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Kinetic and Mechanistic Requirements for Helping CD8 T Cells

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The requirements for the generation of fully competent long-lived memory CD8 T cells and in particular the role and the mechanisms of help from CD4 T cells remain ill-defined. Memory CD8 T cells generated in the absence of CD4 T cell help often have an impaired recall proliferation and are thus unable to confer protection against certain pathogens. However, the timing and the mechanisms involved in the delivery of help are still unclear and differ between various experimental systems. In this study, we investigated the role of CD4 T help in generating memory CD8 T cells in a defined heterologous prime-boost system, consisting of priming with replication incompetent virus-like particles and challenge with recombinant vaccinia virus, both sharing only a common lymphocytic choriomeningitis virus-derived CD8 T cell epitope. We show in this system that delivery of help is only essential during the challenge phase for recall proliferation of memory CD8 T cells. Furthermore, we show that generation of proliferation-competent memory CD8 T cells is independent of CD40 and CCR5 and that in vivo IL-2 supplementation neither during priming nor during challenge was able to rescue recall proliferation of “unhelped” memory CD8 T cells. *The Journal of Immunology, 2008, 180: 1517–1525.

The CD8 T cells play a central role in the defense against intracellular pathogens. After pathogen encounter, naive CD8 T cells become activated, start to proliferate, and acquire effector functions. Once the pathogen is cleared, only 5–10% of CD8 T cells survive the contraction phase, becoming long-lived memory cells. These cells exhibit increased responsiveness after reencounter with the same pathogen, thereby being able to confer protection (reviewed in Refs. 1 and 2). Understanding the critical factors involved in the generation and maintenance of memory CD8 T cells is therefore crucial for the development of T cell-based vaccines (3). Among other parameters such as the level of inflammation (4) as well as the amount and duration of Ag availability (5–10), Th cells can play a crucial part in the differentiation of memory CD8 T cells (11–16).

Initial experiments suggested that the help conferred by CD4 T cells to generate a pool of memory CD8 T cells was only required in the case of noninflammatory immunogens, such as cell-associated minor histocompatibility Ags. In these situations, CD4 T cells deliver secondary stimuli that activate APCs, mostly via CD40-CD40 ligand (CD40L)3 interaction and thus “license” the APCs to become a potent inducer of CD8 T cells (17–19). In contrast, infectious agents were thought to circumvent the requirement for CD4 T cells by directly activating APCs, mostly via signaling through pattern recognition receptors (20–25). Recent studies showed, however, that whereas primary expansion of effector CTLs is CD4 T cell-independent, memory CD8 T cells generated in the absence of Th cells show an impaired secondary expansion after recall stimulation (14–16). Although the phenomenon of poor secondary expansion of “unhelped” CD8 T cells held true for many experimental in vivo systems, there are also some situations in which unhelped CD8 T cells are not impaired in their recall proliferation potential (26).

The exact timing and mechanisms that are involved in delivering help to CD8 T cells are still controversial and currently, with respect to the timing, there are two different models:

1. The first one, the programming model, proposes that T help is only conferred to CD8 T cells during the early phases of the priming (12, 13, 27). The second model, the maintenance model, suggests that CD4 T cells are required during the entire phase of memory CD8 T cell differentiation and are thus important for the maintenance of memory CD8 T cells (16). With respect to the mechanisms by which help is conferred, a number of potential pathways were described. Several studies have addressed the role of CD40-CD40L interaction in general for the priming and differentiation of effector and memory CD8 T cells and, in particular, in immunizations with noninfectious Ags the CD40-CD40L interaction seems to play an important role (5, 17–19, 27, 28). In most studies, CD40L-interaction with CD40 on APCs was responsible for their full activation. One report indicated that CD40L on the surface of CD4 Th cells can directly interact with CD40 expressed by CD8 T cells (27) and it was proposed that this direct interaction between CD4 and CD8 T cells was a prerequisite for the help-receiving mechanism of CD8 T cells. However, these findings could not be confirmed in other experimental systems (24, 25, 29).

In a recent study using intravital microscopy, it was suggested that CD4 T cells directly, or indirectly via activation of dendritic cells (DCs), produce the chemokines CCL3 and CCL4. These chemokines then may specifically attract CCR5-expressing CD8 T cells to the Ag-presenting DC which had interacted with Th cells (30).
Finally, it has been shown that IL-2R signaling is implicated in generating proliferation-competent memory CD8 T cells in acute/resolved viral infections (31, 32).

