OX40 Costimulation Promotes Persistence of Cytomegalovirus-Specific CD8 T Cells: A CD4-Dependent Mechanism[1]

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The mechanisms that regulate CMV-specific T cell responses in vivo are poorly understood. During murine CMV infection of B6 mice, primary responses in the spleen are dominated by CD8 T cells reactive with antigenic epitopes in M45, M57, and m139 murine CMV gene products. However, during the later persistent phase of infection, CD8 T cell responses to epitopes in m139 and M38 viral gene products predominate. The basis for this shift in CD8 T populations is unknown. In this study, we demonstrate that OX40, a TNFR superfamily member, specifically regulates the accumulation of CD8 T cells reactive with the persistent-phase epitopes. Defective CD8 T cell responses in OX40$^{-/-}$ mice were replicated in MHC class II$^{-/-}$ mice implying that CD4 T cells in part controlled the differentiation of the CD8 T cell clones responsive to these epitopes during persistent infection. Furthermore, treatment of infected mice with an agonist OX40 Ab induced expansion of protective primary virus-specific CD8 T cells independent of CD4 T cell help, but CD4 T cells were crucial for anti-OX40 to promote CD8 T cells reactive to the persistent dominant epitopes. Collectively, these results indicate manipulation of OX40 may be useful in improving cellular immunotherapy regimes for treatment of persistent virus infections. *The Journal of Immunology,* **2007, 179: 2195–2202.**

H uman CMV (HCMV)[3] is a β-herpesvirus that causes life-long, species-specific infection in vertebrate hosts. HCMV infection in humans is generally asymptomatic in healthy individuals, but results in multiorgan disease in the immunologically immature (e.g., congenital infection), and in the immune compromised (e.g., organ transplant recipients and AIDS patients) (1).

A critical role for CD8 T cells in the control of HCMV is suggested by the correlation between virus reactivation in immune-compromised patients and defective CD8 T cell responses (2, 3). Moreover, transfer of HCMV-specific CD8 T cells can protect immune-suppressed individuals from virus reactivation (4–6). However, despite the efficacy of this treatment, it is not widely adopted due to extensive technical and financial demands of ex vivo cell culture. Another important aspect of HCMV-specific T

cell responses is the unusually high frequency of virus-specific CD4 and CD8 T cells in healthy seropositive individuals (7). This expansion of cells increases with age (8) and is associated with defective T cell responses to heterologous Ags (9, 10), suggesting that HCMV infection may impair protective immunity to other pathogens.

Mouse CMV (MCMV) serves as a useful model to investigate the mechanisms controlling T cell responses to a viral pathogen with latent and persistent properties. After resolution of the primary infection, CD8 T cells control virus reactivation from latency (11), whereas CD4 T cells control persistent virus replication in the salivary gland (12). Similar to HCMV, MCMV-specific memory CD8 T cells expand to very high frequencies as infection progresses (13, 14), and transfer of either primary effector or memory CD8 T cells from MCMV immune donors can inhibit virus replication and disease in immune-suppressed recipients (15–18).

Although a role of CD8 T cells in controlling MCMV infection is established, the mechanisms that regulate expansion of MCMV-specific CD8 T cells and maintenance of the pool of memory T cells are poorly understood. In particular, the role of cosignaling pathways that operate during Ag recognition that determine expansion of effector cells and memory development have not been investigated. The TNFR superfamily member OX40 (CD134) is an inducible cosignaling molecule expressed by T cells (19–21). OX40 was initially described as a costimulatory receptor for CD4 T cells regulating primary and memory responses (19, 20, 22–24). In contrast, early reports using OX40- and OX40 ligand (OX40L)-deficient mice indicated that OX40 was dispensable for primary CD8 T cell responses to influenza virus, lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), and Theiler's murine encephalomyelitis virus (25–27). However, more recently, in response to rechallenge with influenza infection (28), CD8 T cell priming

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[3] Abbreviations used in this paper: HCMV, human CMV; MCMV, murine CMV; OX40L, OX40 ligand; LCMV, lymphocytic choriomeningitis virus; VSV, vesicular stomatitis virus.

was found to be impaired in the absence of OX40-OX40L interactions. Furthermore, CD8 T cell responses were also impaired when OX40 was targeted in mouse models of contact hypersensitivity (26) and reaction to tissue allografts (29), and OX40 was shown to directly signal CD8 T cells and control primary (30, 31) and secondary CD8 T cell responses (32) to Ag presented in adjuvant, in adenoviral vectors, or expressed by tumors cells.

We now report a critical role for the OX40 cosignaling pathway in the regulation of MCMV-specific CD8 T cells during the persistent phase of virus replication. Using genetic and pharmacological approaches, we demonstrated that OX40 signaling promoted accumulation of MCMV-specific CD8 T cell populations after the acute phase of infection and this required the participation of CD4 T cells. By contrast, activation of the OX40 cosignaling pathway induced a massive expansion of MCMV-specific CD8 T cells during the initial infection, independent of CD4 T cells. These findings substantiate the role of OX40 in regulating CD8 T cell responses that are both independent and dependent on CD4 T cells and provide insight into the mechanisms that regulate the expansion and survival of MCMV-specific T cells in vivo.

