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Th1 and Th2 Cells Help CD8 T-Cell Responses[▽]

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Help from CD4 T cells is often important for the establishment of primary and memory CD8 T-cell responses. However, it has yet to be determined whether T helper polarization affects the delivery of help and/or whether responding CD8 T cells helped by Th1 or Th2 cells express distinct effector properties. To address these issues, we compared CD8 T-cell responses in the context of Th1 or Th2 help by injecting dendritic cells copulsed with the major histocompatibility complex class I-restricted OVA peptide plus, respectively, bacterial or helminth antigens. We found that Th2 cells, like Th1 cells, can help primary and long-lived memory CD8 T-cell responses. Experiments in interleukin-12 (IL-12)$^{-/-}$ and IL-4$^{-/-}$ mice, in which polarized Th1 or Th2 responses, respectively, fail to develop, indicate that the underlying basis of CD4 help is independent of attributes acquired as a response to polarization.

CD8 T cells play a critical role in protection against viral and intracellular bacterial and protozoan infections and are important in tumor and graft rejection. After activation, naive antigen (Ag)-specific CD8 T cells are able to proliferate quickly and differentiate into potent effector cells capable of rapid cytokine production and cytolytic killing of target cells (10). Early studies suggested that CD4 T-cell help is required for priming CD8 T-cell responses in the absence of inflammation (2, 22, 24), whereas CD8 T-cell responses to highly inflammatory Ag develop independently of CD4 help (4, 21, 34). Recently, it has become clear that CD4 Th cells present during the initiation stages of the immune response are essential for the development and/or maintenance of memory CD8 T cells even in conditions where primary CD8 responses are able to develop independently of help (3, 12, 25, 28). However, it is also apparent that CD4 T cells are required for the maintenance of memory CD8 T cells after acute infection (29).

After appropriate activation, naive Th cells differentiate into Th1 or Th2 effector cells. Th1 cells characteristically produce gamma interferon (IFN-γ) and are important for resistance to intracellular pathogens, whereas Th2 cells secrete interleukin-4 (IL-4), IL-5, IL-9, and IL-13 and play protective roles against helminths and other extracellular pathogens (19, 27). Despite the wealth of knowledge about the direct roles of Th1 versus Th2 cells in protection and immunopathology, there is little information regarding whether Th1 and Th2 cells are equally capable of providing help for CD8 T-cell responses. To specifically address this question, we developed a system for CD8 priming by dendritic cells (DCs) that have been conditioned to induce Th1 or Th2 responses.

In addition to being highly capable of activating naive T cells, DCs are able to interpret pathogen-inherent signals and influence the characteristics of the T-cell responses that they induce (13). In this context, we have shown that murine bone marrow-derived DCs exposed to the gram-positive bacterium *Propionibacterium acnes* induce strong *P. acnes*-specific Th1 responses when injected into naive wild-type (WT) mice, whereas DCs exposed to Ag from the parasitic helminth *Schistosoma mansoni* (SEA) induce SEA-specific Th2 responses (7, 16–18). For the experiments described here, mice were injected with DCs pulsed with *P. acnes* or SEA in combination with the major histocompatibility complex class I-restricted ovalbumin peptide SIINFEKL (OVA_{pep}), and thus CD8 T-cell priming occurred in the context of help from Th1 or Th2 cells, respectively. The data indicate that Th1 and Th2 cells are capable of providing help for CD8 T-cell priming and memory development, that the effector properties of CD8 T cells that develop with Th1 help are similar to those that develop with Th2 help, and that the underlying basis of CD4 help is independent of Th1 and/or Th2 polarization.

MATERIALS AND METHODS

Mice. Female 6- to 8-week-old C57BL/6 (B6) mice, and B6 IL-12p35$^{-/-}$, IL-4$^{-/-}$, and major histocompatibility complex class II$^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All experiments were conducted according to the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee.

In vivo depletion of CD4 T cells. CD4$^+$ T cells were acutely depleted by subcutaneous administration of 1 mg of monoclonal antibody GK1.5 (BioExpress, West Lebanon, NH) per mouse on days −2 and +2 surrounding the injection of the DCs. Flow cytometric analysis revealed that depletion was >90% effective. Control mice received equivalent doses of purified normal rat immunoglobulin G (IgG; Sigma-Aldrich, St. Louis, MO).