Based on the heterogeneity of the proposed mechanisms of how and when CD4 T cells are involved in the generation of memory CD8 T cells, we sought to investigate the relative importance of the different mechanisms proposed in one defined heterologous prime-boost system. This experimental system consists of CD8 T cell priming with replication-incompetent virus-like particles (VLP) followed by a challenge infection with a recombinant vaccinia virus (VVG2). The only determinant that is shared between the two Ags is the CD8 T cell epitope lymphocytic choriomeningitis virus (LCMV) gp33–41. We have shown previously that VLP priming of CD8 T cells occurs in the absence of T help and that CD8 T cells primed in the absence of T help are impaired in their recall proliferation potential in a T help-deficient environment (11, 25). In this report, we show that the secondary proliferation of unhelped CD8 T cells could be restored in presence of T help only during the challenge phase. Furthermore, we observed neither a general need of signaling through the CD40 molecule nor a requirement of CD40 expression by CD8 T cells. We could also exclude a role for CCR5 expression on CD8 T cells, since the level of primary and secondary CD8 T cell expansion was comparable in CCR5-deficient and wild-type mice. Finally, we show that in vivo supplementale of IL-2 either during the priming or the challenge phase cannot compensate for the absence of T help in inducing proliferation-competent memory CD8 T cells.

**Materials and Methods**

Mice, viruses, VLPs, and peptides

C57BL/6, MHC class II-deficient (33), CD4-deficient (22), CD40-deficient (34), CCR5-deficient (The Jackson Laboratory (35)), and Ly5.1+327 TCR-transgenic (36) mice were maintained in a specific pathogen-free facility. Mice were immunized between 6 and 12 wk of age. Animal experiments were performed according to the regulations of the cantonal veterinary office. Recombinant vaccinia virus expressing the LCMV glycoprotein (VVG2) was originally obtained from Dr. D. H. L. Bishop (Oxford University, Oxford, U.K.) and was grown on BSC40 cells at a low multiplicity of infection; quantification was performed as previously described (37). Mice were infected i.p. with 5 × 10^6 PFU of VVG2.

gp33 VLPs, based on peptide gp33 coupled to VLPs derived from the bacteriophage Qβ, have been described previously (38). Packaging of CpG oligonucleotides (5′-GGGTTACCACTGGTACCGGG-3′, thioester stabilized) into the gp33 VLPs was performed as described previously (38). The LCMV glycoprotein peptide aa 33–41 (gp33 peptide, KAVYN FATM) was purchased from NeoMPS.

Immunization

Mice were immunized s.c. with 150 μg of gp33 VLPs. At least 30 days after immunization, mice were challenged i.p. with 5 × 10^6 PFU of VVG2. MHC class II-deficient or CD4-depleted C57BL/6 mice were coinjected i.d. daily with a mix of 50 μg of anti-IL-2 (clone 4B6; American Type Culture Collection) and 1.5 μg of recombinant mouse IL-2 (BD Biosciences) or with a mix of 10 μg of anti-IL-2 and 0.3 μg of recombinant mouse IL-2 (five times diluted mix).

In vivo CD4 T cell depletion

When indicated, mice were injected i.p. with 0.2 mg of purified anti-mouse CD4 YTS 191.1 mAb. For continuous depletion, mice were injected 3 and 1 days before immunization and then weekly. For depletion only during priming, mice were treated at days 3 and 1 before immunization. For depletion only during challenge, mice were treated at day −3 and −1 before challenge with VVG2. For depletion of CD25+ cells, mice were injected i.p. with 0.25 mg of anti-mouse CD25 mAb (PC61) at days 3 and 1 before immunization (a generous gift from Dr. A. Gallimore, Cardiff University, Cardiff, U.K. (39)).

Adoptive transfer

For adoptive transfer experiments, 10^6 splenocytes isolated from naive Ly5.1+ gp33-specific TCR-transgenic mice were adoptively transferred into naive recipient mice (C57BL/6 or MHC class II-deficient mice). At least 30 days after gp33 VLP immunization, lymphocytes were harvested from spleen and draining lymph nodes and CD8 T cells were purified by MACS according to the instructions of the manufacturer (Miltenyi Biotec). Before transfer, the frequency of memory Ly5.1+ CD8 T cells was determined by Ly5.1 surface staining and the numbers of donor cells transferred were normalized to contain an equal number of Ly5.1+ CD8 T cells (∼5.5 × 10^6 Ly5.1+ cells).

To study the role of CD40 on CD8 T cells, 10^6 splenocytes from L5.1+ TCR-transgenic CD40+/− or Ly5.1+ TCR-transgenic CD40−/− mice were adoptively transferred into naive C57BL/6 mice.

