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Recall Responses by Helpless Memory CD8+ T Cells Are Restricted by the Up-Regulation of PD-1

Shinichiro Fuse,* Ching-Yi Tsai,* Michael J. Molloy,* S. Rameez Allie,† Weijun Zhang,* Hideo Yagita,‡ and Edward J. Usherwood2*

CD4 help is crucial for memory CD8+ T cell development, yet the mechanisms of CD4 help and why (CD4) helpless memory CD8+ T cells elicit poor recall responses are currently not well understood. In this study we investigated these questions using an in vivo acute virus infection model. We show herein that CD4 help during priming is required for memory CD8+ T cell differentiation, and that stimulation of CD40 during priming rescues the helpless defects in the absence of CD4+ T cells. The defective recall response by helpless memory cells did not correlate with the amount of cell death and was independent of TRAIL. However, helpless memory cells excessively up-regulated the inhibitory receptor PD-1 (programmed cell death-1), and PD-1 blockade enhanced the recall response of helpless memory cells. Furthermore, providing IL-2 signaling in vivo during the recall response reduced PD-1 expression and rescued the recall response of helpless memory cells. Our study identifies molecular pathways involved in CD4 help for memory CD8+ T cell generation that are independent of TRAIL, and it provides therapeutic implications that helpless memory cell function can be restored at multiple stages through various immunological interventions. The Journal of Immunology, 2009, 182: 4244–4254.

Memory CD8+ T cells provide protective immunity against secondary infections, and on information on the signals required for their differentiation is valuable for designing effective vaccination strategies (1). Help mediated by CD4+ T cells has been shown to be crucial for memory CD8+ T cell differentiation in various models (2–6). However, there is great controversy on the timing of CD4 help required for memory CD8+ T cell differentiation. Depending on the model examined, CD4 help can be required during priming, maintenance, or recall, or in some cases it can be dispensable (2–4, 7–13).

Furthermore, the molecular and cellular mechanisms of how CD4+ T cells deliver help have not been resolved. One model postulates that CD4 help is mediated through the CD40L-CD40 pathway (14–16). In this model, activated CD4+ T cells up-regulate CD40L and engage CD40 on dendritic cells (DCs), resulting in full maturation and “licensing” of DCs and stimulation of CD8+ T cells (17). In these initial studies, agonistic anti-CD40 mAb substituted for the lack of CD4+ T cells. However, studies investigating the role for CD40 signaling in the memory CD8+ T cell differentiation have reported contradictory results, where in several models fully functional memory cells were observed in the absence of CD40 (6, 13, 18–22). Furthermore, stimulation of CD40 during priming has been shown to impair CD8+ T cell memory in some models, further complicating the interpretation (23, 24). The second hypothesis states that IL-2 released by CD4+ T cells recognizing cognate Ag in the vicinity of CD8+ T cells mediates CD4 help. IL-2 signals during priming from a paracrine source have been shown to be critical for differentiation of memory CD8+ T cells (25, 26), although no direct evidence has been shown that the IL-2 is derived from CD4+ T cells. Another study has shown that direct ligation of CD40L on CD4+ T cells and CD40 on CD8+ T cells is required for memory CD8+ T cell development (27). However, subsequent reports using infection models and virus-like particles (VLPs) have shown that CD40+/−CD8+ T cells develop into fully functional memory cells, suggesting that CD40 expression on CD8+ T cells is not required (12, 19, 20). Finally, a study using OVA-specific transgenic T cells has shown that the interaction of CD4+ T cells with DCs results in the production of CCL3 and CCL4, recruits OVA-specific CD8+ T cells through CCR5, and results in efficient priming (28). Blocking of CCL3 and CCL4 during priming impaired memory CD8+ T cell generation. However, CCR5 has been shown to be dispensable for memory differentiation in other models (12).