Materials and Methods

Mice

Wild-type C57BL/6 (B6) mice were purchased from The Jackson Laboratory and MHC class II-deficient mice (33) were purchased from Taconic Farms. OX40$^{-/-}$ knockout mice (27) on the B6 background were bred in-house. All experiments were conducted following the guidelines of the La Jolla Institute for Allergy and Immunology's Institutional Animal Care and Use Committee.

Virus, mouse infections, and treatments

MCMV Smith strain (American Type Culture Collection (ATCC)) was prepared in BALB/c salivary glands, and titered on 3T3 cells as described previously (34). Mice were infected i.p. with 5×10^4 PFU MCMV. Mice were injected at the time of infection with 100 μg of either rat IgG (Chemicon International) or anti-OX40 (clone OX86 (35)). In other experiments, mice were irradiated (700 rad), infected i.p. with 5×10^3 PFU of MCMV and injected i.v. with 1×10^5 CD8$^+$ T cells purified from spleens of MCMV-infected mice (8 days postinfection) by MACS separation, according to manufacturer's instructions (Miltenyi Biotec).

Intracellular cytokine staining and flow cytometry

To evaluate CD8 T cell responses, splenocytes were isolated from MCMV-infected mice and incubated at 5×10^6 cells/ml in the presence of 2 μg/ml brefeldin A (Sigma-Aldrich) and 2 μg/ml MCMV-derived peptides (Genemed Synthesis) for 5 h at 37°C. The peptides used were the H-2D^b-restricted M45-derived peptide HGIRNASFI, and the H-2K^b-restricted peptides derived from the M38 (SSPPMFRVP), M57 (SCLEFWQRV), and m139 (TVYGFCLL) open reading frames, as previously described (36). Cells were then washed and incubated with Fc block (BD Pharmingen). Cells incubated with peptide (or medium-treated controls) were then surface stained with anti-CD8α-PE-Cy7. In some experiments, cells were also stained with anti-CD43-PE and anti-CD44-CyChrome or anti-CD122-FITC and anti-CD127-PE. For evaluation of CD4 T cell responsiveness, splenocytes were stimulated with 1 μg/ml anti-CD3 (clone 145-2C11) and 1 μg/ml anti-CD28 (clone 37.51; BD Pharmingen) for 5 h at 37°C in the presence of brefeldin A, and stained with anti-CD8α-PE-Cy7 and anti-CD4 Pacific Blue (all Abs purchased from BD Pharmingen). All cells were then fixed with 3% formalin, permeabilized with saponin buffer (PBS, 2% FCS, 0.05% sodium azide, and 0.5% saponin), and stained with anti-IFN-γ-allophycocyanin (BD Pharmingen) and, in some experiments, with anti-IL-2-PE (eBioscience) or anti-human granzyme B-PE (Caltag Laboratories/Invitrogen Life Technologies). In other experiments, splenocytes were immediately stained with anti-CD8α-PE-Cy7, anti-CD4-Pacific Blue, and OX40-PE (Serotec). All data were acquired on an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo software.
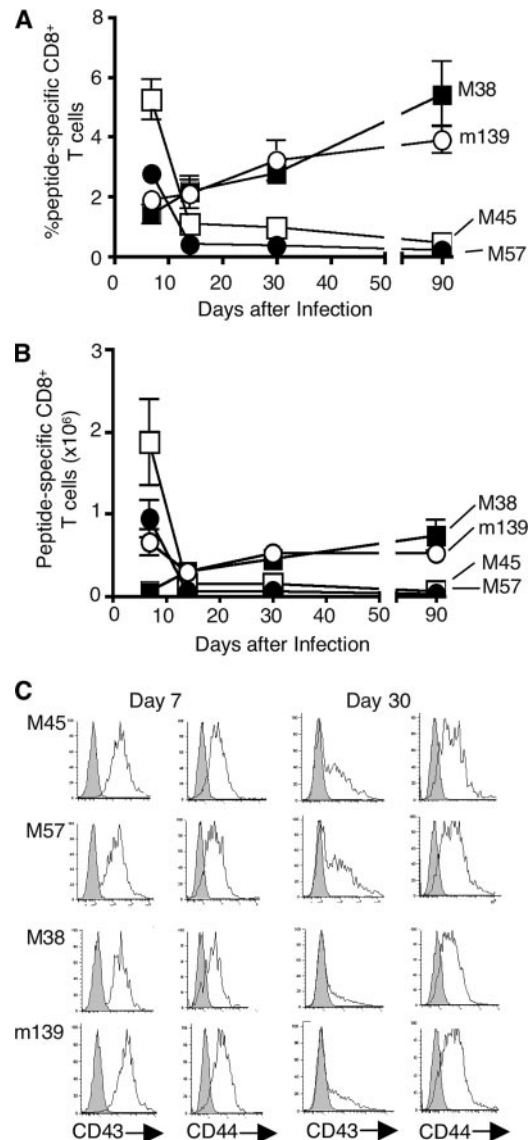