DC preparation. DCs were generated from murine bone marrow cultured in the presence of granulocyte-macrophage colony-stimulating factor (PeproTech, Rocky Hill, NJ) as described previously (15). SIINFEKL ($OVA_{257-264}$, OVA_{pep})

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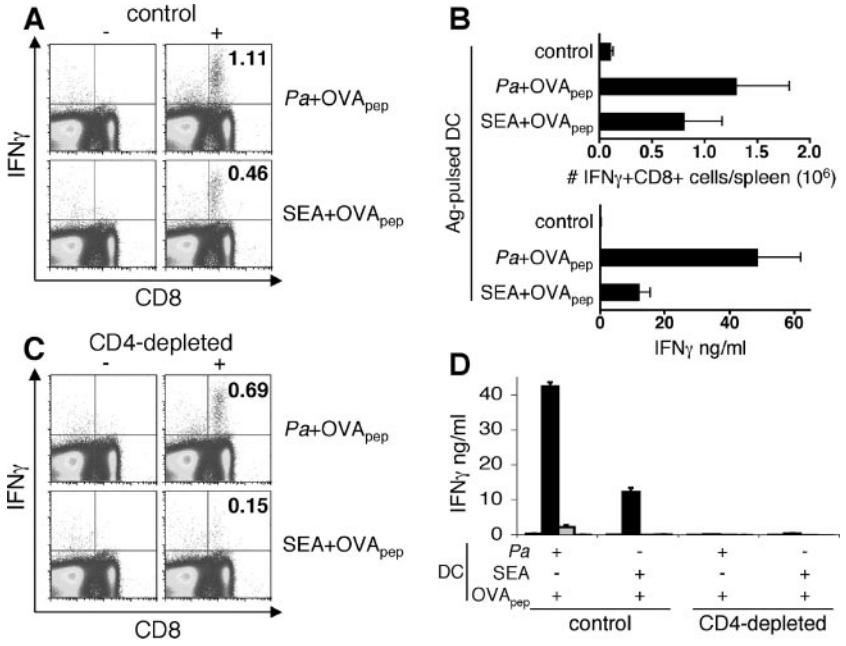

FIG. 1. CD4 help is required for the development of IFN-γ-producing CD8 T cells during immunization with peptide-pulsed DCs. DCs pulsed with *P. acnes* or SEA in combination with OVA_{pep} were injected into naive WT mice. One week after immunization, splenocytes were harvested, and IFN-γ production was measured by ICS and ELISA after ex vivo incubation with OVA_{pep}. (A and C) Splenocytes from mice primed with OVA_{pep}+*P. acnes*- or OVA_{pep}+SEA-pulsed DCs were restimulated for 5 h in medium alone (−) or with OVA_{pep} (+) and then stained for intracellular IFN-γ. The FACS plots are from a representative mouse, and the numbers in the quadrants are the percentages of CD8 T cells producing IFN-γ. The values in panel A are from mice treated with a control rat IgG; the values in panel C are from mice depleted of CD4 cells by injection with GK1.5. (B) Splenocytes from untreated mice primed with DCs alone (control) or with *P. acnes*+OVA_{pep}- or SEA+OVA_{pep}-pulsed DCs were restimulated for 5 h with OVA_{pep}. CD8 cells were stained for intracellular IFN-γ by ICS (upper panel), or splenocytes were stimulated for 72 h with OVA_{pep}, after which the culture supernatants were assayed for IFN-γ by ELISA (lower panel). The data represent mean values ± the standard error of the mean from seven separate experiments. (D) IFN-γ levels as measured by ELISA from groups of three individual DC-immunized (shown on the *x* axis) control (normal rat IgG treated) or CD4 T-cell depleted mice and restimulated with medium (hatched bars), OVA_{pep} (black bars), *P. acnes* (gray bars), or SEA (empty bars). Little or no detectable IFN-γ was made in medium- or SEA-restimulated cultures. Values are means ± the standard deviation of three analyses. These experiments were repeated twice with similar results.

was synthesized by Invitrogen (Carlsbad, CA). Heat-killed *P. acnes* was obtained from The Van Kampen Group (Hoover, AL), whereas endotoxin-free SEA was prepared as previously described (17). DCs were pulsed with the appropriate Ag (1 μg of OVA_{pep}/ml plus 50 μg of SEA/ml or 20 μg of *P. acnes*/ml) for the final 18 h of incubation, after which they were washed and resuspended in Hanks balanced salt solution (Mediatech, Inc., Herndon, VA) and injected intraperitoneally (5×10^5 cells per mouse) into naive mice.

