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Dendritic Cells and CD28 Costimulation Are Required To Sustain Virus-Specific CD8\(^+\) T Cell Responses during the Effector Phase In Vivo

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Although much is known about the initiation of immune responses, much less is known about what controls the effector phase. CD8\(^+\) T cell responses are believed to be programmed in lymph nodes during priming without any further contribution by dendritic cells (DCs) and Ag. In this study, we report the requirement for DCs, Ag, and CD28 costimulation during the effector phase of the CD8\(^+\) T cell response. Depleting DCs or blocking CD28 after day 6 of primary influenza A virus infection decreases the virus-specific CD8\(^+\) T cell response by inducing apoptosis, and this results in decreased viral clearance. Furthermore, effector CD8\(^+\) T cells adoptively transferred during the effector phase fail to expand without DC, CD28 costimulation, and cognate Ag. The absence of costimulation also leads to reduced survival of virus-specific effector cells as they undergo apoptosis mediated by the proapoptotic molecule Bim. Finally, IL-2 treatment restored the effector response in the absence of CD28 costimulation. Thus, in contrast to naive CD8\(^+\) T cells, which undergo an initial Ag-independent proliferation, effector CD8\(^+\) T cells expanding in the lungs during the effector phase require Ag, CD28 costimulation, and DCs for survival and expansion. These requirements would greatly impair effector responses against viruses and tumors that are known to inhibit DC maturation and in chronic infections and aging where CD28\(^-\) CD8\(^+\) T cells accumulate. The Journal of Immunology, 2011, 186: 000–000.

CD28 was the original costimulatory molecule characterized to amplify T cell proliferation in response to CD3 or antigenic stimulation (8). The CD28 stimulus is responsible for increased proliferation, Ag sensitivity, and survival, as well as decreased activation threshold and doubling time, in the presence of sufficient or suboptimal TCR/CD3 engagement (9–11). CD28 is required in vivo for optimal primary CD8\(^+\) T cell responses to occur in most models of infection (12–16). Historically, it has been believed that CD28 costimulation is required during the initial priming of the CD8\(^+\) T cell response in the draining LNs. Previous reports have showed that priming of CD8\(^+\) T cells may occur within extralymphoid structures known as induced BALT (iBALT), which is an aggregation of activated DCs and effector T cells induced in the lungs during influenza infections, and the integrity of these structures are maintained by the presence of chemokines such as CXCL13 and CCL21 (17). Given the large influx of DCs during infections in inflamed effector sites such as the lung and the liver, where they form effector lymphoid tissue (18) and iBALT (17), the question arises as to whether these DCs provide CD28 costimulation at the effector site and what purpose such costimulation may serve late in the response after initiation and programming has occurred.

Our study investigates whether effector CD8\(^+\) T cells retain the programmed response acquired during the original encounter of naive cells with Ag and examines the requirement for Ag, DC, and CD28 costimulation by CD8\(^+\) T cells during the effector phase of the immune response to influenza virus. We show that depleting DCs as late as day 6 post influenza virus infection significantly decreases the Ag-specific CD8\(^+\) T cell response. Because DCs influxing into the lung express CD80 and CD86, we tested whether DCs were providing CD28 costimulation. Indeed, depriving effector CD8\(^+\) T cells of CD28 costimulation during the effector phase by blocking CD28 or transferring effector CD8\(^+\) T cells into infected CD80\(^-\)/CD86\(^-\)/ mice results in markedly decreased virus-specific CD8\(^+\) T cell responses. Lack of CD28

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; DT, diphtheria toxin; DTR, diphtheria toxin receptor; iBALT, inducible BALT; KLRG1, killer cell lectin-like receptor G1; LCMV, lymphocytic choriomeningitis virus; LN, lymph node; MFI, mean fluorescence intensity; MLN, mediastinal lymph node; MPEC, memory precursor effector cell; NP, nucleoprotein; PR8, A/Puerto Rico8/34; RNA U, RNA unit; SLEC, short-lived effector cell; WSN, influenza A/WSN/33 virus; WT, wild-type.

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signaling causes upregulation of proapoptotic Bim and apoptosis of CD69+ virus-specific CD8+ T cells. This apoptosis is independent of CD4 T cell help or Fas signaling. Furthermore, we show that specific Ag is required for effector CD8+ T cell expansion during the effector phase. Thus, we have uncovered a novel role for Ag, DCs, and the CD28 signaling during the effector stages beyond the initial programming of the CD8+ T cell response. This dependency has important implications for effector CD8+ T cell responses against viruses (19–22) and tumors (23, 24) that suppress DC maturation and CD28 costimulation. These studies demonstrate that DCs not only initiate CD8+ T cell responses in the LN but also are required together with Ag and costimulation to maintain the response at the effector site late in the response. Thus, the effector phase of the CD8+ T cell response is not subject to Ag-independent programming, which may only apply to early naive T cell activation and expansion.