FIGURE 1. The hierarchy of immunodominance and phenotype of virus-specific CD8 T cells changes during MCMV infection. B6 mice were infected i.p. with MCMV. *A*, On days 7, 14, 30, and 90, splenic CD8 T cells specific for M38 (■), M45 (□), M57 (●), and M139 (○) peptides were detected by staining for intracellular IFN-γ after restimulation in vitro. Results are expressed as mean percent (*A*) and total numbers (*B*) of peptide-specific CD8 T cells ± SEM of four mice per group. *C*, CD43 and CD44 expression was analyzed by gating on IFN-γ^+ MCMV peptide responding CD8 T cells taken 7 (*left panels*) and 30 (*right panels*) days postinfection. Results representative of four mice per group.

Results

OX40 is required for CD8 T cells that dominate the persistent phase of MCMV infection

MCMV-specific CD8 T cells were analyzed in virus-infected B6 mice by intracellular staining for IFN-γ following ex vivo stimulation of splenocytes with class I MHC-restricted peptides derived from M38, M45, M57, and m139 proteins. Given the H-2-restricted nature of these peptides, only CD8 T cells are activated by this in vitro stimulation. The MCMV-reactive CD8 T cell populations analyzed represent ~60% of the MCMV-specific CD8 T cell response during the initial infection (36). Similar to data reported previously (37), the initial T cell response at 7–8 days

FIGURE 2. Reduced MCMV-specific CD8 T cell responses during persistent infection of OX40$^{-/-}$ mice. B6 (■) and OX40$^{-/-}$ (□) mice were infected with MCMV. *A*, Weight loss was monitored daily and results are expressed as percent of original weight. *B–D*, Peptide-specific CD8 T cells in the spleens of B6 and OX40$^{-/-}$ mice were quantified by intracellular IFN-γ staining on days 7 (*B*), 34 (*C*), and 90 (*D*) after MCMV infection. *E–H*, Accumulation and persistence of M45 (*E*), M57 (*F*), M38 (*G*), and m139 (*H*) specific CD8 T cells on days 0, 7, 34, and 90 days after infection. Results in *B–H* are derived from the same experiment and are shown as mean numbers ± SEM of four mice per group and representative of three independent experiments. Significance is ∗, $p < 0.05$ with Student's *t* test.
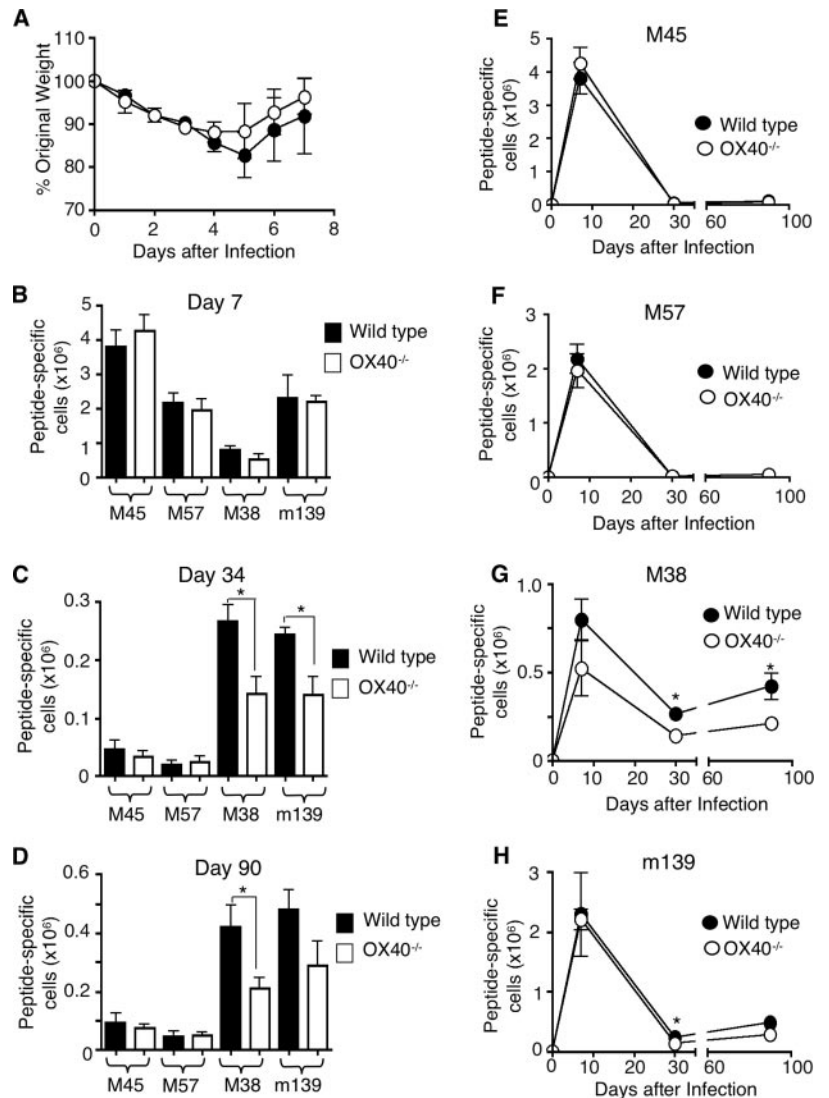
postinfection consisted primarily of M45-specific CD8 T cells (~6% of all CD8 T cells; Fig. 1, *A* and *B*). Significant populations of M57- and m139-specific cells were also detectable, with M38-reactive CD8 T cells present at the lowest frequency (<2% of CD8 T cells). However, during the persistent phase of infection (>30 days), the CD8 T cell response in the spleen shifted to being dominated by m139- and M38-specific CD8 T cells (~10% of all CD8 T cells,; Figs. 1, *A* and *B*, and 2). The progressive decrease in expression of CD43 (marker for primary effector cells), while retaining CD44, suggested that these cells transitioned over this time away from an effector state (Fig. 1*C*).