One of the hallmarks of helpless memory cells is the defective recall response upon secondary challenge with Ag (2–6). The mechanism behind this defective recall response is unclear. The expression of the transcription factor T-bet, which suppresses IL-7Ra expression, is increased with the absence of CD4+ T cell help, and deletion of T-bet has been shown to reverse the helpless phenotype (29). It is currently unclear why overexpression of T-bet limits the recall response in helpless memory CD8+ T cells. Using an in vivo cross-priming system and a lymphocytic choriomeningitis virus infection model, Janssen et al. provided evidence that helpless memory CD8+ T cells up-regulate TRAIL upon restimulation in vitro (30). This led to an increased cell death during recall, leading to an abortive response. Inhibition of TRAIL function through blockade or genetic deletion, as well as inhibiting cell death through the use of caspase inhibitors, reversed the impaired sence of CD40 (6, 13, 18–22). Furthermore, stimulation of CD40 during priming has been shown to impair CD8+ T cell memory in some models, further complicating the interpretation (23, 24). The second hypothesis states that IL-2 released by CD4+ T cells recognizing cognate Ag in the vicinity of CD8+ T cells mediates CD4 help. IL-2 signals during priming from a paracrine source have been shown to be critical for differentiation of memory CD8+ T cells (25, 26), although no direct evidence has been shown that the IL-2 is derived from CD4+ T cells. Another study has shown that direct ligation of CD40L on CD4+ T cells and CD40 on CD8+ T cells is required for memory CD8+ T cell development (27). However, subsequent reports using infection models and virus-like particles (VLPs) have shown that CD40+/−CD8+ T cells develop into fully functional memory cells, suggesting that CD40 expression on CD8+ T cells is not required (12, 19, 20). Finally, a study using OVA-specific transgenic T cells has shown that the interaction of CD4+ T cells with DCs results in the production of CCL3 and CCL4, recruits OVA-specific CD8+ T cells through CCR5, and results in efficient priming (28). Blocking of CCL3 and CCL4 during priming impaired memory CD8+ T cell generation. However, CCR5 has been shown to be dispensable for memory differentiation in other models (12).

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recall response (30). However, in a separate study, TRAIL was shown to only delay the helpless defect, implicating the existence of a TRAIL-dependent and -independent mechanism for the helpless defect. Furthermore, another report has indicated that the helpless defect is completely independent of TRAIL (31). Therefore, the underlying mechanism for the helpless defect is not well understood and, furthermore, whether the function of the incorrectly “programmed” memory cells in the absence of CD4 help could be repaired after the primary response is uncertain.

Herein we characterize the molecular mechanism of CD4 help in the generation of memory CD8+ T cells in vivo by nontransgenic endogenous CD8+ T cells upon infection with acute cytopathic viral infection through a mucosal route. Intranasal (i.n.) infection by vaccinia virus Western Reserve strain (VV-WR) represents a natural route of infection by poxviruses and results in a pathic viral infection through a mucosal route. Intranasal (i.n.)/H11001genic endogenous CD8+/H11001mice were purchased from The National Cancer Institute (Bethesda, MD).

Materials and Methods

Mice and virus infections

C57BL/6 (B6) and congenic B6-Ly5.2-Cr (which are Ly5.1/CD45.1+/H9262) mice were purchased from The National Cancer Institute (Bethesda, MD). CD40+/H11001 and TRAIL−/−+H9262 mice were generously provided by Dr. Randolph J. Noelle (Dartmouth Medical School, Lebanon, NH) and Amgen, respectively, and were used with permission. The Animal Care and Use Program of Dartmouth College approved all animal experiments. For infections, mice were infected with 10^7 PFU of VV-WR (originally obtained from Dr. William R. Green, Dartmouth Medical School, Lebanon, NH) through the i.n. route, and loss of body weight was measured as previously described (32). Murine gammaherpesvirus-68 (MHV-68) virus (clone G2.4) was originally obtained from Dr. A. A. Nash (University of Edinburgh, Edinburgh, U.K.) and was propagated and titrated as previously described (32). A recombinant vaccinia virus expressing the open reading frame (ORF68487–495/Derture of MHV-68 (vVV-ORF68) was obtained from Dr. Peter Doherty (St. Jude Children’s Research Hospital, Memphis, TN). For MHV-68 infection, 400 PFU was given i.n.

Ab treatments

For depletion of CD4+ T cells, mice were treated with 500 μg of anti-CD4 (GK1.5) on days −1 and 0 of infection, followed by 250 μg on day 3 postinfection (p.i.) and twice weekly thereafter, resulting in <0.1% CD4+ T cells (data not shown). Control mice were either untreated or given rat IgG (R-IgG; Jackson ImmunoResearch Laboratories). No differences in T cell responses were observed with R-IgG injection (data not shown). The hybridoma producing anti-CD40 mAb (FKG115) was kindly provided by Dr. Randolph J. Noelle. Anti-CD40 mAb (100 μg) was injected on days 1 and 7 p.i., and 500 μg of anti-PD-1 (RMP1–14) was injected on days 0, 2, and 4 during challenge. Injection of IL-2 immune complexes has been described previously (32, 33). Briefly, a mixture of 50 μg of anti-IL-2 mAb (S4B6) and 1.5 μg of murine IL-2 (eBioscience) was injected i.p. daily from day 0 to 4 postchallenge.