**Abs and peptide MHC class I tetramers**

PE-conjugated peptide-MHC class I tetrameric complexes were generated as previously described (40). The following mouse mAbs were purchased from BD Biosciences: anti-CD8 (PerCP), anti-CD127 (IL-7Ra) (FITC), anti-CD62L (allophycocyanin), anti-CD4 (FITC or Pacific Blue), anti-CD54.1 (PE), anti-CD25 (FITC), and anti-IFN-γ (allophycocyanin).

**Immunofluorescence staining and analysis**

For stainings, whole blood or single-cell suspensions from spleens and ovaries were used. Cells were washed in FACS buffer (PBS, 2% heat-inactivated FCS, 5 mM EDTA, and 0.02% sodium azide) and surface stained with directly labeled Abs or peptide-MHC class I tetramer complexes for 20 min at 4°C. Thereafter, erythrocytes were lysed using 1 ml of FACS Lyse (BD Biosciences) for 10 min at room temperature. Cells were washed and resuspended in PBS containing 1% paraformaldehyde.

For intracellular cytokine stainings, cells were stimulated with 1 μg/ml gp33 peptide in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich) at 37°C for 6 h. Cell surface staining was done as described above, followed by fixation and permeabilization using 500 μl of FIX/Perm solution (FACS Lyse diluted to 2× concentration and 0.05% Tween 20) for 10 min at room temperature. Cells were washed and stained with directly labeled Abs against IFN-γ.

Data were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**ELISA**

Thirty-five days after VLP immunization, serum was analyzed for Qβ-specific IgG. Ninety-six-well plates (Greiner Bio-One) were coated overnight with Qβ protein (4 μg/ml) in coating buffer (0.1 M NaHCO3, pH 9.6) at 4°C. Plates were washed with PBS-0.05% Tween 20 (PBST) and blocked for 2 h at room temperature (RT) with 2% BSA in PBST. After washing, serial dilutions of serum samples (prediluted 1/150 and subsequently serially diluted 1/3) were added to the wells and plates were incubated for 2 h at RT. Plates were washed and incubated with HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich) diluted 1/1000 in 2.5% FCS-PBS/3 mM NaN3. After 1 h, plates were washed five times and developed with ABTS (20 mg and 4 μl of H2O2, in 100 ml of 0.1 M Na2PO4/H2O (pH 4)). Plates were incubated for 30 min at RT and OD was determined at 405 nm with an ELISA reader (VICTOR3; PerkinElmer).

**Results**

Secondary expansion of memory CD8 T cells is impaired in the absence of T help

We studied the primary and secondary expansion of epitope-specific memory CD8 T cells in the presence or absence of T help. For this purpose, we compared effector and memory cells generated in C57BL/6 mice, MHC class II-deficient, CD4-deficient mice, or C57BL/6 mice continuously treated with a depleting CD4 Ab. Thirty-five days after priming with replication-incompetent VLPs carrying the LCMV gp33–41 peptide epitope (gp33 VLP), mice were challenged with recombinant vaccinia virus (VVG2) expressing the same gp33 epitope. Expansion and maintenance of gp33-specific CD8 T cells was analyzed weekly by tetramer staining in the blood. Extending our earlier results (11, 25), we show that in our prime-boost system recall proliferation of memory CD8 T cells is impaired in the three situations with absent CD4 T cells (Fig. 1A).
To confirm the absence of “classical” T help in CD4-depleted animals as well as in MHC class II-deficient animals, we measured the Qβ-specific IgG response upon VLP immunization, since the VLPs used in this study consist of the Qβ capsid protein of the bacteriophage Qβ. Thirty-five days after gp33 VLP priming, high titers of anti-Qβ-IgG were present in the serum of C57BL/6 mice. Thus, the number of CD4 T cells required to generate proliferation-competent memory CD8 T cells was abolished, similar to the situation observed in the CD4 and MHC class II-deficient mice (Fig. 2B, left panel). In contrast, when depletion of CD4 T cells was performed using a slightly less efficient batch of depleting Abs and CD4 T cell percentages ranged between 0.1% and 0.9% (Fig. 2A, right panel), gp33-specific memory CD8 T cells were able to expand upon VVG2 challenge (Fig. 2B, right panel). Thus, the number of CD4 T cells required to generate fully competent memory CD8 T cells is extremely low.

The titer of the anti-Qβ IgG response was indistinguishable in C57BL/6 and CD4-deficient mice (Fig. 1B), which is in line with previous reports showing a CD4-negative MHC class II-restricted population of functional Th cells in CD4-deficient mice (41–43). In contrast, in MHC class II-deficient mice as well as in CD4-depleted mice, the amount of anti-Qβ-IgG Abs was close to background (Fig. 1B). Thus, classical T help-mediating isotype switching is absent in MHC class II-deficient and CD4-depleted mice, whereas it is present in CD4-deficient mice.