To investigate whether OX40-OX40L interactions regulated MCMV-specific CD8 T cells, wild-type B6 or OX40$^{-/-}$ mice were analyzed. Deletion of OX40 had no significant effect on weight loss induced by MCMV (Fig. 2*A*). Importantly, the generation of the primary CD8 T cell response to MCMV was also unaffected by the absence of OX40 (Fig. 2*B*). Numbers of NK cells (NK1.1$^+$CD3$^-$) and IFN-γ-expressing NK cells on days 3 and 7 postinfection were also comparable in wild-type and OX40$^{-/-}$ mice (data not shown). Furthermore, virus replication in the spleen and liver at these time points was also unaltered in the absence of OX40 (data not shown), similar to reports in mice infected with LCMV, VSV, influenza, and Theiler's murine encephalomyelitis

virus, where no major role for OX40 was found in the acute antiviral response (25–27).

In contrast, the OX40 deficiency led to a defect in the number of CD8 T cells responding to late dominant Ags, M38 or m139, detectable at 34 and, in the case of M38, 90 days postinfection (Fig. 2, *C* and *D*). Plotting peptide-specific CD8 T cells against time revealed reduced accumulation of the OX40$^{-/-}$ T cells specific for M38 and, to a lesser extent, m139 peptides (Fig. 2, *E–H*). The OX40 deficiency did not alter expression of IL7Rα chain (CD127) or IL-15R β-chain (CD122) on virus-specific T cells, suggesting that impaired CD8 T cell responses in OX40$^{-/-}$ mice were not due to reduced responsiveness to IL-7 or IL-15 (Table I). Furthermore, wild-type and OX40$^{-/-}$ CD8 T cells expressed comparable levels of granzyme B, indicating that the absence of OX40 did not affect the cytolytic potential of these cells (Table I).

OX40 regulates CD4 T cell help during the late phase of MCMV infection

To determine whether OX40 regulated CD4 T cells that might promote MCMV-specific CD8 T cells, CD4 T cell responsiveness was determined in the early (acute) and late (chronic) phases of infection. The epitopes of MCMV recognized by CD4 T cells have not been described in B6 mice, but the numbers of

Table I. *OX40 deficiency does not alter granzyme B, CD127, or CD122 expression by MCMV-specific CD8 T cells*[a]

	Granzyme B (MFI)		IL-7R (MFI)		CD122 (MFI)	
	Wild type	OX40$^{-/-}$	Wild type	OX40$^{-/-}$	Wild type	OX40$^{-/-}$
M38	12 ± 0.4	11 ± 0.4	80 ± 0.5	73 ± 10	60 ± 3	59 ± 7.5
m139	10.5 ± 0.6	10 ± 0.9	99 ± 29.5	72.9 ± 18.3	79 ± 19	62 ± 12

[a] MCMV-specific CD8 T cells were detected after 34 days of infection by IFN-γ expression following ex vivo stimulation with M38 and m139 peptides and coexpression of granzyme B, CD127, and CD122 was measured by flow cytometry. Results are expressed as mean fluorescent intensity (MFI) of four mice per group from two experiments. No significant differences were detected.

virus-reactive CD4 T cells can be reliably estimated with a short-term cytokine expression assay that detects Ag-primed T cells using limiting anti-CD3 stimulation. In OX40$^{-/-}$ mice, significantly lower numbers of IFN-γ- and IL-2-producing CD4 T cells were visualized 34 (Fig. 3*B*), but not 7 (Fig. 3*A*), days after MCMV infection. This data implied that the defect in M38- and m139-reactive CD8 T cell populations in OX40$^{-/-}$ mice might be related to a poor sustained CD4 T cell response. In line with this hypothesis, priming of M38- and m139-specific

FIGURE 3. MCMV-induced CD4 T cell responses are impaired in OX40$^{-/-}$ mice. *A* and *B*, IL-2- and IFN-γ-producing CD4 T cells were enumerated by intracellular staining 7 (*A*) and 34 (*B*) days after MCMV infection of wild-type (■) and OX40$^{-/-}$ (□) mice following in vitro stimulation with anti-CD3/CD28. Results are mean numbers ± SEM of four to eight mice per group and representative of three independent experiments. *C*, IFN-γ-producing CD8 T cells generated in B6 (■) and MHC class II$^{-/-}$ (□) mice were quantified 33 days after infection by intracellular staining following in vitro stimulation with MCMV peptides. Results represent mean ± SEM of 12 mice/group from three experiments. Significance is *, $p < 0.05$ with Student's *t* test.