MHC/peptide tetramer, Ab, intracellular staining, and flow cytometric analysis

Single-cell suspensions of spleen and lung lymphocytes were prepared as described previously (32). MHC/peptide tetramer for the VV-WR epitope B8R20–27(TSYKFESV)/Kb or ORF68487–495(AGPHNDMEI)/Db conjugated to allophycocyanin was obtained from the National Institutes of Health Tetremer Core Facility (Emory University, Atlanta, GA). Cells were stained for 1 h at room temperature and were further stained with PerCP-conjugated anti-CD8α (53-6.7; BD Pharmingen) along with Abs against surface markers (32). For PD-1 expression, PE-conjugated anti-PD-1 (J43; BD Pharmingen) was used. Staining with PE-conjugated anti-CTLA-4 (UC10-4F10-11; BD Pharmingen) and anti-Bcl-2 (BD Pharmingen) expression was performed at 4°C for 30 min after staining with tetramer, anti-CD8, and FITC-conjugated anti-CD45.2 (clone 104; eBioscience), followed by cell fixation with 1% formaldehyde (Ted Pella) and permeabilization with staining buffer containing 0.5% saponin (Sigma-Aldrich). An-

FIGURE 1. CD4 help does not affect the initial disease and CD8+ T cell kinetics during VV infection. A, Weight loss after infection was measured. Numbers indicate surviving mice. B and C, The VV-B8R-specific CD8+ T cell response was measured by tetramer staining on day 10 (B) and day 69 (C) p.i. Total numbers of CD8+ tetramer+ cells in each organ are graphed. Open bars indicate undepleted; black bars, CD4-depleted. Representative data from two independent experiments with four mice per group are shown. Error bars indicate SEM.
cytokine staining was performed after restimulation of splenocytes with 1 μg/ml peptide, 10 U/ml IL-2, and 5 μg/ml brefeldin A for 5 h at 37°C. IFN-γ production in control wells with no peptide was subtracted as background, as previously described (32).

Adoptive transfer and challenge experiments

Similar to our previous study (32), CD8+ T cells were purified (typically >95% purity) from spleens of VV-WR-infected mice (40+ days p.i.), and equal numbers of donor-derived CD8+ T cells (2 to 4 × 10^6 cells) were injected into naive congenic B6-CD45.1 (B6-Ly5.2-Cr) mice i.v. Before transfer, cells from each group were stained to determine the percentage of CD8+ T cells and tetramer+ cells (similar frequencies were observed in all experiments; see Fig. 1C), and the data were used to determine the exact number of virus-specific memory CD8+ T cells injected into each individual recipient. One day after transfer, mice were challenged i.p. with 2 × 10^6 PFU of VV-WR. Spleens were harvested 5 days postchallenge and stained with VV-B8R-tetramer, anti-CD8, and anti-CD45.2, and the total number of donor-derived VV-specific CD8+ T cells was calculated. To calculate fold expansion of donor-derived VV-specific CD8+ T cells in each individual recipient, the total number of virus-specific memory CD8+ T cells 5 days postchallenge was divided by the total number of virus-specific CD8+ T cells initially transferred. Four to seven recipients per group were used in each experiment.

For experiments involving MHV-68-specific memory CD8+ T cells, CD8+ T cells were purified from infected mice, and equal numbers of

**FIGURE 2.** Helpless VV-specific memory CD8+ T cells are impaired in function. A, Phenotype of VV-B8R-specific memory CD8+ cells in undepleted and CD4-depleted mice were analyzed at day 69 p.i. by tetramer staining. Plots are gated on CD8+tetramer+ cells. B, Phenotype of lung-resident memory cells. C, Cytokine production by VV-specific memory cells was examined by intracellular cytokine staining. Plots are gated on CD8+ T cells producing IFN-γ (left), or percentage of IFN-γ+ cells that produce TNF-α (middle) or IL-2 (right). D, The recall response of helped and helpless memory cells were analyzed by an adoptive transfer approach (see Materials and Methods for details). CD8+ T cells were purified from undepleted or CD4-depleted VV-infected mice 4 mo p.i. and were transferred into naive congenic (CD45.1+) recipients. One day later, recipients were challenged i.p. with 2 × 10^6 PFU of VV-WR, and 5 days postchallenge expansion of donor-derived VV-specific memory CD8+ T cells was analyzed by tetramer staining before and after (lower panel) challenge. E, Results of the adoptive transfer experiment. The total numbers of donor-derived VV-specific memory CD8+ T cells was analyzed by tetramer staining before transfer and 5 days postchallenge are graphed. Fold expansion of tetramer+ cells was calculated for each recipient, and the number shown indicates the average fold expansion for each group. Each circle (●, undepleted → B6; ○, CD4-depleted → B6) represents an individual recipient. Representative plots from two to three independent experiments using three to six to seven recipients per group are shown. In D, four donor mice per group and six to seven recipients per group were used. *, p < 0.05 and **, p < 0.01.