In the experiments described so far, gp33-specific CD8 T cells were induced from the endogenous pool of naive CD8 T cells. To assess whether impaired secondary expansion may be linked to a selection of certain CD8 T cell clones in Th-deficient hosts with different functional or phenotypic properties, we repeated these experiments with adoptive transfer of a low number (10^6) of monoclonal Ly5.1+ TCR-transgenic CD8 T cells specific for the gp33-3-4 epitope before VLP immunization. TCR-transgenic CD8 T cells also failed to undergo secondary in vivo expansion in hosts lacking CD4 T cells (data not shown), demonstrating that the impaired secondary proliferation also holds for a monoclonal CD8 T cell population.

**FIGURE 1.** Impaired recall response of memory CD8 T cells in the absence of T help. C57BL/6, MHC class II+−, CD4+−− mice, and C57BL/6 mice treated continuously with depleting anti-CD4 Abs were immunized s.c. with 150 μg of gp33 VLPs. Thirty-five days later, mice were challenged i.p. with 5 × 10^6 PFU of VVG2. A, Frequencies of gp33+− specific CD8 T cells in the blood. B, Thirty-five days after gp33 VLP immunization, serum was analyzed by ELISA for the presence of anti-Qβ IgG. A first serum predilution 1/150 (predilution) was followed by serial 3-fold dilutions. As control, serum from a naive C57BL/6 was included. Each symbol represents the average of three mice per group. Error bars indicate the SD within a group. Data are representative of two independent experiments.

**FIGURE 2.** Low percentage of CD4 T cells is sufficient to confer full help to CD8 T cells. C57BL/6 mice were treated continuously with two batches of CD4-depleting mAbs. Mice were immunized with 150 μg of gp33 VLPs and 35 days later challenged i.p. with 5 × 10^6 PFU of VVG2. A, Frequencies of CD4 T cells in blood of mice treated with a more efficient (left panel) or with a less efficient (right panel) batch of depleting Abs. B, Frequencies of gp33+− specific CD8 T cells in the blood of mice treated with a more efficient batch (left panel) or mice treated with a less efficient (right panel) batch of depleting Abs. Triangles show timing of Ab injections. Each symbol represents the average of three mice per group. Error bars indicate the SD within a group. Data are representative of two independent experiments.

**Very low numbers of CD4 T cells can confer help to CD8 T cells**

Next, we assessed the threshold frequencies of CD4 T cells required to generate proliferation-competent memory CD8 T cells. To this end, we compared two different batches of CD4-depleting Abs which differ slightly in their effectiveness of depletion of CD4 T cells was performed using a slightly less efficient batch of depleting Abs and CD4 T cell percentages ranged between 0.1% and 0.9% (Fig. 2A, right panel). gp33-specific memory CD8 T cells were able to expand upon VVG2 challenge (Fig. 2B, right panel). Thus, the number of CD4 T cells required to generate fully competent memory CD8 T cells is extremely low.
FIGURE 3. Transient depletion of CD4 T cells during the priming or during the challenge does not affect memory CD8 T cell generation. C57BL/6 mice were immunized s.c. with 150 μg of gp33 VLPs and 36 days later challenged by i.p. infection with $5 \times 10^6$ PFU of VVG2. Mice were mock treated or treated with CD4-depleting Abs at the time of priming (d-3 and d-1), at the time of challenge (d33 and d35), or continuously. Frequencies of gp33-specific CD8 T cells in the blood of untreated mice (A), mice continuously treated (B), mice treated at the time of priming (C), and mice treated at the time of challenge (D). Numbers in bold represent the percentage of CD4 T lymphocytes in the blood at the indicated time points (d8, d36 + 1, and d36 + 7). Triangles show timing of Ab injections. Each symbol represents the average of three mice per group. Error bars indicate the SD within a group.

T help restricted to the challenge infection is sufficient for memory CD8 T cell recall proliferation

So far, we showed that continued absence of CD4 T cells during primary and secondary CD8 T cell responses abolished the recall proliferation of CD8 T cells. To address the question at which time point T help is required for the generation of fully competent memory CD8 T cells, CD4 T cells were depleted only at the time of priming, only at the time of challenge, or continuously. Only when CD4 T cells were depleted continuously, memory CD8 T cells failed to proliferate upon VVG2 challenge (Fig. 3B). Neither depletion during priming alone or during challenge alone abolished the proliferative capacity of memory CD8 T cells (Fig. 3, C and D). It should be mentioned, however, that depletion of CD4 T cells from primed mice is less efficient compared with depletion of CD4 T cells from naive mice. In fact, the percentage of CD4 T cells on day 7 after VVG2 challenge was 0.5% when depletion was performed just before challenge, whereas it remained at 0.07% when CD4 T cells were continuously depleted (Fig. 3, C and D).