**Materials and Methods**

**Mice and infections**

Wild-type (WT) C57BL/6J, B6.MRL-Fas+/−/J, B6.FVB-Tg(Igα-1-DTR/EGFP)57lan/J mice, B6.PL-Thy1+Cy5 mice, B6.129S4-Cd80tm1Shr/Cd86tm1Shr/J (B7+2) (The Jackson Laboratory), and B6.129-H-2Ab1tm1GruN12/N12 Farms (Taconic Farms) were kept in an American Association for the Accreditation of Laboratory Animal Care-certified barrier facility at the Drexel University College of Medicine Queen Lane Campus animal facility. Animal work was carried out according to approved Institutional Animal Care and Use Committee protocols. Mice were infected at 8–10 wk of age. Mice were completely anesthetized with 2-2-2-tri-bromoethanol (250 mg/kg i.p.; Acris) before intranasal inoculations. Mice were infected by intranasal administration of X31 influenza virus (a recombinant viral strain of A/Aichi/2/68 and A/Puerto Rico/8/34 [PR8 strain], A/HKX31, H3N2) in a total volume of 20 μl. In some experiments, PR8 viral strain (H1N1; generous gift of Dr. W. Gerhard, Wistar Institute, Philadelphia, PA) and influenza A/WSN/33 virus (WSN)-OVA strain, an OT-I peptide (OVA257–264, SIINFEKL), expressing WSN strain (H1N1; a kind gift from Dr. David Topham, University of Rochester), were used. The primary response was examined by harvesting cells from lung, mediastinal LNs (MLN), spleen, and blood on various days postinfection. Infected mice were treated i.p. with 100 μg anti-CD28 mAb (clone 37.51, functional grade; eBiosciences) or anti-CD80 (16-10A1) and anti-CD86 (GL-1) mAbs (both a generous gift from Dr. Stephen Schoenberger, La Jolla Institute of Allergy and Immunology) on days 6 and 8 postinfection. For some experiments, CD80/CD86- and age-matched WT mice were i.p. injected with 2000 IU recombinant human IL-2 (Roche).

**Isolation of pulmonary lymphocytes**

Pulmonary lymphocytes were isolated from individual mice by removing each lobe individually and mincing into smaller pieces. The tissue was then digested for 2 h at 37°C with 3.0 mg/ml collagenase D and 0.15 mg/ml DNase I (Roche) in RPMI 1640 (Mediatech) containing 5% heat-inactivated FBS (Life Technologies), 2 mM l-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (Mediatech). The digested tissue was then run through a 40-μM cell strainer (Falcon) and washed in the same media as above. Total mononuclear cells were separated over a density gradient of Lymphocyte-M (Cedarlane Laboratories) centrifuged at 1300 × g for 20 min at room temperature. Media gradient interface and washed before cells were counted in ethidium bromide/acridine orange under UV light.

**Flow cytometry**

Influenza virus nuclear protein (NP)366–374-specific CD8+ T cells were detected using MHC class I tetramers. Tetramers were prepared by linking four biotinylated monomeric H-2Kb class I MHC molecules reoxidized in the presence of equimolar amounts of β2-microglobulin and excess peptide representing the immunodominant NP epitope (366–374, ASNENMETM, NP366–374) and OVA epitope (257–264, SIINFEKL, OVA257–264) to allophycocyanin- or PE-labeled streptavidin (Molecular Probes). Cells were costained with anti-mouse CD8α conjugated to PerCP (Pharminingen), anti-mouse CD69 conjugated to PE or FITC (eBioscience), and anti-mouse CD25 conjugated to allophycocyanin (BD Pharmingen). PE-conjugated anti-Thy1.1 and PE-Cy7-conjugated anti-Thy1.2 (eBioscience) Abs were used in adoptive transfer experiments to distinguish donor and host CD8+ T cell responses. For some stains, mAbs anti-CD44–FITC (eBioscience), anti-CD202–PE (eBioscience), anti-killer cell lectin-like receptor G1 (KLRG1)–FITC (Cell Lab: Beckman Coulter), and anti-CD127–PE (eBioscience) were used. For Bim staining, purified rat anti-mouse/human Bim (Millipore) was used with an anti-rat IgG–FITC secondary Ab (eBioscience). For cell death assessment, Annexin V Cy5.5 (eBioscience) was used. All surface stains were done on ice to prevent internalization. Cells were fixed in 1% paraformaldehyde (Fisher Scientific) before flow cytometric analysis. For Annexin V stains, 2.5 mM calcium chloride was added to all buffers used during staining. Data was collected on a FACScalibur using Cell Quest software or an FACSaria using FACSDiva software (BD Biosciences). Analysis was performed using FlowJo software (Tree Star).