CD8 T cells was also partially impaired in MHC class II-deficient mice after 33 days of infection (Fig. 3*C*) approximating the defect found in OX40$^{-/-}$ mice.

OX40 activation enhances accumulation of MCMV-specific CD8 T cells

Given that endogenous OX40 signaling augmented the frequency of persisting MCMV-specific CD8 T cells following development of the acute response, we hypothesized that targeting of OX40 with agonist Ab would enhance the accumulation and longevity of CD8 T cells, particularly reactive with M38 and m139 Ags that dominated the late phase of the infection. Administration of anti-OX40 at the time of infection strongly enhanced the numbers of IFN-γ-expressing CD8 T cells at early (Fig. 4*A*) and late (Fig. 4*B*) time points, as detected following ex vivo stimulation with anti-CD3 and anti-CD28. Moreover, anti-OX40 also enhanced the accumulation of MCMV-reactive CD8 T cells during the early response (Fig. 4*C*), and promoted a pronounced accumulation of M38 and m139 CD8 T cell populations as late as 34 days after infection (Fig. 4*D*). When these responses were plotted over time, all populations expanded at about the same rate (Fig. 4, *E–H*), but OX40 signaling only affected the long-term persistence of M38- (Fig. 4*G*) and m139-(Fig. 4*H*) specific CD8 T cells. OX40 targeting did not appreciably maintain the M45- and M57-reactive cells (Fig. 4, *E* and *F*). By 90 days postinfection, all CD8 T cell populations were equivalent in anti-OX40-treated vs untreated mice. Anti-OX40 treatment had no effect on granzyme B expression during early infection (Fig. 4*I*). Furthermore, CD8 T cells derived 7 days after infection from anti-OX40-treated mice afforded comparable protection from virus replication following transfer into irradiated MCMV-infected recipients compared with CD8 T cells derived from control-treated MCMV-infected donors (Fig. 4*J*). These results indicate that the CD8 T cell frequency rather than the antiviral potential of these cells was the primary target of OX40 action. Collectively, these data imply that CD8 T cell populations that react to different MCMV epitopes are regulated in alternate manners. OX40 can participate in the response of M45- and M57-reactive cells but does not normally play a major role, whereas OX40 strongly influences M38- and m139-reactive cells after the acute phase of infection.
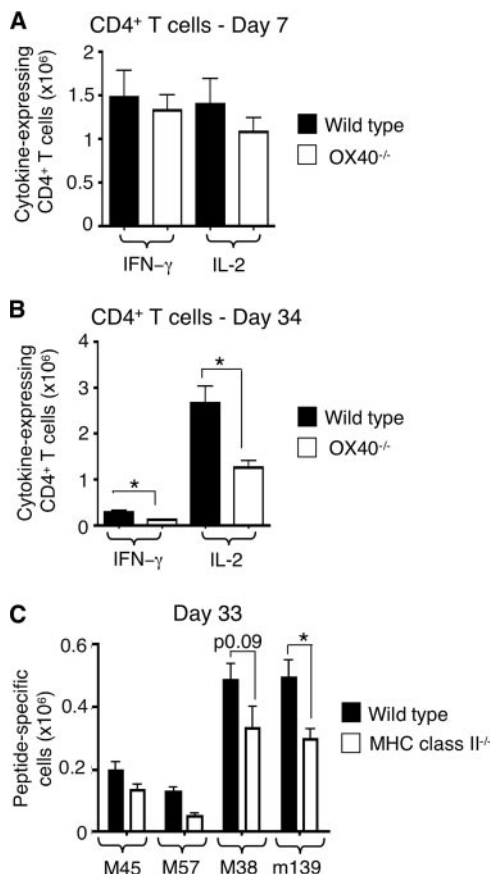
CD4 T cells are dispensable for early but not prolonged MCMV-specific CD8 T cell responses induced by OX40