Infections. Approximately 2 months after injection with Ag-pulsed DCs, mice were challenged intravenously with 3×10^4 CFU/mouse of a recombinant *Listeria monocytogenes* that expresses the SIINFEKL epitope (rLmOVA) (26).

ELISA. Spleens were aseptically removed, and single-cell suspensions were prepared as described previously (17). Splenocytes were resuspended at 10^7 cells/ml in RPMI 1640 (Mediatech) supplemented with 30 mM HEPES, 100 U of penicillin/ml, and 100 μg of streptomycin (Life Technologies, Gaithersburg, MD)/ml, 50 μM 2-mercaptoethanol, and 3% normal mouse sera (Sigma-Aldrich) and restimulated ex vivo in 96-well flat-bottom plates (Becton Dickinson, Franklin Lakes, NJ) with OVA_{pep} (100 μg/ml), *P. acnes* (20 μg/ml), or SEA (50 μg/ml) for 72 h at 37°C in 5% CO_2. Culture supernatants were then collected, and the cytokine levels were measured by capture enzyme-linked immunosorbent assays (ELISAs) (17).

ICS and fluorescence-activated cell sorting (FACS). For intracellular cytokine staining (ICS), splenocytes were resuspended at 2×10^7 cells/ml in complete RPMI plus 10% fetal calf serum (Gemini Bio-Products, Woodland, CA) and restimulated ex vivo with OVA_{pep} in 96-well U-bottom plates with human rIL-2 (10 U/ml) and brefeldin A (4 μg/ml; BD Pharmingen) for 5 h at 37°C in 5% CO_2. The splenocytes were then fixed, permeabilized, and stained with fluorochrome-conjugated MAbs specific for CD8, IFN-γ, or tumor necrosis factor alpha (TNF-α; BD Pharmingen). Events were acquired by using a FACSCaliber flow cytometer and CellQuest (BD Biosciences, San Jose, CA) and then analyzed with FlowJo software (Tree Star, San Carlos, CA).

In vivo cytotoxic-T-lymphocyte assay. The in vivo cytotoxicity assay was performed as described previously (1). Briefly, splenocytes from B6 mice were labeled with high (1 μM) or low (0.1 μM) doses of CFSE (Molecular Probes, Eugene, OR). $CFSE^{high}$ cells were then incubated with 1 μg of OVA_{pep}/ml for 45 min at 37°C in complete RPMI 1640 supplemented with 3% normal mouse serum. Equal numbers (5×10^6) of high or low CFSE cells were resuspended in Dulbecco phosphate-buffered saline (Mediatech) and injected intravenously into DC-primed recipient mice at 5 days after rLmOVA challenge. One hour later the splenocytes were removed, and two million events were analyzed by flow cytometry. The percent killing was calculated as described previously (1).

RESULTS

CD4 T-cell help is required for the optimal development of IFN-γ-producing CD8 T cells. There is little information regarding whether Th2 polarized cells provide help to or inhibit the priming of CD8 T-cell responses. To address this issue, mice were immunized with DCs that were copulsed with either *P. acnes* (a Th1-polarizing Ag) or SEA (a Th2-polarizing Ag) in combination with OVA_{pep}. One week later, splenocytes were harvested, and IFN-γ production by CD8 T cells in response to ex vivo stimulation with OVA_{pep} was measured by ICS and flow cytometry or by ELISA. These analyses showed that the magnitude of the OVA_{pep}-specific primary CD8 T-cell response, as measured by the percentage (Fig. 1A) or absolute numbers (Fig. 1B) of IFN-γ-producing OVA_{pep}-specific CD8

T cells or the amount of IFN-γ secreted during prolonged culture (Fig. 1B) was greater when mice were primed with OVA_{pep}+*P. acnes*-pulsed DCs than when mice were primed with OVA_{pep}+SEA-pulsed DCs or DCs alone (control). Low or undetectable levels of IFN-γ were made in the absence of $OVA_{pep.}$ (Fig. 1A). These data indicate that both Th1 and Th2 cells can help increase the magnitude of the primary CD8 T-cell response, but Th1 cells are more capable in this regard.

