELISA as described previously (24). The detection limit of this assay was 13.7 pg/ml. Uninfected APCs or cultures containing infected APCs alone were used as controls. All reagents were obtained from PharMingen.

ELISA for Virus-Specific Antibody. Serum antibody titers were determined by ELISA. Nunc Maxisorp plates were coated overnight at 4°C with a 1/100 dilution of sucrose gradient purified MHV-68 in 0.1 M sodium bicarbonate (pH 9.0). Plates were blocked with PBS containing 1% BSA and were incubated for 1 h at room temperature with various dilutions of serum from animals sampled 50 days after infection with MHV-68. Sera from uninfected mice and positive control sera were included in each assay. Bound antibody was detected by using peroxidase-conjugated anti-mouse antisera (Southern Biotechnology Associates) and 2,2′-azino-di-(3-ethyl)benzthiazoline-6-sulfonic acid substrate. The absorbance was measured at 405 nM. The titer of a serum sample was taken as the $-\log_{10}$ of the highest dilution that gave a reading >0.1.

Cytotoxic T Cell Assays. Virus-specific cytotoxic T cell activity was determined by using a chromium-release assay on suspensions of bronchoalveolar lavage (BAL) or spleen cells from MHV-68-infected animals. Mouse embryonic fibroblast (MEF)-1 cells (ATCC CRL 2214) were infected with 2–5 pfu MHV-68/cell for 2 h at 37°C before loading the cells with ^{51}Cr (Dupont/NEN) for 1 h at 37°C. After washing, 5×10^3 targets were incubated with graded numbers of spleen or BAL cells (in duplicate) for 6–8 h at 37°C. Supernatants were then harvested for gamma-counting. Total release (T) was determined by adding Triton X-100 (2%) to the targets. Spontaneous release (S) was determined from targets cultured in the absence of effector cells. The level of specific chromium release was calculated from the formula: % specific lysis = $(E - S)/(T - S)$, where E is the experimental chromium release in the presence of cytotoxic cells.

Intracellular Staining for IFN-γ. The frequencies of MHV-68-specific CD8 T cells in spleen or BAL was determined by intracellular staining for IFN-γ, as described by Stevenson *et al.* (25), after a 6-h restimulation with MHV-68 peptides p56 (AGPHNDMEI from ORF6) and p79 (TSINFVKI from ORF 61), which have previously been identified as CD8 T cell epitopes (26, 27). All reagents were obtained from BD PharMingen and were used according to protocols supplied by the manufacturer. Cell suspensions were incubated with the peptides (1 μM) for 6 h at 37°C in the presence of monensin (2 μM) before cell surface staining with FITC-conjugated anti-CD8a (clone 53–6.7). The cells were then fixed with paraformaldehyde and permeabilized by using saponin in a single step (using Cytofix/Cytoperm reagent; PharMingen, San Diego, CA), before intracellular staining with phycoerythrin-conjugated anti-IFN-γ (clone XMG1.2) and flow cytometric analysis.

Results

Agonistic Antibodies to CD40 Prevent Viral Reactivation in MHC Class II −/− Mice. Lung virus titers were determined 7, 15, 35, 50, or 60 days after infection with MHV-68. Anti-CD40- and isotype-control-treated MHC Class II −/− mice showed similar high levels of virus in their lungs 7 days after infection, and no virus was detected by day 15 after infection (Fig. 1). As expected, all MHC Class II −/− mice treated with control antibody showed significant viral reactivation (Fig. 1) at days 35–60 after infection. Anti-CD40 treatment, however, was very effective in preventing viral reactivation, and the majority of treated mice showed no significant viral reactivation. As reported previously (15), wild-type mice did not show viral reactivation at days 35, 50, or 60 after infection (data not shown).

Anti-CD40 Treatment Does Not Prevent the Establishment of Latency. Levels of latent virus were also determined by using an infectious centers assay. This was performed on splenocytes harvested at day 50 after infection. The frequency of latently infected cells was not significantly different in anti-CD40-treated MHC Class II −/− mice and those treated with control Ig. The mean number of infectious centers/10^7 splenocytes ± SEM for duplicate determinations from four separate experiments was 18 ± 4 for the control group (n = 14) and 15 ± 5 for the anti-CD40 treated group (n = 13). n is the total number of animals sampled. These data indicate that, like CD4 T cells, anti-CD40 treatment assists in controlling viral reactivation but does not prevent the establishment of latency. As expected, no lytic virus was detected in splenocyte homogenates.