Of note, we observed an increased primary expansion of Ag-specific CD8 T cells when CD4 T cells were depleted before priming (Fig. 3C). We hypothesized that this increased expansion might be related to the absence of regulatory T cells after depletion of all CD4 T cells. To determine whether this was the case, we depleted mice of CD25+ cells before VLP immunization, which resulted in similarly enhanced primary expansions of gp33-specific CD8 T cells as in CD4-depleted mice (data not shown). Thus, regulatory T cells seem to negatively impact on the primary expansion of gp33-specific CD8 T cells upon gp33 VLP immunization.

Since transient CD4 depletion before VVG2 challenge in VLP-immunized mice was not as efficient as in continuously depleted mice and since small amounts of CD4 T cells seem to support recall proliferation of CD8 T cells (Fig. 2), we addressed the same question using a different experimental setup, which was not dependent on in vivo depletion of CD4 T cells. We adoptively transferred T cells from Ly5.1+ gp33-specific TCR-transgenic mice into Ly5.2+ C57BL/6 or MHC class II-deficient recipients before immunization with gp33 VLP and then followed the expansion of Ly5.1+ CD8 T cells. As observed before, primary expansion of transferred gp33-specific CD8 T cells was comparable in both hosts 7 days after immunization (Fig. 4A) and at later time points (data not shown). Forty-seven days after immunization, mice were sacrificed and CD8 T cells were purified from the spleen and the draining lymph nodes. Equal numbers of CD8 Ly5.1+ cells were then transferred into naive C57BL/6 or MHC class II-deficient recipients and the new hosts were subsequently challenged with VVG2. The secondary expansion of the transferred memory CD8 T cells was analyzed in blood, spleen, and ovaries. In blood and spleen, robust secondary expansion was observed for TCR-transgenic CD8 T cells in T help-sufficient C57BL/6 hosts, irrespective of whether they had been primed in the presence (Th3→Th+)$\rightarrow$ or absence (Th3→Th+) of T help (Fig. 4B). In contrast, reduced secondary expansion of helped (Th3→Th+) or unhelped (Th3→Th+) CD8 T cells occurred in MHC class II-deficient hosts. A similar trend was observed in the ovaries. In line with the secondary expansion data, CD8 T cells in Th help-competent hosts showed increased down-regulation of CD62L in the blood compared with T help-deficient hosts (Fig. 4C). Concordant with previous results (11), we observed that in the ovaries, CD62L was down-regulated in all hosts, consistent with an effector phenotype of cells that migrate to peripheral tissues (Fig. 4C).

T help conferred to CD8 T cells does not depend on CD40 expression on CD8 T cells

Next, we initiated a series of experiments aimed at addressing the mechanisms by which T help is conferred to memory CD8 T cells. Based on the reported finding that CD8 T cells can transiently express the CD40 molecule upon activation, potentially allowing direct interaction between CD8 and CD4 T cells through CD40-CD40L binding (27), we tested the role of CD40 expression on CD8 T cells in our experimental prime-boost system. For this purpose, we transferred CD8 T cells from Ly5.1+ CD40+/+ or Ly5.1+ CD40−/− gp33-specific TCR-transgenic mice into Ly5.2+ C57BL/6 hosts and analyzed primary and secondary expansion of the transferred cells after VLP priming and VVG2 challenge. Both after priming with VLP and after challenge with VVG2, the expansion of the CD40++ and CD40−/− CD8 T cells in the blood was comparable (Fig. 5A). Also in spleen and in ovaries, we could not detect any difference in the frequency of CD40++ and CD40−/− gp33-specific CD8 T cells after VVG2 challenge (Fig. 5, B and C). Thus, CD40 expression on CD8+ T cells is
not required for primary or secondary CD8+ T cell responses in our prime-boost system. We have previously shown that the primary and secondary proliferation capacity of CD8 T cells was not impaired in complete absence of CD40 in this experimental system (25).