**FIGURE 3.** Functional VV-specific memory cells require CD4 help during priming and CD40 signaling. A, Mice were infected with VV-WR and were untreated or CD4-depleted during priming (days 1, 0, and 3). One hundred days p.i., the recall response of VV-specific memory CD8+ T cells was analyzed by adoptive transfer experiments described in Fig. 2 (●, early CD4-depleted → B6). B, B6 or CD40−/− mice were infected with VV-WR and the recall response of VV-specific memory CD8+ T cells was analyzed by adoptive transfer and challenge (●, B6 → B6; ○, CD40−/− → B6). Representative data from two independent experiments are shown using four donors per group and four to eight recipients per group. Each circle represents an individual recipient. Fold expansion of tetramer+ cells was calculated for each recipient, and the number shown indicated the average fold expansion for each group. ***, p < 0.01.
In most viral infection models, CD4 help does not affect the primary virus-specific CD8+ T cell response, but in some cases help is required for maintaining the memory population. Therefore, we examined the VV-specific CD8+ T cell response in the lung and the spleen using MHC/peptide tetramers. CD4+ T cells did not affect the primary virus-specific CD8+ T cell response in the lung and the spleen (Fig. 1B), which occurs at day 10 in this model (32). Thus, CD4+ T cells play no role in the early events that occur during this infection. At 2 mo postinfection, we observed a slight decrease in the frequency of virus-specific memory CD8+ T cells in the lung of CD4-depleted mice (data not shown); however, the total numbers of virus-specific memory CD8+ T cells in the spleen and lungs were unaffected by the absence of CD4+ T cells (Fig. 1C). Overall, CD4 help does not affect the kinetics of the virus-specific CD8+ T cell response during VV-WR infection.

Helpless memory CD8+ T cells are severely impaired in function

Numerous studies have provided evidence for a crucial role of CD4 help in memory CD8+ T cell differentiation (7). Thus, although the kinetics of the virus-specific memory CD8+ T cell response was unaffected by the absence of help, we suspected that the helpless memory cells may be functionally impaired. First, we investigated the phenotype of VV-specific memory CD8+ T cells in the spleen and the periphery (lungs) in the absence of CD4 help. Helpless memory cells in the spleen expressed lower levels of CD127, CD62L, and CD27 as measured by mean fluorescent intensity (MFI), and there were lower percentages of CD127high, CD62Lhigh, and CD27high cells (Fig. 2A). The helpless memory cells in the lung also expressed lower levels of CD44 and CD122 (Fig. 2B). We observed minimal
expression of CD25 and CD69 in the untreated and CD4-depleted groups in both organs, indicating that the cells had a resting phenotype (data not shown). Second, we tested the ability of helpless memory cells to produce cytokines in response to restimulation by intracellular cytokine staining. In the absence of CD4 help, memory cells produced lower levels of IFN-γ, as measured by MFI (Fig. 2C). The frequency of IFN-γ-producing CD8+ T cells was slightly reduced, but was not statistically significant (data not shown). Furthermore, lower frequencies of IFN-γ-producing memory cells produced TNF-α and IL-2 (Fig. 2C).

Finally, we measured the ability of the helpless memory cells to elicit a robust recall response by utilizing an adoptive transfer approach used previously (Fig. 2D) (32). CD4 help is required for the induction of neutralizing Abs; thus, directly rechallenging helped and helpless mice would result in different doses of virus infecting the host. Therefore, we purified CD8+ T cells from infected helped or helpless mice and transferred them into naive congenic (CD45.1+) hosts, which were challenged 1 day later with 2 x 10^6 PFU of VV-WR i.p. Before transfer, we calculated the exact number of virus-specific CD8+ T cells transferred into each host by tetramer staining (see Materials and Methods). Five days later the recall response was analyzed in the spleen through tetramer staining, and the total number of donor-derived virus-specific CD8+ T cells was calculated. This approach allows us to accurately measure the fold expansion of virus-specific CD8+ T cells during recall in vivo in each individual animal, through dividing the total number of donor-derived virus-specific CD8+ T cells 5 days post-challenge by the total number initially transferred. VV-specific memory CD8+ T cells that received CD4 help expanded an average of 39-fold upon challenge, while helpless memory cells expanded on average ~10-fold (Fig. 2E). Thus, VV-specific memory CD8+ T cells that developed in the absence of CD4 help were severely impaired in their ability to mount a recall response upon viral challenge (Fig. 2E). Although the magnitude of reduction varied between experiments, we consistently observed reduction of the recall response by helpless memory cells in all experiments, typically 2- to 4-fold, similar to numbers reported in other models (3, 11, 22, 30, 37). The data clearly demonstrate the requirement of CD4 help for memory CD8+ T cell differentiation during acute VV infection.