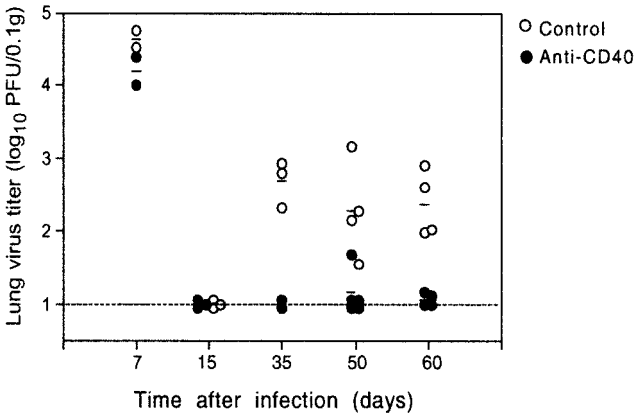

Fig. 1. Agonistic antibodies to CD40 can substitute for CD4 T cells in preventing reactivation of MHV-68 in CD4 T cell-deficient mice. MHC Class II −/− mice were infected with 2×10^4 pfu MHV-68 intranasally, and 100 μg FGK-45 anti-CD40 was injected i.v. on days 1 and 15 after infection. Control animals received Rat Ig (day 35) or IgG2a isotype control antibody (all other time points). Lung virus titers were determined by plaque assay at day 7, 15, 35, 50, and 60 days after infection. The limit of detection was 10 pfu/0.1 g lung tissue. Data are expressed as log_{10} pfu/0.1 g of lung tissue for individual mice.

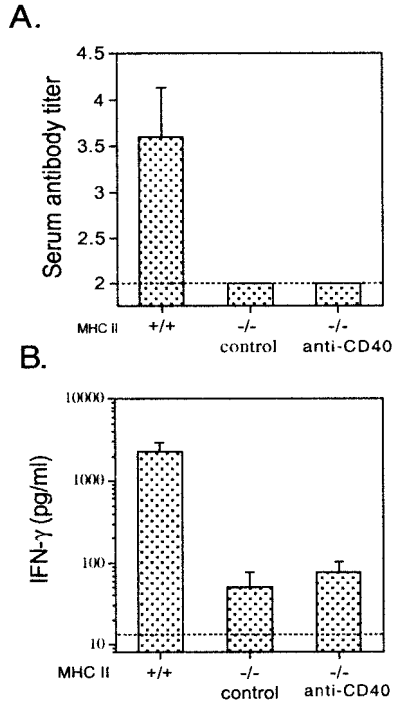

Fig. 2. Treatment of MHC Class II −/− mice with agonistic antibodies to CD40 does not increase virus-specific antibody responses or IFN-γ production. MHC Class II −/− or wild-type mice were infected with MHV-68 and treated with anti-CD40 or an isotype control antibody as described in the legend to Fig. 1. Mice were killed 50 days after infection. (*A*) Virus-specific serum antibody titers were determined by ELISA. The secondary antibody detects IgM + IgG + IgA (heavy and light chains). Data are expressed as mean serum antibody titers for 8–10 individual mice from three separate experiments. Two to four mice were sampled in each experiment. (*B*) IFN-γ production. Splenocytes were restimulated *in vitro* with virus-infected APCs, and IFN-γ in the supernatants was determined by ELISA. Data are expressed as mean IFN-γ concentrations + SEM for 4–6 mice from two separate experiments. Peak values (which were usually attained after 72-h culture) are reported. Duplicate cultures from two to three mice were sampled in each experiment. The detection limit was 13.7 pg IFN-γ/ml.