**Adoptive transfer experiments**

For adoptive transfer experiments, 106 OT-I splenic lymphocytes were injected i.v. into mice and infected with WSN-OVA influenza virus the same day. On day 8 postinfection, lung lymphocytes were isolated as above, and 8 × 106 effector OT-I CD8+ T cells were transferred i.v. into uninfected animals or animals that had been infected 5 d earlier with either PR8 or WSN-OVA virus. In some experiments, effector OT-I CD8+ T cells were transferred into B7−/− mice. Naive OT-I cells (8 × 106 cells) isolated from spleens of uninfected animals were also i.v. transferred and served as controls.

**CFSE labeling of effector CD8+ T cells**

Day 8 postinfection with WSN-OVA, the lungs were isolated and processed. The whole lung cells were then labeled with 5 μM CFSE at a concentration of 2 × 106 cells/ml in CFSE buffer consisting of PBS and BSA (0.01%). The CFSE-labeled cells were incubated for 10 min at 37°C and were then observed under a microscope. Postincubation, the cell suspension was under laid with FBS and centrifuged at room temperature at 1500 rpm. This allowed the cell clumping of the excessive dye. The CFSE-labeled cells were then washed with media and i.v. injected into recipient mice.

**Determination of viral load**

On day 10 postinfection, the lungs of the mice were harvested, weighed, and frozen at −80°C in TRIzol (Invitrogen). RNA was isolated by the Qiagen RNeasy kit (Qiagen). The isolated RNA was then used for cDNA synthesis, and virus was measured by real-time PCR as previously described (25). Viral loads of the experimental samples were calculated using a standard curve of a stock PR8 virus with known concentration of virus 50% tissue culture-infective dose per milliliter. Virus RNA quantities in lungs were expressed as RNA units (RNA U)/100 mg. One RNA U was the amount RNA that resulted in equal amplification to 1% 50% tissue culture-infective dose stock PR8 virus in the real-time PCR assay.

**Statistical analysis**

Mann–Whitney U test, ANOVA, ANOVA, Wilcoxon signed-rank test, and Shapiro-Wilk W test for normality were used for statistical analysis with the JMP statistical analysis program (SAS Institute, Cary, NC). All p values <0.05 were considered significant.

**Results**

**Depletion of DCs during the effector phase inhibits the CD8+ T cell response to influenza virus**

Based on the observation that large numbers of CD80- and CD86-expressing DCs infiltrate the lung at late time points during the CD8+ T cell response to influenza virus (data not shown), we sought to determine the role of these DCs in CD8+ T cell responses. Previous work has shown that CD11c+ DCs in the lung of influenza-infected animals are both organized into lymphoid structures and dispersed throughout the lung parenchyma (17). We have confirmed the presence of CD11c+ DCs in the lung tissue during later stages of influenza virus infection as well as their expression of high levels of CD86 (Fig. 1A). To understand the role of these DCs in the effector phase of the CD8+ T cell response to influenza virus, we depleted DCs in CD11c-diphtheria toxin receptor (DTR) transgenic mice during the later stages of the response and measured the CD8+ T cell response against the immunodominant influenza NP366–374 epitope. When CD11c-DTR transgenic mice were treated with DT
on days 6 and 8 postinfection, DCs were efficiently depleted from lungs of infected animals (>85% depletion of CD11c+ cells, data not shown). This depletion of CD11c+ cells led to a significant decrease in the frequency and absolute number of NP366-374-specific CD8+ T cells in the lungs at the peak of the response on day 10 (Fig. 1B, 1C). Absolute numbers of NP366-374-specific CD8+ T cells were decreased to 1.05 ± 0.3 × 10^5 (mean ± SEM) in the lungs of DT-treated CD11c-DTR mice compared with untreated CD11c-DTR mice (6.16 ± 1.3 × 10^5), untreated WT mice (5.35 ± 1.79 × 10^5), and DT-treated WT mice (7.08 ± 0.99 × 10^5) (Fig. 1C). We should note that CD11c was never detected on CD8+ T cells in the lungs of infected WT animals (data not shown); therefore, our DT depletions only targeted DCs. To further exclude that low levels of CD11c present on CD8+ T cells in the CD11c-DTR mice result in their deletion with DT and to directly demonstrate that effector CD8+ T cells require interaction with DCs, we performed adoptive transfers of day 8 lung effector OT-I CD8+ T cells isolated from WSN-OVA influenza virus [expresses the OVA257-264 peptide]-infected mice. These day 8 effector cells were transferred into day 5 WSN-OVA–infected CD11c-DTR mice treated with DT on days 4 and 6. The expansion of donor effector OT-I cells was greatly reduced when DCs were depleted (0.55 ± 0.3 × 10^6 cells) compared with untreated CD11c-DTR mice (2.4 ± 0.08 × 10^6 cells), DT-treated WT mice (4.9 ± 0.7 × 10^6 cells), and untreated WT mice (3.9 ± 0.8 × 10^6 cells). The above shows that signals from DCs are required during the effector phase of the CD8+ T cell response for the expansion of effector CD8+ T cells. To assess whether the depletion of DCs had an effect on the clearance of the virus, we quantified the viral RNA load from the day 10 lungs of the PR8-infected and DT-treated and untreated CD11c-DTR and WT animals by real-time PCR. We observed that the lung viral load in the absence of the DCs in the CD11c-DTR mice (3206 ± 1501 RNA U/100 mg) was 3-fold higher than the untreated CD11c-DTR (1062 ± 870 RNA U/100 mg) and WT animals (1166 ± 958 RNA U/100 mg). Compared to the DT-treated WT mice (439 ± 344 RNA U/100 mg), the DT-treated CD11c-DTR had 7-fold higher viral RNA loads. Therefore, depletion of DCs at the effector phase impairs viral clearance.