During lymphocytic choriomeningitis virus infection, lack of CD4 help or CD40L-CD40 interactions leads to viral resurgence at ~2 mo postinfection, which causes deterioration of memory...
CD8$^+$ T cell function (38). However, we found no evidence of viral persistence in CD4-depleted mice (data not shown), suggesting that the defects observed were a direct effect of CD4 help and not an indirect effect of CD8$^+$ T cell exhaustion caused by viral persistence.

Role of CD40 signaling in helping memory CD8$^+$ T cell differentiation

The requirement for CD4 help in memory CD8$^+$ T cell differentiation has been documented in various infection models, yet the timing of help and the signals that mediate help have been a subject of debate (7, 8). Therefore, we utilized our adoptive transfer system to investigate the required timing and the molecular nature of CD4 help for VV-specific memory CD8$^+$ T cells to acquire the ability to mount a robust recall response. First, we tested whether CD4 help is required during priming, by injecting the anti-CD4 mAb only on days 1, 0, and 3. VV-specific memory CD8$^+$ T cells from mice that received early CD4 depletion were defective in mounting a recall response (Fig. 3A). The data support the requirement for CD4$^+$ T cells during priming, although they do not rule out an additional role of CD4 help during the maintenance phase. Next, due to the importance of CD40L-CD40 interactions in mediating CD4 help (14–16), we investigated the role of CD40 signaling in memory cell development by transferring VV-specific memory CD8$^+$ T cells from infected B6 or CD40$^{-/-}$ mice. Upon adoptive transfer and challenge with VV-WR, memory CD8$^+$ T cells that developed in CD40$^{-/-}$ mice showed a significantly reduced recall response (Fig. 3B). Therefore, during VV-WR infection CD40 signaling significantly impacts memory CD8$^+$ T cell differentiation.

The requirement for CD4 help during priming and CD40 signaling prompted us to assess whether providing CD40 stimulation during priming in the absence of CD4$^+$ T cells can reverse the defects of helpless memory cells. Agonistic anti-CD40 mAb was injected into CD4-depleted mice at days 1 and 7 p.i., and at 49 day p.i., memory CD8$^+$ T cells were analyzed for their phenotype (Fig. 4). The aberrant expression of CD44, CD127, CD27, and CD62L observed in CD4-depleted mice was restored by CD40 signaling during priming. Furthermore, the defects in cytokine production, particularly TNF-α and IL-2, were restored by CD40 ligation back to levels similar to memory cells that received CD4 help (Fig. 5, A and B). IFN-γ production was also slightly increased, although...
complete restoration of production of this cytokine was never observed. Finally, the defect in the secondary response by the helpless cells was reversed by administering the agonistic anti-CD40 mAb during priming (Fig. 5C). The results show that CD40 stimulation during priming can rescue helpless CD8⁺ T cell memory differentiation, and may indicate a role for CD40 in CD4 help. However, administering CD40 stimulation during priming slightly enhanced recall responses of helped memory CD8⁺ T cells in undepleted mice (Fig. 5D), implying that CD40 signaling may have a nonoverlapping role with CD4⁺ T cell help.

The helpless defect is independent of TRAIL.

Although our data demonstrate that CD4 help is required for VV-specific memory CD8⁺ T cells to acquire the ability to mount a robust recall response, the molecular mechanism of why helpless memory cells elicit defective recall responses remains elusive. Evidence provided by Janssen et al. suggested that the up-regulation of TRAIL by helpless memory cells upon recall leads to increased cell death and an impaired recall response (30). Therefore, we hypothesized that the absence of CD4 help leads to increased cell death of memory CD8⁺ T cells during recall. VV-specific memory CD8⁺ T cells from undepleted, CD4-depleted/R-IgG-treated, or CD4-depleted/anti-CD40-treated mice were challenged with VV-WR upon adoptive transfer into naive recipients (as described in Fig. 5C), and 5 days later, donor tetramer⁺ cells were analyzed for cell death by annexin V staining. No differences in the percentage of virus-specific CD8⁺ T cells undergoing cell death were observed among the three groups (Fig. 6A). Furthermore, we found no difference in the expression of the antiapoptotic molecule Bcl-2 by tetramer⁺ cells (Fig. 6A). Thus, the data imply that the reason for the impaired recall response by helpless memory cells is not due to increased cell death. We next examined whether TRAIL deficiency could reverse the defective recall response by helpless memory cells, as previously shown (30, 37). As shown in Fig. 6B, VV-specific memory CD8⁺ T cells from CD4-depleted mice were impaired in their recall response, and this defect was not restored by TRAIL deficiency. Recently, TRAIL has been shown to possess antiviral function against dengue virus (39); however, we observed no defects by TRAIL⁻/⁻ mice in the ability to control i.n. VV-WR infection (data not shown). Collectively, the results imply that the
impaired recall response by helpless memory cells in VV-WR infection is not due to increased cell death and is independent of TRAIL.