Anti-CD40 Treatment Has No Effect on Virus-Specific Serum Antibody Titers or IFN-γ Production. The effect of treatment with agonistic antibodies to CD40 on serum antibody titers and IFN-γ production was also determined. Virus-specific antibody titers were assayed by ELISA on serum samples taken 50 days after infection. Anti-CD40 treatment could not restore the virus-specific antibody response in MHC Class II −/− mice. Neither anti-CD40 nor isotype control-treated groups of MHC Class II −/− mice had detectable MHV-68-specific titers of total Ig, IgG1, IgG2a, or IgG2b (all were <1/100), whereas wild-type mice had titers ranging from 1/1,000–1/100,000 (Fig. 2*A* and data not shown). Titers of virus-specific IgM, IgA, and IgG3 were at or below the detection limit (1/100) for wild-type, anti-CD40-treated MHC Class II −/− and isotype control-treated MHC Class II −/− mice (data not shown).

When splenocytes from MHC Class II −/− mice treated with anti-CD40 or control antibody were restimulated *in vitro* with virus-infected APCs at day 50 after infection, there was no significant difference between the two groups in the levels of IFN-γ produced (Fig. 2*B*, $P = 0.46$, Student's t test). Splenocytes from MHV-68-infected wild-type mice produced much higher levels of IFN-γ under the same conditions (Fig. 2*B*), probably because of the contribution of CD4 T cells to production of this cytokine.

Because there was no significant increase in serum antibody titers or IFN-γ production, our data suggest that the anti-CD40 treatment does not work via either of these pathways.

CD8 T Cells Are Required for the Effect of Anti-CD40 on Viral Reactivation. To determine whether CD8 T cells played an essential role in the effect of anti-CD40 treatment, this subset was depleted by administration of anti-CD8 antibodies *in vivo*. Because CD8 T cells are required for initial viral clearance in MHC Class II −/− mice, depletion commenced at day 18, which is a time-point after initial viral clearance, but before viral reactivation is observed. Depletion of CD8 T cells completely blocked the preventative effect of anti-CD40 treatment on viral reactivation, showing the essential role of this T cell subset (Fig. 3). Thus, whereas anti-CD40-treated MHV-68-infected MHC Class II −/− mice did not have significant levels of infectious virus in their lungs, CD8-depleted anti-CD40-treated animals showed significant viral reactivation (Fig. 3). Lung virus titers in a group of MHC Class II −/− mice depleted of CD8 T cells and treated with an isotope-matched control antibody were not significantly different from those in CD8-depleted anti-CD40-treated mice (data not shown) but, as previously reported (15), were significantly higher than for undepleted MHV-68-infected MHC Class II −/− mice (mean ± SD 257 ± 200 vs. 39 ± 47 pfu/0.1 g lung tissue, $P = 0.01$, Student's t test). This result suggests that, in the absence of "help," the CD8 T cells function to some extent, but are inefficient in preventing viral reactivation.

To determine whether anti-CD40 treatment resulted in an expansion of virus-specific CD8 T cells, the frequencies of virus-specific CD8 T cells were determined at day 50 after infection, by intracellular staining for IFN-γ after a brief restimulation with viral peptides, p56 and p79, which were previously characterized as prominent CD8 T cell epitopes (26, 27). Cytolytic activity against MHV-68-infected fibroblast targets was also determined. Despite the obvious requirement for CD8 T cells in the effect of anti-CD40 treatment on viral reactivation, there was no detectable increase in the frequency of virus-specific CD8 T cells in the anti-CD40-treated group compared with the control antibody-treated group (Table 1). The frequencies detected in both anti-CD40- and control antibody-treated mice were in accordance with those previously reported by Stevenson *et al.* for MHV-68-infected MHC Class II −/− mice

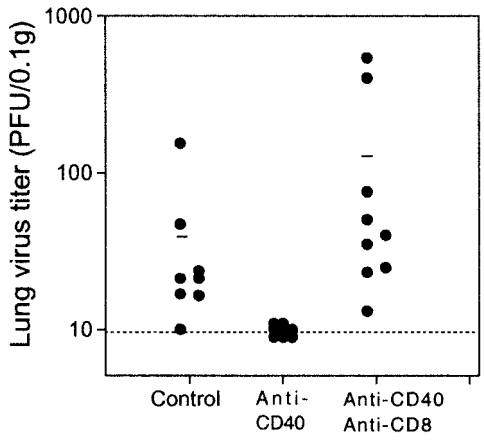

Fig. 3. Agonistic antibodies to CD40 cannot prevent viral reactivation in the absence of CD8 T cells. MHC Class II −/− mice were infected with MHV-68 and treated with anti-CD40 or an isotype control antibody as described in the legend to Fig. 1. A group of anti-CD40-treated mice was also depleted of CD8 T cells by treatment with mAb 2.43 commencing 18 days after infection. Lung virus titers were determined 50 days after infection. Data are expressed as $\log_{10}$ pfu/0.1 g of lung tissue for individual mice and are derived from two separate experiments.