**Absence of CCR5 does not influence primary and secondary CD8 T cell responses**

It was proposed that activated CD4 T cells interact with Ag-presenting DCs and induce the release of chemokines CCL3 and CCL4, which then specifically attract CCR5-expressing CD8 T cells to the Ag-presenting DCs (30). To test this possibility, we analyzed whether the CCL3/4-CCR5 interaction was relevant in our experimental system. For this purpose, we primed C57BL/6 or CCR5-deficient mice with gp33 VLP and challenged them after 36 days with VVG2. Absence of CCR5 did not affect the priming of the gp33-specific effector cells nor recall responses of the memory CD8 T cells after VVG2 challenge in both secondary lymphoid organs and in the ovaries (Fig. 6).

**In vivo supplementation of IL-2 does not rescue secondary expansion of unhelped memory CD8 T cells**

IL-2 is a crucial cytokine in activation and differentiation of CD8 T cells (reviewed in Refs. 44 and 45) and recently it was shown that IL-2 signals are required for the differentiation of proliferation-competent memory CD8 T cells during viral infections (31, 32). We therefore tested the effect of daily injections of IL-2 along with anti-IL-2 Ab on unhelped memory CD8 T cells during the challenge phase with VVG2. We observed that injection of a mix of IL-2 and anti-IL-2 (at concentrations used in earlier studies)
therefore decided to use smaller amounts of IL-2/anti-IL-2 complexes. We immunized C57BL/6 or CCR5-deficient mice s.c. with 150 μg of gp33 VLP and 36 days later mice were infected i.p. with 5 × 10⁶ PFU of VVG2. A. Frequencies of gp33-specific CD8 T cells in the blood. Seven days after VVG2 challenge, the frequency of gp33-specific CD8 T cells was determined in the spleen (B) and in the ovaries (C). Each symbol represents the average of three mice per group. Error bars indicate the SD within a group.

During VVG2 challenge resulted in a generally increased activation and expansion of CD8 T cells (presumably also via activation of naive CD8 T cells (47, 48)), leading to an overall 3.5-fold increase of total CD8 T cells in spleen (data not shown). However, this treatment did not rescue the impaired proliferation of unhelped memory gp33-specific CD8+ T cells (data not shown). The extent of overall CD8 T cell activation after IL-2/anti-IL-2 administration might have secondary effects on reactivation of memory gp33-specific CD8 T cells, e.g., competition for APC interactions. We therefore decided to use smaller amounts of IL-2/anti-IL-2 complexes (five times diluted) during the challenge phase. In this case, overall CD8 T cell numbers were comparable to those of untreated mice, but also in this situation the secondary expansion of the memory gp33-specific CD8 T cells generated in the absence of help was not rescued (Fig. 7A).

We next addressed the question whether IL-2 supplementation during the priming phase would allow the differentiation of proliferation-competent memory CD8 T cells in a T help-deficient host. We therefore treated mice with IL2/anti-IL-2 complexes during the first 7 days of gp33 VLP immunization. Also in this case, treatment did not rescue the secondary expansion of memory CD8 T cells generated in absence of help (Fig. 7B).

**Discussion**

In this study, we analyzed the generation of memory CD8 T cells and their capacity to re-expand upon restimulation in the presence or absence of CD4 Th cells using a defined prime-boost regimen. We observed that help is required essentially during the challenge phase and not during the priming phase for robust secondary expansion to occur. Moreover, we analyzed whether a series of currently proposed mechanisms are involved in the delivery of help. We demonstrate that help does not depend on CD40 expression on CD8 T cells, we show that priming and generation of proliferation-competent CD8 T cells is not reduced in the absence of CCR5 and that in vivo supplementation of IL-2 does not rescue the proliferation incompetence of unhelped CD8 T cells.

In the last years, it became apparent that Th cells often play a central role in generating proliferation-competent memory CD8 T cells even after pathogen infections (12, 14–16, 25, 49). However, due to the complexity and the differences among the diverse models studied, there are still controversies concerning the time points and the mechanisms that are implicated in the delivery of help for CD8 T cells.

We chose to study the role of CD4 T cells in generating proliferation-competent memory CD8 T cells in a defined heterologous prime-boost regimen that we have described previously (25, 38). Priming with replication-incompetent gp33 VLP has the advantage to exclude possible Ag persistence even in mice lacking CD4 T cells.

**FIGURE 6.** CCR5 is not required for generating fully competent memory CD8 T cells. C57BL/6 or CCR5-deficient mice were immunized s.c. with 150 μg of gp33 VLP and 36 days later mice were infected i.p. with 5 × 10⁶ PFU of VVG2. A. Frequencies of gp33-specific CD8 T cells in the blood. Seven days after VVG2 challenge, the frequency of gp33-specific CD8 T cells was determined in the spleen (B) and in the ovaries (C). Each symbol represents the average of three mice per group. Error bars indicate the SD within a group.