Excessive up-regulation of PD-1 on helpless memory cells impairs the recall response

Data suggesting that the defective recall response of helpless memory CD8⁺ T cells is independent of TRAIL-mediated cell death prompted us to investigate other pathways that may mediate the helpless defect. We hypothesized that during recall, helpless memory cells express higher levels of receptors that inhibit T cell proliferation, reducing the magnitude of the recall response. We explored two immunoregulatory receptors in the B7-CD28 superfamily, CTLA-4 and PD-1, which are known for their potent inhibitory role in the T cell response. VV-specific memory CD8⁺ T cells from undepleted, CD4-depleted/R-IgG-treated, or CD4-depleted/anti-CD40-treated mice were challenged upon transfer, and 5 days later they were assessed for the expression of these molecules. The expression of CTLA-4 during recall did not correlate with the ability of the memory cells to mount a recall response (data not shown), indicating that the molecule does not account for the difference in proliferative capacity between helped and helpless memory cells. Next, we examined the expression of PD-1 on memory CD8⁺ T cells during the recall response. The expression of PD-1, both in terms of percentage of VV-specific CD8⁺ T cells expressing the receptor and the MFI, was significantly increased on memory CD8⁺ T cells that did not receive CD4 help (Fig. 7, A–C). Furthermore, restoring the recall response in CD4-depleted mice by administering agonistic anti-CD40 mAb resulted in the down-regulation of PD-1 expression during the recall response.

**FIGURE 10.** PD-1 up-regulation by MHV-68-specific helpless memory CD8⁺ T cells and restoration by IL-2 in vivo. A, Helpless MHV-68-ORF6-specific memory CD8⁺ T cells were adoptively transferred and challenged (see Materials and Methods for details) and were treated with R-IgG or the IL-2 immune complex. Five days postchallenge, transferred memory cells were analyzed for expansion as described in Fig. 2D. Numbers indicate average fold expansion of MHV-68-specific memory cells for each group. B, Donor-derived ORF6 tetramer⁺ cells at 5 days postchallenge were stained for PD-1 expression. Representative plots gated on CD45.2⁺ CD8⁺ cells are shown. Numbers indicate average MFI. C, MFI of PD-1 expression by donor-derived VV-specific CD8⁺ T cells is graphed. Representative data from two independent experiments with four to six recipient mice per group are shown. Error bars indicate SEM. ***, p > 0.01.
Thus, expression of PD-1 during the recall response correlated with CD4 help and CD40 signaling, as well as the proliferative capacity of helped vs helpless memory cells during recall. PD-1 expression was not observed on resting memory cells before recall in all groups (Fig. 7D).

Because the expression of PD-1 was higher on helpless memory cells during recall compared with helped cells, we hypothesized that the level of PD-1 expression may play a role in blunted recall responses in the absence of CD4 help. To investigate the functional significance of PD-1 expression, we inhibited PD-1 on helpless memory cells during the recall response by Ab blockade. Treatment with anti-PD-1 during recall significantly enhanced the recall response of helpless memory cells, to the levels of helped memory cells (Fig. 8A). Interestingly, treatment with anti-PD-1 had no effect on annexin V staining (Fig. 8B) or Bcl-2 expression (data not shown) on the virus-specific CD8\(^+\) T cells, implying that PD-1 inhibits the recall response without affecting cell survival. PD-1 blockade also enhanced recall responses of helped memory cells (Fig. 8C), which is in line with our observation of PD-1 up-regulation on helped memory cells during the recall response (Fig. 7A, second panel). These data indicate that PD-1 signaling acts as an important brake for recall responses by VV-specific memory CD8\(^+\) T cells. Furthermore, they support our hypothesis that the blunted recall response by helpless cells may be caused by excessive up-regulation of PD-1 by helpless memory cells during recall compared with helped cells.

**IL-2 signaling in vivo rescues the recall response of helpless memory cells**

Previous studies by Carter et al. have shown that PD-1 inhibits T cell proliferation and IL-2 production, and this inhibition can be overcome by augmenting IL-2 in vitro (40). Interestingly, helpless memory cells exhibit defective proliferation upon recall and are unable to produce IL-2 upon restimulation, and both of these defects are restored by CD40 signaling during priming (Fig. 5, A and B). Thus, the ability to mount a robust recall response correlates with their ability to produce IL-2. We therefore tested whether supplying IL-2 signaling in vivo could rescue the defective recall response by helpless memory cells. IL-2 signaling was administered by daily injections of immune complexes (combination of IL-2 and anti-IL-2 mAb S4B6), which have been shown to deliver a strong IL-2 signal to T cells (25, 33). We have previously shown that injection of the IL-2 immune complex could restore defective recall responses by memory CD8\(^+\) T cells that failed to receive CD28 costimulation (32). Delivering IL-2 signaling during recall revived the blunted recall response by helpless memory cells during recall (Fig. 9A). Furthermore, treatment with the IL-2 immune complex resulted in a significant down-regulation of PD-1 expression on responding helpless cells (Fig. 9, B and C). Furthermore, augmenting IL-2 signaling to helped memory cells did not enhance recall responses by helped memory cells (Fig. 9D). In summary, these data imply that IL-2 signaling allows the unleashed memory cells to override inhibition by PD-1, likely through down-regulation of the receptor.