To determine whether CD4 T cells were required for the action of anti-OX40 in promoting MCMV-reactive CD8 T cell populations, MCMV-infected wild-type and MHC class II-deficient mice were treated with control Ig or anti-OX40. Expansion of CD8 T cells following OX40 targeting was associated with a dramatic increase in the number of CD4 T cells producing
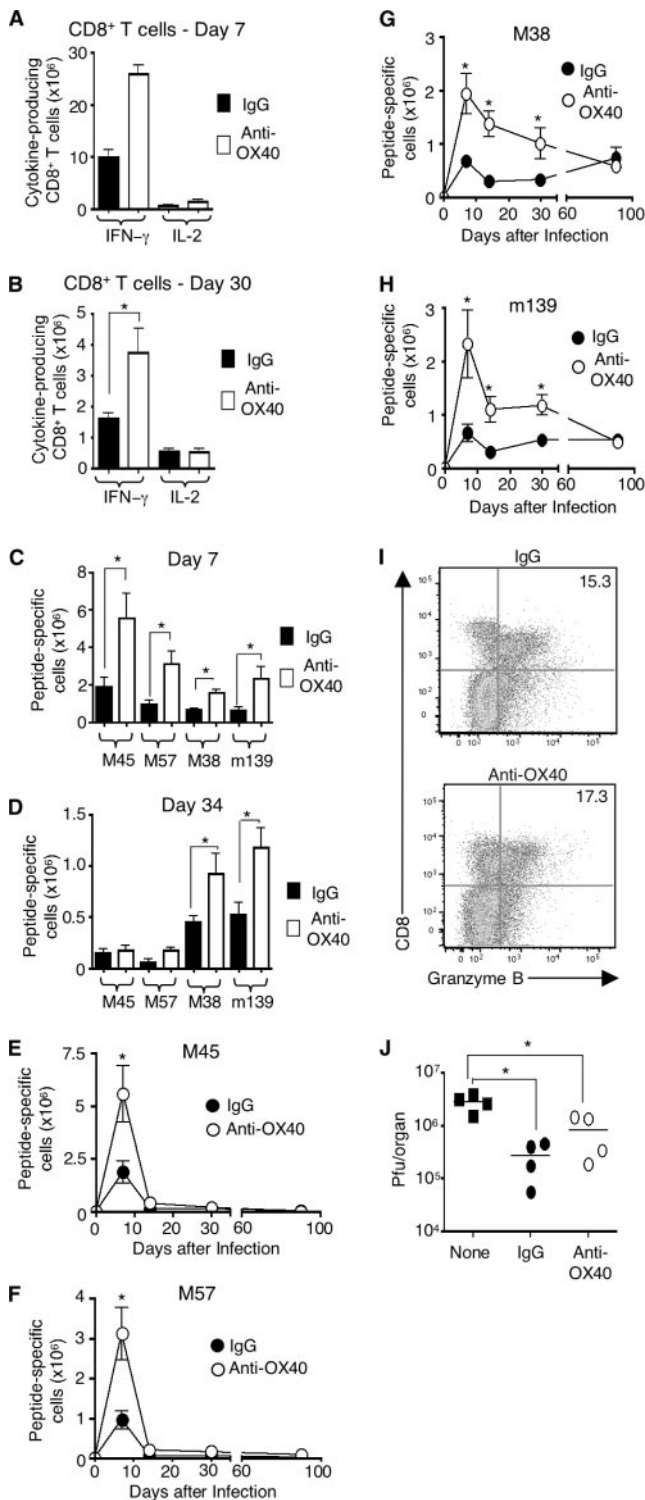
FIGURE 4. OX40 stimulation enhances expansion of protective CD8 T cells during MCMV infection. B6 mice were infected with MCMV and treated with rat IgG (closed bars or symbols) or anti-OX40 (open bars or symbols) Ab. *A* and *B*, On days 7 (*A*) and 30 (*B*) postinfection, IFN-γ- and IL-2-expressing CD8 T cells were quantified following ex vivo anti-CD3 and anti-CD28 stimulation. *C* and *D*, Peptide-specific IFN-γ-producing CD8 T cells were quantified 7 (*C*) and 34 (*D*) days after infection. *E–H*, Accumulation and persistence of M45 (*E*), M57 (*F*), M38 (*G*), and m139 (*H*) specific CD8 T cells measured on days 0, 7, 14, 34, and 90 days after infection. All results are derived from the same experiment. Results are mean numbers ± SEM of four mice per group. *I*, Granzyme B expression by unstimulated CD8 T cells from MCMV-infected control IgG (*top*) or anti-OX40 (*bottom*) treated mice,

IFN-γ and IL-2 at 7 (Fig. 5*A*) and 30 (Fig. 5*B*) days postinfection. By analyzing MCMV-infected wild-type and class II-deficient mice, we found that CD4 T cells were not required for the primary MCMV-specific CD8 T cell responses (Fig. 5*C*). Compared with wild-type controls in untreated mice, the numbers of MCMV-specific CD8 T cells at day 7 were equivalent or even increased in MHC class II$^{-/-}$ mice. Interestingly, anti-OX40-induced primary expansion of all CD8 T cell populations was not abrogated in MHC class II$^{-/-}$ mice and was equivalent to increases seen in wild-type mice (Fig. 5*C*).