An alternative interpretation of the data shown in Fig. 1 is that DCs can activate CD8 cells independently of CD4 T-cell help and that *P. acnes*-activated mature DCs are better Ag-presenting cells for CD8 T cells than are SEA-pulsed DCs or OVA_{pep}-pulsed DCs, which retain an immature phenotype (15; data not shown). To address this issue, we injected OVA_{pep}-pulsed DCs into WT mice that had been depleted of CD4 T cells. At 1 week postinjection, OVA_{pep} stimulation of splenocytes from these mice revealed that the primary CD8 T-cell response was significantly impaired by the absence of help, especially under Th2 conditions (Fig. 1C versus 1A). The extent of this impairment was made clear by the measurement of IFN-γ in the supernatants of ex vivo cultured OVA_{pep}-restimulated cells (Fig. 1D). Whereas significant amounts of IFN-γ were produced by CD8 T cells from immunized intact (control) mice, little if any of this cytokine was made when priming occurred in the absence of CD4 T cells (Fig. 1D). Taken together, these results suggested that CD4 help was required during priming of the CD8 OVA-specific response and that Th1 help induced a more robust primary response to OVA_{pep}.

Given that, like CD8 T cells, Th1 cells play an important role in immunity to intracellular pathogens (27), a role for this subset of CD4 cells as helpers during CD8 T-cell responses is relatively easy to envisage. However, recent evidence has highlighted the importance of IL-4, a major Th2 product, in the development of CD8 T-cell responses against the malaria parasite (6), a finding that fits with earlier reports indicating that IL-4 is a helper factor for CD8 T cells (20, 32). Thus, production of IL-4 may provide a mechanism by which Th2 cells can help CD8 T-cell responses. To directly compare lineage-specific components of Th1- versus Th2-mediated help, WT mice and mice deficient for IL-4 and IL-12p35, which exhibit defects in Th2 and Th1 response development, respectively, after priming with Ag-pulsed DCs (16), were injected with DCs copulsed with OVA_{pep}+*P. acnes* or OVA_{pep}+SEA. One week later, the Th1 (IFN-γ) and Th2 (IL-5) cytokine levels were measured ex vivo in the supernatants of *P. acnes*- or SEA-restimulated splenocytes from WT and cytokine-deficient mice. *P. acnes* restimulation of spleen cells from OVA_{pep}+*P. acnes*-pulsed DC-immunized WT mice was found to induce measurable production of IFN-γ, a finding consistent with the Th1-polarizing effects of *P. acnes* (Fig. 2A). Notably, the levels of IFN-γ were less than those induced by restimulation with OVA_{pep} (Fig. 2C). In this system, IFN-γ made in response to *P. acnes* was produced by CD4 T cells, whereas IFN-γ made in response to OVA_{pep} was produced by $CD8^+$ T cells (data not shown). As anticipated, spleen cells from IL-12p35$^{-/-}$ recipients of OVA_{pep}+*P. acnes*-pulsed DCs were unable to produce IFN-γ in response to restimulation with *P. acnes* (Fig. 2A), illustrating the loss of Th1 responsiveness in the absence of IL-12 (16). Nevertheless, OVA_{pep} restimulation did lead to