We further investigated whether the up-regulation of PD-1 by helpless memory CD8\(^+\) T cells during the recall response and the restoration by IL-2 signaling are unique features of VV-WR infection, or if they can also be seen in other infection models. MHV-68 causes a latent infection characterized by a low-level viral persistence. MHV-68-specific CD8\(^+\) T cells elicit robust recall responses upon challenge with rVV-expressing MHV-68 Ags (41). In the absence of CD40-mediated CD4 help, CD8\(^+\) T cell-mediated immune surveillance of the persistent virus is lost (42, 43). We measured the ability of helpless memory CD8\(^+\) T cells specific for the MHV-68 ORF6\(_{487-495}/Db\) epitope upon transfer into naive recipients and rVV-ORF6 challenge. Similar to VV-specific memory CD8\(^+\) T cells, helpless MHV-68-specific memory CD8\(^+\) T cells showed a severely impaired recall response (Fig. 10A), and the helpless cells expressed significantly higher levels of PD-1 during the recall response (Figs. 10, B and C). Furthermore, administering the IL-2 immune complex during the recall response resulted in the down-regulation of PD-1 and the restoration of the recall response (Fig. 10). Thus, the observation made in the VV-WR model that helpless memory CD8\(^+\) T cells express higher levels of PD-1 is not a unique feature of VV-WR infection but is also seen in other models where CD4 help plays a critical role for memory CD8\(^+\) T cell differentiation.

**Discussion**

Help by CD4\(^+\) T cells has been shown to be pivotal for memory CD8\(^+\) T cell differentiation in most infection models studies (7). However, the discrepancies in mechanisms of CD4 help reported among the model studied are perplexing. One such controversy is the involvement of the CD40 pathway. Fully functional memory CD8\(^+\) T cells develop in the absence of CD40 during *Listeria monocytogenes* infection and following immunization with VLPs (6, 19, 21). In contrast, CD40 on non-T cells is required for full differentiation of memory CD8\(^+\) T cells following influenza infection (20), and loss of CD8-mediated control of MHV-68 infection occurs in the absence of CD40-mediated CD4 help (42–44). The requirement for CD40 in our model may suggest that during infection through the i.n. route, there is a general requirement for CD40. This may be due to priming by distinct populations of DCs in the lung (45) or the unique lung environment (46). Outliers to this generalization exist, as recall CD8\(^+\) T cell responses after priming with systemic *Listeria* infection in BALB/c mice have been shown to be dependent on CD40L (22), suggesting that the location of priming does not completely account for the requirement for CD40.

In our model, administering agonistic CD40 during priming restored the phenotype, cytokine production, and the recall response in the absence of CD4\(^+\) T cells (Figs. 4 and 5). The results directly contrast with recent work suggesting that early CD40 ligation results in the deterioration of T cell memory (23, 24). On the contrary, CD40 stimulation has been shown to replace CD4 help and restore CD8-mediated control of MHV-68 (43). Furthermore, immunization with CD40 stimulation in combination with a TLR agonist results in the generation of functional CD8\(^+\) T cell memory to a tumor/self Ag, which is otherwise poorly immunogenic (47), showing that CD40 could provide beneficial signals for memory generation.

The requirement for CD4 help during priming and CD40 signaling contrasts with a recent study using immunization with VLPs, which did not require CD4 help during priming and was CD40-independent (12). The discrepancy is particularly interesting, since both models use i.p. infection of VV during recall, and thus the only difference is the method used to prime the CD8\(^+\) T cell response. There are notable differences between the VLP immunization and the VV-WR infection used in our model, which may explain the disparities observed. Such differences include route of priming (s.c. immunization vs i.n. infection), duration of Ag (nonreplicating VLPs vs live virus infection), and degree of inflammation. Although the immunization using VLPs included a TLR agonist, inflammation induced by live virus infection exceeds the immunization both in degree and duration. Inflammation has been shown to negatively regulate generation of memory precursors and memory CD8\(^+\) T cells (48, 49), and the effects of CD40...
signaling may shift the balance in favor of generating potent memory under inflammatory conditions. Similar requirements for early CD4 help have been reported upon systemic VV infection (11).