Most significantly, when M38- and m139-reactive populations were examined after 30 days of infection, we found that anti-OX40 had no effect on prolonging the longevity of these CD8 T cells in MHC class II$^{-/-}$ mice (Fig. 5*D*). Collectively, these data show that the initial expansion of MCMV-specific CD8 T cells occurred regardless of CD4 T cells, and could be augmented in a CD4 T cell-independent manner by activating OX40. However, prolonged persistence of CD8 T cells that dominated the early chronic phase of infection partially required CD4 T cells and was dependent on naturally occurring OX40 signals. Furthermore, these late responses could be prolonged by exogenous stimulation of OX40 in a CD4 T cell-dependent manner.

Discussion

The mechanisms that regulate CMV-specific responses in vivo are not understood. Using MCMV, we now report that OX40 cosignaling promotes virus-specific CD8 T cell responses during late stages of MCMV infection, in a manner dependent upon CD4 T cell help. Furthermore, we show that ligation of OX40 enhances initial expansion of MCMV-specific CD8 T cells independently of CD4 T cells and prolongs CD8 T cell persistence in a CD4 T cell-dependent manner. This represents the first study describing a role for OX40 signaling in the regulation of T cell responses during a chronic viral infection and illustrates the notion that the time and use of costimulatory receptors might vary depending on the nature of the Ag and its availability over time. Furthermore, these results provide new insights into the mechanisms that drive expansion of CMV-specific T cells and have potentially exciting implications for the improvement of current HCMV immunotherapy strategies.

MCMV-specific CD8 T cells varied in their responsiveness to OX40 stimulation. Whereas endogenous and exogenous OX40 stimulation promoted CD8 T cells reactive to M38 and m139 epitopes, only exogenous OX40 signals influenced the accumulation of CD8 T cells recognizing M45 and M57 peptides, and did not promote their long-term accumulation. Why these populations of CD8 T cells behave differently is not clear, but it is likely related to recognition or availability of the antigenic peptides over time. Thus, the early M45 and M57 responses might contract dramatically due to a lack of Ag recognition, perhaps because of inhibition of MHC class I expression by MCMV (38) or alternatively due to poor processing or availability of M45 and M57 proteins during the chronic phase of infection. This is supported by the finding that

8 days postinfection. Quadrants were set using isotype control Abs. Results represent four mice per group from three experiments. *J*, Seven days after MCMV infection and Ab treatment, 1×10^5 total splenic CD8 T cells were recovered and injected into irradiated MCMV-infected recipients. No cells (■), control IgG CD8 T cells (●) or anti-OX40 CD8 T cells (○). Seven days later, replicating virus in the spleen was detected by plaque assay. Individual mice plus mean PFU are shown. All results represent two to three similar experiments. Significance is *, $p < 0.05$ with Student's *t* test in all experiments.

FIGURE 5. Prolonged accumulation of MCMV-specific CD8 T cells following OX40 stimulation requires CD4 T cell help. B6 mice were infected with MCMV and treated with rat IgG or anti-OX40. *A* and *B*, On days 7 (*A*) and 30 (*B*) postinfection, IL-2- and IFN-γ-expressing CD4 T cells were quantified. *C* and *D*, B6 and MHC class II$^{-/-}$ mice were infected with MCMV and treated with IgG or anti-OX40. On days 7 (*C*) and 30 (*D*), IFN-γ-producing peptide-specific CD8 T cells were quantitated in wild-type mice treated with IgG (■) or anti-OX40 (□), and in MHC class II$^{-/-}$ mice treated with IgG (▨) or anti-OX40 (▤). All results are mean numbers ± SEM of four to eight mice per group and are representative of three independent experiments. Significance is ∗, $p < 0.05$ with Student's *t* test.
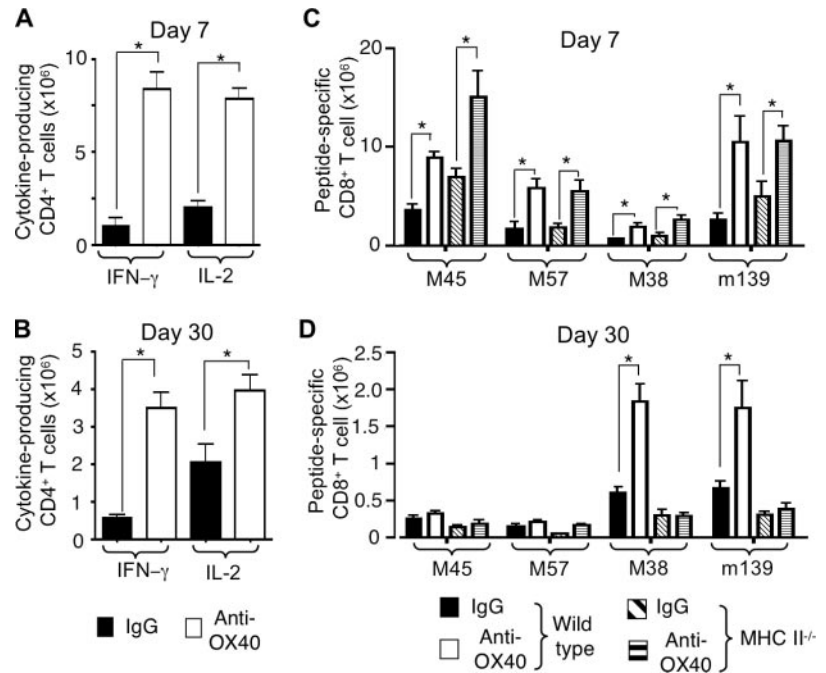
anti-OX40 enhanced and prolonged M38 and m139 responses, but could not prolong the M45 and M57 responses. Functional effects of OX40 signaling on T cells are likely dependent on concomitant TCR signals, in line with it being a costimulatory molecule, and hence in the absence of Ag recognition, OX40 signals might not be expected to rescue the decline of M45 and M57 anti-viral CD8 T cell populations.