**FIGURE 7.** In vivo IL-2 supplementation does not rescue unhelped CD8 T cells. C57BL/6 (T help⁺), MHC class II⁻/⁻ (A), or CD4⁻/⁻ (B) (T help⁻) mice were immunized s.c. with 150 μg of gp33 VLPs and 34 or 35 days later mice were challenged i.p. with 5 × 10⁶ PFU of VVG2. A. One group of T help⁺ mice was injected during the challenge phase with IL-2/anti-IL-2 complexes. B. One group of T help⁻ mice was injected during the priming phase (days 0–7) with IL-2/anti-IL-2 complexes. Control mice were injected at the same time points i.p. with PBS. The numbers in bold indicate the percentage of tetramer-positive (tet⁺) CD8 T cells among total CD8 and numbers in parentheses indicate the percentage of tetramer-positive CD8 T cells among total lymphocytes in the blood. Right panels, Total numbers of tetramer-positive cells per spleen are shown. Three mice per group were tested and error bars indicate the SD within a group.
cells (and hence isotype-switched Abs) (38, 50). As challenge Ag we choose a recombinant vaccinia virus which shares with the priming immunogen only the LCMV-derived gp33 peptide. Therefore, potentially confounding effects of other memory cells or of Abs could be excluded. In the VLP prime-VVG2 boost system, CD4 T cells are required for the generation of proliferation-competent memory CD8 T cells (11, 25).

In contrast to our results, other studies (12–15) observed a decrease in the secondary proliferation of CD8 T cells when CD4 T cells were only absent for a few days during the priming phase. Janssen et al. (12, 13) proposed that CD4 T cells program CD8 T cells during the first 3–4 days after immunization. However, we showed by transient depletion of CD4 T cells before priming and with adoptive transfer experiments that memory CD8 T cells generated in the absence of help can proliferate well when challenged in the presence of help. This demonstrates that they are not irreversibly programmed during VLP priming in the absence of T help. The discrepancies between our results and the findings by Janssen et al. (12, 13) might be explained by differences in the experimental systems; while Janssen et al. (12, 13) primed CD8 T cells with noninflammatory cell-associated Ags and restimulated memory cells in vitro, we primed CD8 T cells with VLPs loaded with TLR9 ligands and challenged in vivo with a viral vector. In line with our results, it was shown that after LCMV infection CD4 T cells are dispensable during the priming phase for the generation of proliferation-competent CD8 T cells, as long as the challenge was performed in a Th cell-sufficient host (16).

In analogy to our adoptive transfer experiments, Sun and Bevan (15) performed similar adoptive transfer experiments using recombinant *Listeria monocytogenes* as Ag. They observed a significantly decreased proliferation capacity of CD8 T cells primed in the absence of help and challenged in the presence of CD4 T lymphocytes. We observed, however, for challenge with recombinant vaccinia virus that the presence of T help during the challenge period completely restored the proliferation capacity of memory CD8 T cells initially primed in the absence of T help. One possible explanation for these differences could be that viral and bacterial challenge systems differ in their need for help. Furthermore, Sun and Bevan (15) primed and challenged the mice with recombinant *L. monocytogenes*. Therefore, the new recipient received, additionally to OVA-specific memory cells, other memory CD8 T cells specific for different *Listeria* epitopes. Moreover, there is the possibility that in MHC class II-deficient hosts some bacteria might persist at low levels, keeping CD8 T cells in a more activated state, which might influence their proliferation potential.

We observed a very low threshold for CD4 T cell numbers to deliver T help for the generation of proliferation-competent memory CD8 T cells. Even in vivo, CD4 T cell depletion below 1% was not sufficient to generate an unhelped CD8 T cell phenotype. In fact, in vivo depletion had to be below 0.1% of CD4 T cells to get the unhelped CD8 T cell phenotype. In line with our results, it was previously shown that CD8 T cell responses against some viruses, including LCMV, were not affected in CD28-deficient mice even though CD4 T cell help is severely reduced in these mice (51, 52). Considering these results and the fact that the priming and challenge Ags in our experimental system do not share any CD4 T cell epitopes, it is highly unlikely that T help is conferred in an Ag-specific, cognate manner. This is further supported by our adoptive transfer experiments, in which primed CD8 T cells are transferred into hosts exhibiting a naive CD4 T cell compartment. Also in this situation, efficient recall responses occurred upon challenge with VVG2. A further interesting point to mention is the fact that proliferation-incompetent memory CD8 T cells were also generated in CD4-deficient mice. Although lacking the CD4 molecule, these mice are largely normal with respect to classical Th functions such as cognate help for B cells (Refs. 41–43 and our own data; Fig. 1B). MHC class II-restricted CD4-negative T cells are present in these mice, which are fully competent of helping B cells but which apparently fail to mediate help for the priming of proliferation-competent CD8 T cells.