In our experiments, helpless memory cells did not exhibit higher levels of cell death during recall, and the defect was independent of TRAIL (Fig. 6). During lymphocytic choriomeningitis virus infection, TRAIL only mediated the helpless defects observed early (~60 days), but at later time points (~90 days) the impairment occurred in a TRAIL-independent manner (37). Furthermore, TRAIL deficiency did not restore the function of helpless memory cells during Listeria infection or responses to help-dependent Ags (31). Our recall experiments were performed at 36 days p.i., thus indicating that the helpless defect in our model may be completely TRAIL-independent. Our data provide evidence that the TRAIL-independent impairment is mediated through the PD-1 pathway. PD-1 is a potent negative costimulatory molecule that inhibits T and B cell responses (50). The molecule is highly expressed on exhausted T cells during chronic viral infections, and blocking this pathway reverses functional exhaustion (51). Upon resolution of acute VV infection, no PD-1 expression was observed on resting memory cells, regardless of whether they received CD4 help (Fig. 7D). Upon recall, both helped and helpless memory cells up-regulated PD-1; however, helpless memory cells expressed significantly higher levels of PD-1 compared with helped cells (Fig. 7A–C). The up-regulation of PD-1 is likely due to a lack of CD40 signaling and CD4 help, since administering agonistic anti-CD40 mAb during priming reversed PD-1 expression. This up-regulation was selective, since expression of CTLA-4 was not affected. Furthermore, the excessive up-regulation of PD-1 by helpless memory cells during the recall response compared with helped cells was not a unique characteristic of VV-WR infection and was also observed in helpless MHV-68-specific memory CD8+ T cells (Fig. 10, B and C). Blocking PD-1 during the recall response completely restored the response by the helpless memory cells (Fig. 8A). Note that in our experiments, CD8+ T cells are purified and transferred into naive recipients, where the cells are challenged. Thus, memory CD8+ T cells from both groups (helped and helpless) are challenged in the same environment, and the only difference is the transferred CD8+ T cells themselves. Therefore, the most likely explanation is that the helpless cells are “imprinted” along the differentiation pathway through CD40 signaling during priming, to up-regulate PD-1 during recall. CD4 help has been shown to influence epigenetic remodeling of the IFN-γ and IL-2 promoter and enhancer regions (52), which links the inability of helpless memory cells to rapidly produce these cytokines. We hypothesize that one of the important roles for CD4 help through CD40 during priming may be the epigenetic imprinting of the PD-1 locus, in which upon recall is tightly regulated, enabling these cells to rapidly proliferate and mount a robust recall response. The lack of CD4 help could result in loosening of this tight regulation, causing excessive up-regulation of PD-1 compared with helped cells and inhibiting the recall response. Our future experiments will test this hypothesis.

Another intriguing finding is that supplying IL-2 in vivo through the use of immune complexes restores recall response by helpless memory cells (Figs. 9A and 10A). The restoration of the recall response coincided with the down-regulation of PD-1 (Figs. 9 and 10), suggesting this may be one of the mechanisms in which IL-2 restores the response by helpless memory cells. The PD-L1-PD-1 interaction results in the inhibition of proliferation of both CD4+ and CD8+ T cells, and addition of exogenous IL-2 has been shown to overcome this inhibition and restore proliferation of polyclonal T cells in vitro (40). An interesting similarity with the study by Carter et al. (40), our previous study (32), and our present report is that the intrinsic ability of CD8+ T cells to produce IL-2 correlates with their proliferative capacity (Fig. 5). Furthermore, in all three studies, providing IL-2 signaling in vitro or in vivo restores the impaired proliferation by these cells (Figs. 9 and 10) (32, 40).

Thus, one reason helpless memory cells may be defective is their inability to produce IL-2, through the epigenetic alteration of their promoter (52). Interestingly, a recent study reported a correlation between reduced CD27 expression on helpless memory cells and its inability to produce IL-2 and mount a recall response (53). We also observed reduced CD27 expression on VV-specific helpless memory cells (Fig. 2), and its expression was restored by CD40 stimulation during priming, along with IL-2 production and recall potential (Fig. 4). Thus, reduced CD27 expression, impaired IL-2 production, and excessive PD-1 expression during recall may act in concert to restrict secondary expansion of helpless memory cells.

Our data reveal a previously uncharacterized mechanism for why memory CD8+ T cell differentiation is impaired in the absence of CD4 help. Furthermore, we provide evidence that the helpless defects are reversible at multiple stages through various interventions (CD40 stimulation during priming, and PD-1 blockade or IL-2 signaling during recall). The information may be valuable especially in designing vaccinations in settings where CD4+ T cells are unavailable or dysfunctional, such as in the context of chronic HIV infection.

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Disclosures
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