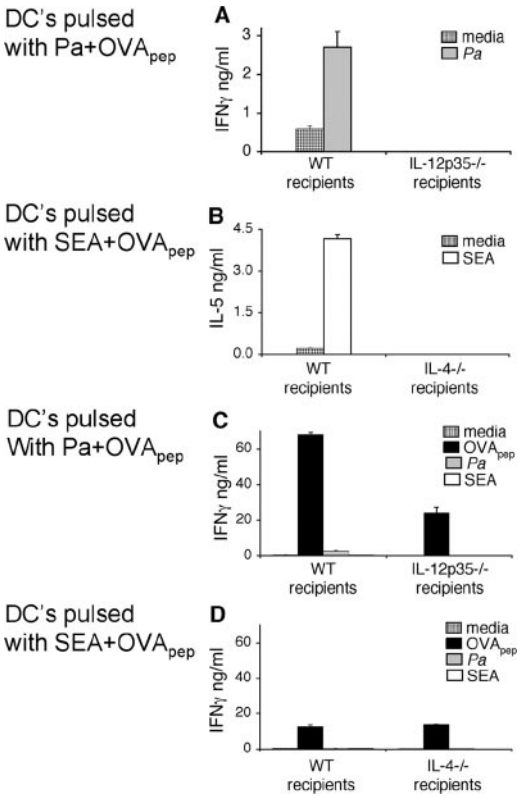

FIG. 2. Effect of Th1 and/or Th2 polarization on CD4 help for primary CD8 T-cell responses. Naive WT, IL-12p35-deficient, or IL-4-deficient mice were injected with Ag-pulsed DCs. One week after injection, splenocytes were harvested from individual mice and cultured in medium alone, Pa, SEA, or OVA_{pep}. Th1 responses are shown in panel A, and Th2 responses are shown in panel B. Panels C and D include IFN-γ production by CD8 T cells in response to restimulation with OVA peptide. Panels C and D utilize different scales compared to panel A, since the amounts of IFN-γ produced by CD8 T cells are so much greater than those produced by Th1 cells (A). Cytokine levels in 72 h culture supernatants were measured by ELISA. Two experiments were performed with similar results.

IFN-γ production in the absence of IL-12, but at levels lower than in the WT controls (Fig. 2C) and closer in magnitude to those seen in WT recipients of OVA_{pep}+SEA-pulsed DCs (Fig. 2D). There is no evidence for a Th2 response to *P. acnes* in the OVA_{pep}+*P. acnes*-pulsed DC-immunized IL-12p35$^{-/-}$ mice (16; data not shown). Although the absence of IL-4 resulted in the failure of OVA_{pep}+SEA-pulsed DCs to induce an SEA-specific Th2 response, as measured by IL-5 production (Fig. 2B), and did not result in a default SEA-specific Th1 response (16; data not shown), it had little effect on the development of OVA_{pep}-specific IFN-γ production (Fig. 2D). Altogether, these data allow us to draw several conclusions. First, the help provided by CD4 cells for maximal OVA_{pep}-specific CD8 T-cell responses in mice immunized with OVA_{pep}+SEA-pulsed DC is independent of a requirement for Th2 polarization. Second, while a component of the help provided by CD4 cells after immunization with OVA_{pep}+*P. acnes*-pulsed DCs does appear to be dependent upon attributes of Th1 cells, there is an underlying mechanism of help that operates independently of Th1 response polarization.

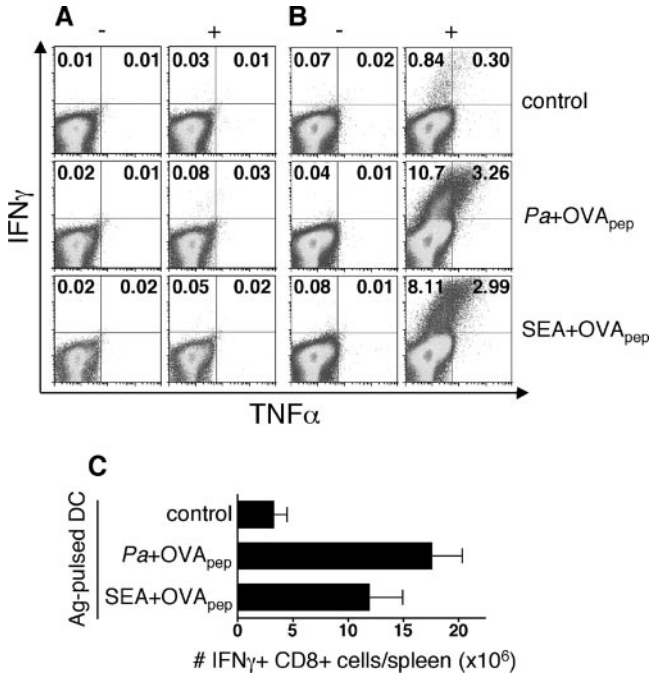

FIG. 3. Th1 and Th2 cells provide help for memory CD8 T-cell development. Naive mice were primed with Ag-pulsed DCs. Sixty days later, mice were challenged with rLmOVA or injected with PBS. Six days after rLmOVA challenge, splenocytes were harvested and restimulated with OVA_{pep} for 5 h, and the IFN-γ and TNF-α levels were measured by ICS. Representative FACS plots show IFN-γ versus TNF-α (gated on live CD8 T cells) production by unstimulated (−) or OVA_{pep}-stimulated (+) splenocytes from unchallenged (A) and rLmOVA-challenged (B) mice. The numbers represent the percentages of CD8 T cells producing cytokines. (C) Bar graph showing mean ± the standard deviation of the numbers of splenic CD8 cells that made IFN-γ in response to in vitro stimulation with OVA_{pep}. Cells cultured in the absence of OVA_{pep} failed to make IFN-γ. These data were pooled from nine individual experiments.