We furthermore assessed several potential mechanisms which might be relevant for the delivery of help from CD4 T cells. After recognition of the peptide-MHC class II complex on DCs, CD4 T cells express CD40L on their surface and can thereby subsequently contribute to the activation of DCs via CD40-CD40L interaction, in particular, in situations where DC activation does not or only marginally occurs via the immunogen. Indeed, in many experimental systems, an important contribution of CD8 T cell priming was shown to be mediated via CD40-CD40L interactions (17–19). Therefore, we sought to determine the importance of signaling via CD40 in generating memory CD8 T cells in our experimental system. However, the presence of CD40 was entirely dispensable in our prime-boost system, as primary and secondary CD8 T cell responses were comparable in wild-type and in CD40-deficient mice (25). This is in line with previous studies, showing that in many pathogen infections CD40 and CD40L are dispensable for induction of CD8 T cell responses (24, 25, 29). Furthermore, CD8 T cells can transiently express CD40 upon activation and might therefore directly interact with CD40L-expressing Th cells (27). We also tested whether specific absence of CD40 on CD8 cells would have any influence on the priming of primary and secondary CD8 T cell responses in our system. However, absence of CD40 on CD8 T cells did not impair generation of memory CD8 T cells, allowing us to exclude that help is conferred by direct interaction between CD4 and CD8 T cells via CD40-CD40L. In line with our results, a dispensable role of CD40 on CD8 T cells was also shown in a system with recombinant *Listeria* priming/LCMV challenge (29). It might be possible that help is delivered by direct interaction of CD4 and CD8 T cells via other molecules than CD40. However, this hypothesis seems to be unlikely, since at the time of challenge, a proportion of one CD4 T cell for more than 10 memory CD8 T cells is sufficient for memory recall proliferation (Fig. 2B). This would imply that one Th cell would have to subsequently interact with >10 CD8 T cells to confer help.

An additional mechanism by which CD4 T cells might impact on CD8 T cell priming involves enhanced recruitment of CCR5-expressing CD8 T cells to APCs which have interacted with CD4 T cells. In this scenario, CD4 T cells or activated DCs secrete chemokines such as CCL3 and CCL4, and CCR5-expressing CD8 T cells are attracted by these chemokines to the Ag-presenting DCs (30). We tested whether CCR5 plays a role in the primary or secondary CD8 T cell responses in our VLP priming-VVG2 boosting system. However, we observed no role of the CCR5 chemokine receptor in generating memory CD8 T cells and in their proliferation capacity upon rechallenge. These different outcomes are probably explained by differences in the experimental systems, since we investigated priming of endogenous CD8 T cells upon VLP immunization and Castellino et al. (30) used Ag-loaded DCs in conjunction with high numbers of adoptively transferred TCR-transgenic cells. Furthermore, absence of CCL3/CCL4 signaling via CCR5 is likely to be compensated by other chemokines produced upon viral infection.
We finally addressed the question whether the help might be delivered by soluble factors instead of direct cell interactions. A good candidate to test was IL-2, because this cytokine is produced by CD4 T cells and is implicated in the induction of proliferation of CD8 T cells. Moreover, it has recently been shown that in the case of LCMV infection, signaling through the IL-2R was necessary for the development of proliferation-competent memory CD8 T cells (31, 32). Because we observed a dependence on T help during the challenge phase, we postulated that CD4 T cells directly secrete or activate other cells to secrete IL-2 during the challenge phase. However, we were not able to rescue unhelped memory cells in vivo supplementation of IL-2 neither during the challenge phase nor during the priming phase. It seems that at least for our prime-boost system that CD4 T cell and signaling through IL-2 are not correlated, implicating that both signals have to be present for efficient memory CD8 T cells recall proliferation. Another possible explanation for our results might be that IL-2, instead of directly acting on CD8 T cells, might activate CD4 T cells, either directly or via other mediators. Therefore, supplementation of IL-2 in the absence of CD4 T cells would fail to rescue CD8 T cells.

In conclusion, we demonstrate that in our VLP prime-vaccinia boost system, T help is required during the challenge phase for recall proliferation of memory CD8 T cells. We found no evidence for a role for CD40, CCR5, and IL-2 in delivering help from CD4 cells. Further studies will aim at the elucidation of the nature of the help conferred to memory CD8 T cells in this in vivo prime-boost system.

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Disclosures

M.F.B. and K.S. are Cytos employees and own stocks or stock options of the company.

References