(25). As reported in the latter study, the frequency of CD8 T cells specific for the two viral epitopes tested was not substantially higher in wild-type than in MHC Class II −/− mice. Total BAL and spleen cell numbers per mouse were comparable in anti-CD40- and control-antibody-treated MHC Class II −/− mice (Table 2). Thus, there was no difference in the absolute number of virus-specific CD8 T cells in BAL or spleen. Furthermore, virus-specific cytolytic activity, measured *in vitro* by using MHV-68-infected MEF targets, was not significantly altered in anti-CD40-treated mice (Table 2).

Discussion

Previous studies have shown that CD4 T cells are not required for primary clearance of infectious MHV-68, but are essential for the long-term control of latent virus (15). CD4 or CD8 T cells or antibody can prevent viral reactivation (12, 15). However, the antibody and CD8 T cell responses do not appear to develop efficiently in the absence of CD4 T cells (15). In the present study, we assessed the ability of agonistic antibodies to CD40 to substitute for CD4 T cell function in preventing reactivation of MHV-68. Surprisingly, despite the diverse array of costimulatory molecules present on CD4 T cells, treatment with agonistic antibodies to CD40 was sufficient to prevent reactivation of MHV-68. Thus, anti-CD40-treated MHC Class II −/− mice did not show viral reactivation, whereas mice treated with a control antibody showed significant lung virus titers. The effect of the anti-CD40 antibody lasted for at least 45 days after the second injection. Because reactivation of latent herpesviruses is a significant problem in immunocompromised individuals, who lack effective CD4 T cell function, the observations made in our study may have implications for mobilizing residual immune defenses to prevent viral reactivation in such individuals.

Table 2. Effect of anti-CD40 treatment on MHV-68-specific cytolytic activity in spleen and BAL of MHC Class II −/− mice

		% Specific lysis			
		BAL		Spleen	
Mice	Treatment	10:1*	2.5:1*	50:1*	*n*
−/−	Control	31.0 ± 15.0	19.3 ± 15.4	26.3 ± 10.4	4
−/−	Anti-CD40	25.0 ± 3.1	14.0 ± 2.4	25.0 ± 10.0	4
+/+	None	43.0	14.0	25	1†

MHC Class II −/− or +/+ mice were infected with MHV-68 and treated with anti-CD40 or control antibody as described in the legend to Table 1. BAL and spleen cells harvested 60 days after infection were tested for virus-specific CTL activity in a chromium release assay using MHV-68-infected MEF-1 cells as targets. Data are expressed as mean percent specific lysis ± SD, for the effector to target (E:T) ratios specified. *n*, number of mice tested.
*E:T ratio.
†Cells from two wild-type mice were pooled for CTL assays.

Our studies show that CD8 T cells play an essential role in anti-CD40-mediated control of viral reactivation, as depletion of this subset ablated the effect. In contrast, virus-specific antibody did not appear to be involved and was not detected in anti-CD40-treated mice. However, paradoxically, the effect of anti-CD40 antibody on CD8 T cells appeared to affect their *in vivo* efficacy in virus control, rather than their frequency or their *in vitro* cytolytic activity. This finding differs from observations in previous studies, in which treatment of CD4 T cell-deficient mice with anti-CD40 antibodies was found to increase antigen-specific CTL activity measured *in vitro* (17–19). In the latter studies, no CTL activity could be detected in the absence of anti-CD40 treatment, whereas in the present study, similar levels of CTL activity were measured in cell preparations from anti-CD40 or control antibody-treated MHC Class II −/− mice. However, the data in the present study are consistent with previous studies in the MHV-68 model, in which no difference in the frequency of virus-specific CD8 T cells or CTL activity *in vitro* was observed in wild-type and CD4 T cell-deficient mice (15, 25). In contrast to the data from *in vitro* studies on MHV-68, *in vivo* studies have shown functional differences between CD8 T cells that have developed in the presence or absence of CD4 T cell help (12, 15).