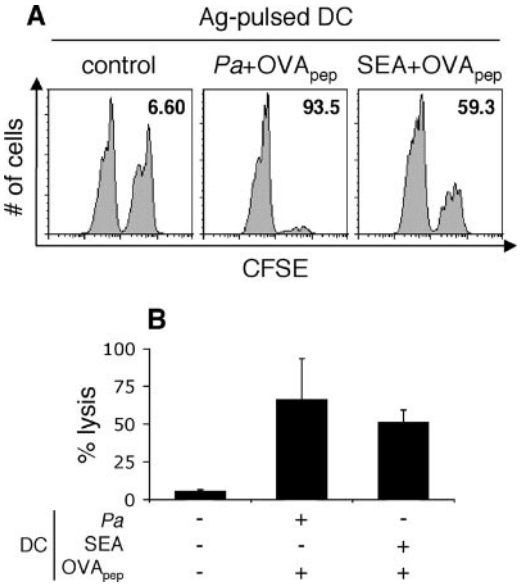

FIG. 4. Cytotoxicity of CD8 T cells helped by Th1 or Th2 cells. Naive mice were primed with Ag-pulsed DCs and >60 days later challenged with rLmOVA. At 5 days postchallenge, the mice were injected with $CFSE^{high}$-OVA_{pep}-pulsed syngeneic target cells and with unpulsed $CFSE^{low}$ syngeneic control cells. One hour later individual spleens were removed, and in vivo cytotoxicity was measured directly by flow cytometry through a comparison of the ratio of $CFSE^{high}$ to $CFSE^{low}$ cells in individual animals. (A) Representative plots gated on CFSE-positive cells. The numbers indicate the percent lysis. (B) Mean values (± the standard error of the mean) from three mice per group for the percent lysis of target cells.

Th1- and Th2-like responses can help CD8 memory cell development. To examine the effectiveness of Th1 versus Th2 help in the development of memory CD8 T-cell responses, we measured the OVA_{pep}-specific CD8 T-cell response in mice that had been immunized >60 days previously. After ex vivo restimulation with OVA_{pep}, very few IFN-γ-producing CD8 T cells were identifiable in these mice (Fig. 3A). Nevertheless, these mice clearly possessed memory CD8 T cells that formed the basis for an effective secondary immune response, since during challenge with rLmOVA there was a dramatic expansion of CD8 T cells capable of making IFN-γ, TNF-α (Fig. 3B and C), and IL-2 (data not shown) in response to ex vivo restimulation with OVA_{pep} in these mice compared to in mice that had seen only control DCs previously. The data from ELISA analyses of IFN-γ secretion supports these conclusions (data not shown). These findings reinforce the view that DCs are able to induce the development of a stable memory population of CD8 T cells that are capable of robust proliferation and cytokine responses upon secondary immune responses (9). In these experiments there was a reproducible trend for the number of responsive memory CD8 T cells to be greater in the context of Th1 than Th2 help.

To further examine the development of CD8 T-cell memory under conditions of Th1 versus Th2 help, we used an in vivo cytotoxicity assay to examine the lytic activity of DC-primed CD8 T cells (1). Specifically, we compared OVA_{pep}-loaded target cell cytolysis in DC-immunized mice after challenge with rLmOVA. Although some target cell lysis was observed in all groups at day 4 after rLmOVA infection, a dramatic increase in cytotoxicity was observed in DC-immunized, rLmOVA-infected mice at day 5 after infection (Fig. 4). In control mice immunized with DCs alone and challenged with rLmOVA, 5.6% ± 0.95% of target cells were lysed. In contrast, in mice immunized with DCs pulsed with OVA_{pep} plus *P. acnes* or SEA, larger numbers of target cells were lysed (66.6% ± 26.9% and 51.4% ± 8%, respectively). These results suggest that both Th2 and Th1 cells can help the development of memory cytotoxic CD8 T cells in DC-immunized mice.