Defective M38- and m139-specific CD8 T cell responses during chronic MCMV infection of OX40-deficient mice were associated with reduced late CD4 T cell activity. The similar defects in these CD8 T cell responses in OX40- and MHC class II-deficient mice further suggested that OX40 at least partially, if not wholly, promoted persistent CD8 T cell responses via sustaining CD4 T cells, and this hypothesis was substantiated by the finding that anti-OX40 could not mediate CD8 T cell persistence in MHC class II-deficient mice. OX40 signaling during the activation and priming of CD4 T cells in a number of model systems has been shown to promote clonal expansion, survival, and memory development (20, 39, 40), through targeting cell cycle and antiapoptotic molecules such as survivin, Bcl-x$_L$, and Bcl-2 (21, 41). This is in line with the idea that endogenous OX40 signaling during the initial to late stages of acute MCMV infection influenced the persistence of virus-reactive CD4 T cells. Furthermore, although we only observed impaired CD4 T cell responses at later stages (day 34) of infection in OX40$^{-/-}$ mice, exogenous OX40 stimulation at the time of infection induced a massive expansion of IFN-γ- and IL-2-producing CD4 T cells during both acute and chronic phases of infection.

A role for CD4 T cells in mediating MCMV-specific CD8 T cell responses further adds to the literature suggesting that the requirement for CD4 T cells in CD8 T cell responses can vary depending on the nature of the Ag and context in which it is presented, and also the time at which responses are analyzed. In different models of CD8 T cell generation, CD4 T cells have been found to be required either during the priming and/or the maintenance of memory CD8 T cell populations (42–45), although a growing number of reports are highlighting that it is long-term CD8 responses that are most dependent on CD4 T cells. With all MCMV-specific populations, we found that their

initial generation was not affected by the absence of CD4 T cells in line with data assessing the induction of primary CD8 T cells to other viral infections (46). In contrast, the persistence of M38 and m139-specific populations after the acute response was influenced by the presence of CD4 T cells, raising parallels with other data in a model of chronic LCMV infection where depletion of CD4 T cells induced a strong decrease in CTL activity when assessed at 5 mo (47).

Given that our data indicates that both OX40 and CD4 T cells can strongly influence the early persistence of MCMV-specific CD8 T cell populations after the acute response, it will be interesting to determine in future studies whether similar regulation takes place in HCMV-infected individuals many months to years after initial infection. OX40 signaling can strongly enhance the clonal expansion of memory CD4 T cells following re-exposure to Ag (48) and has been suggested in some cases to play a role in reactivation of already primed CD8 T cells (28, 32). This implies that periodic OX40 stimulation during late phases of chronic infection could also contribute to maintaining high frequencies of MCMV-reactive cells. Interestingly, CD8 T cell populations present during persistent polyoma infection consist in part of T cells newly recruited from the thymus (49), suggesting that long-term T cell populations in HCMV-infected individuals may also represent a combination of persistent and more recently primed T cells. Consistent with this idea, HCMV-specific T cells display heterogenous surface expression of costimulatory receptors indicative of different stages of memory development (50, 51), and it is highly likely that these cells may differentially express OX40 and/or respond to OX40 signals.

The observation that stimulation of OX40 augments the expansion of CD4 and CD8 T cells during MCMV infection has potential implications for the improvement of existing HCMV immunotherapy regimes. The generation of HCMV-specific CD8 T cell clones is technically and financially demanding. Although HCMV-specific CD8 T cells can be isolated directly from peripheral blood using HLA-peptide tetramers (52), therapeutic approaches that could induce the expansion and/or prolonged survival of these cells in vitro or following transfer into immune-suppressed recipients could significantly enhance the

efficacy of such treatments. Because OX40 can promote allograft rejection (53, 54), there are obvious complications in attempting to target OX40 in vivo following transfer of HCMV-reactive cells into immune-suppressed transplant recipients. However, HCMV-specific cells could be stimulated through OX40 in vitro to allow the generation and transfer of greater numbers of cells. In line with this idea, our recent data in a therapeutic model of adoptive transfer of tumor-reactive CD8 T cells has shown that OX40 signaling in vitro can strongly influence the survival of cells when transferred into a recipient (32). Although the timing and requirement for OX40 stimulation and associated CD4 T cell help requires definition, these types of strategies may be applicable to HCMV immunotherapy in the future.

Acknowledgments

Disclosures

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