There could be several explanations for our observation that anti-CD40 treatment affects the ability of CD8 T cells to prevent viral reactivation *in vivo*, but does not affect the frequency of virus-specific CD8 T cells or *in vitro* cytolytic function: (*i*) Cytolytic activity measured *in vitro* may not reflect that *in vivo*,

Table 1. Treatment with anti-CD40 Ab does not alter the frequency of MHV-68-specific CD8 T cells in BAL or spleen of MHC Class II −/− mice

		Percentage IFN-γ-positive of total $CD8^+$ population				Total cell no./mouse		
		BAL		Spleen				
Mice	Treatment	p56	p79	p56	p79	BAL (×10^5)	Spleen (×10^7)	*n*
−/−	Control	5.0 ± 2.0	6.1 ± 3.1	1.4 ± 0.6	1.0 ± 0.2	4.8 ± 1.8	6.1 ± 2.0	7
−/−	Anti-CD40	4.4 ± 1.8	5.8 ± 4.3	1.5 ± 0.9	1.2 ± 0.3	4.1 ± 2.0	7.4 ± 2.3	7
+/+	None	2.5	1.5	0.5	1.4	2.7	7.3	2

MHC Class II −/− or +/+ mice were infected with 2×10^4 pfu MHV-68, and BAL and spleen cells were harvested 60 days after infection. MHC Class II −/− mice were treated with 100 μg anti-CD40 or control antibody i.v. 1 and 15 days after infection. BAL or spleen cells were stimulated for 6 h with either p56 or p79 peptides in the presence of monensin prior to staining for CD8 and IFN-γ. Results are shown as mean ± SD. *n*, total number of mice analyzed.

because of differential requirements for adhesion, costimulatory or other molecules expressed by CD8 T cells. In addition, different viral peptides may be presented *in vitro* and *in vivo*, because of cell type-specific and temporal differences in viral gene expression. Therefore, it is possible that CTL specific for as-yet unidentified viral epitopes may be important in the long-term control of MHV-68. (*ii*) CD8 T cells may control latent MHV-68 by a non-cytolytic mechanism. Recent evidence has shown that hepatitis B virus (HBV) is cleared from hepatocytes by a non-cytolytic mechanism (28). Furthermore, CD8 T cells have been shown to prevent the reactivation of herpes simplex virus in ganglion cultures without lysing the infected neurons (29). Although IFN-γ played an important role in the clearance of HBV (30), we were unable to detect any change in the frequency of IFN-γ-producing CD8 T cells or in the levels of IFN-γ produced, after anti-CD40 antibody treatment of MHV-68-infected MHC Class II −/− mice. This observation suggests that the effect of anti-CD40 antibodies on MHV-68 reactivation is not mediated by IFN-γ alone, although it is possible that other cytokines play a role. (*iii*) CD8 T cell activity may be unchanged, whereas the anti-CD40 treatment exerts an effect on the environment, which enables the CD8 T cells to function efficiently. This effect might involve up-regulation of MHC or adhesion molecules on virus-infected lung epithelial cells. Alternatively, anti-CD40 antibody might exert a direct anti-viral effect, which would reduce the load of virus present, to levels that could be adequately controlled by CD8 T cells. In this context, vaccinia constructs encoding CD40L have been reported to replicate poorly, indicating an anti-viral activity (30). Furthermore, inflammatory cytokines have been reported to up-regulate CD40 expression on epithelial cells (31). However, in the present study, peak lung viral titers (at day 7 after infection) in anti-CD40- and control antibody-treated mice were very similar. Viral latency also appeared to be unaltered. These data suggest that a direct antiviral activity is unlikely. More work is needed to distinguish between the possible mechanisms outlined above.

In summary, these data show that immunostimulation via CD40 is sufficient for the generation and maintenance of immune mechanisms that prevent viral reactivation. The fact that CD40L −/− (32) and CD40 −/− (S.R.S., unpublished data) mice also show late reactivation of MHV-68 in the lungs confirms that this interaction is essential for preventing viral reactivation and that there are no redundant pathways. Furthermore, the data presented in the current study may point the way to a novel method of immunotherapy to prevent virus reactivation in immunosuppressed individuals.

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