DISCUSSION

The data presented in these experiments demonstrate that both Th1 and Th2 cells can help primary CD8 T-cell responses and the establishment of long-lived CD8 memory. Importantly, the data suggest that the underlying basis of CD4 help for CD8 T cells is unrelated to Th effector phenotype and that CD8 cells helped by Th1 or Th2 cells are similar in terms of their effector functions.

Injection of DCs pulsed with OVA_{pep} in combination with SEA or *P. acnes* induced priming of an OVA-specific CD8 T-cell response that was dependent on the presence of CD4 T cells (Fig. 1). These data support previous findings that the

induction of primary CD8 T-cell responses by Ag-pulsed DCs is highly dependent upon CD4 help (30). The detection of IFN-γ-producing OVA_{pep}-specific CD8 T cells in OVA_{pep}+*P. acnes*-DC primed CD4 T-cell-depleted mice by ICS, but not by ELISA, indicates a role for CD4 cells in the longer-term (72-h) survival and proliferation of CD8 cells in vitro after restimulation. It also supports the view that Toll-like receptor-activated DCs (OVA_{pep}+*P. acnes* pulsed) to a significantly greater extent than less mature DCs (OVA_{pep}+SEA pulsed) are able to activate CD8 T cells (5), although our data emphasize that Th cells are required for full development of the CD8 T-cell response. Recent findings indicate that helped CD8 T cells are far less likely to undergo TNF-related apoptosis-inducing ligand-mediated activation-induced cell death in prolonged restimulation cultures than are helpless CD8 T cells and suggest that this underlies the importance of help for memory development (11).

Studies that compared CD8 T-cell OVA-specific responses in WT, IL-4-deficient, and IL-12p35-deficient mice demonstrated that, under these priming conditions, CD4 help for CD8 T cells was unrelated to Th effector phenotype. As seen in Fig. 2, the help provided by CD4 cells for maximal OVA_{pep}-specific CD8 T-cell responses in mice immunized with OVA_{pep}+SEA-pulsed DCs was independent of a requirement for Th2 polarization. Although a component of the help provided by CD4 cells after immunization with OVA_{pep}+*P. acnes*-pulsed DCs appeared to be dependent upon the attributes of Th1 cells, there was an underlying mechanism of help that operated independently of Th1 response polarization. This suggests the possibility that the presence of CD4 cells is required to shape the developing CD8 response but that this CD4 help is independent of Th polarization.

Potential mediators of Th polarization-independent CD4 help include IL-2 and CD40-CD40L interactions (3). IL-2, an important growth factor for T cells, is made by activated unpolarized Th cells (31) and therefore may mediate CD4 T-cell help in the absence of Th polarization. Indeed, IL-2 has recently been reported to be of central importance in the generation of robust secondary CD8 T-cell responses (33). A role for CD40-CD40L interactions during priming of CD8 T-cell responses has been reported, with the possibility that CD40L acts to license CD40-expressing Ag-presenting cells to become efficient activators of CD8 T cells and/or to directly costimulate CD40-positive CD8 T cells (reviewed in reference 3). Interestingly, prolonged heightened expression of CD40L after activation is a characteristic of Th1 but not Th2 cells (14) and thus might also contribute to the stronger OVA_{pep}-specific CD8 T-cell responses observed here in conditions of Th1 versus Th2 help.

In conclusion, these experiments demonstrate that both Th1 and Th2 cells can help primary CD8 T-cell responses and the establishment of long-lived CD8 memory. Importantly, the data suggest that the underlying basis of CD4 help for CD8 T cells is unrelated to Th effector phenotype and that CD8 cells helped by Th1 or Th2 cells are similar in terms of their effector functions. Interestingly, we saw no evidence for the preferential generation of IFN-γ^+ IL-4^- type 1 (TC1) CD8 T cells under conditions of Th1 help versus the development of IFN-γ^- IL-4^+ type 2 (TC2) cells when help was provided by Th2 cells (23). In this regard, the outcome of Th1 versus Th2 help for CD8 T cells is distinct from that for B cells, where the effector properties of B cells helped by Th1 cells differ extensively from those helped by Th2 cells (8). In relation to vaccine design, the data presented here support the possibility of using Th2-inducing adjuvants, such as alum, to promote efficient help for generating long-lived memory CD8 T cells capable of robust proliferative, cytotoxic, and cytokine responses upon secondary exposure.

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