CD8+ T cell responses at the effector site during the later phase of expansion require DCs

We next sought to determine how DCs regulate the CD8+ T cell response late in infection. Because we knew lung DCs express CD80 and CD86 (Fig. 1A), we hypothesized that DCs may be providing CD28 costimulation to CD8+ T cells. To test this, we blocked CD28 costimulation using a blocking anti-CD28 mAb (26) starting on day 6 postinfection (Fig. 2A), during the late expansion phase, when CD8+ T cell priming and programming has already occurred in the LN (1–3). When animals were treated with anti-CD28 Ab at days 6 and 8, both frequencies and absolute numbers of NP366-374-specific CD8+ T cells responding in the lung were decreased on day 10 postinfection (Fig. 2B, 2C). Day 10 postinfection, absolute numbers of lung NP366-374-specific CD8+ T cells were reduced from 7.48 ± 1.73 × 10^5 in untreated animals to 1.96 ± 0.49 × 10^5 cells when CD28 was blocked (Fig. 2C). NP366-374-specific CD8+ T cells were also reduced in the draining LN and spleen with anti-CD28 Ab treatment (Fig. 2D, 2E). Isotype control Ab treatment had no effect on the virus-specific CD8+ T cell response (Fig. 2C). These data indicate that CD28 is playing a role beyond the initial priming of the immune response in the draining LN and is required by CD8+ T cell responses at the effector site during the later phase of expansion.

Ligands of CD28 may also bind CTLA-4 and provide negative signals to Ag-specific CD8+ T cells. To exclude whether blocking CD28 during the later stages of the CD8+ T cell response was resulting in increased CTLA-4 signaling due to increased availability of CD80 and CD86, we blocked CD80 and CD86 using mAbs. Similar to anti-CD28 treatment, treatment after day 6 with blocking Abs to CD80 and CD86, which disrupt both CD28 and CTLA-4 signals, reduced NP366-374-specific CD8+ T cells (Fig. 2B, 2C). Furthermore, animals treated with anti-CD28, anti-CD80, and anti-CD86 Abs had similarly decreased NP366-374-specific CD8+ T cell responses as those treated with only anti-CD28 Ab (Fig. 2B, 2C). Thus, interaction with APCs expressing costimula-

FIGURE 1. DCs are required for lung Ag-specific CD8+ T cells during the effector phase of the response to influenza virus. A, CD86 expression is increased on lung DCs of influenza virus-infected animals. Representative histogram showing CD86 expression of pulmonary CD11c+CD11b+ DCs from infected and uninfected animals on day 7 postinfection. Horizontal bar and percentage depicts the frequency of CD86+ DCs from the infected animal. B, Depletion of DCs at day 6 postinfection reduces day 10 pulmonary NP366-374-specific CD8+ T cells. Representative FACS plots from pulmonary lymphocytes from WT and CD11c-DTR mice infected with HKX31 strain influenza virus 10 d earlier. Mice were either untreated or treated with 20 ng/g of body weight DT i.p. on days 6 and 8 postinfection. Lung lymphocytes were stained using NP366-374 tetramers. C, Absolute numbers of NP366-374-specific CD8+ T cells isolated from the lungs of day 10 virus-infected WT and CD11c-DTR mice that were either untreated or DT treated are shown. Symbols represent individual animals, with the horizontal line marking the mean for each group (n = 7–8 mice per group). Data are from three independent experiments. D, Effector OT-I T cells from infected day 8 lungs were adoptively transferred into WT or CD11c-DTR mice on day 5 postinfection with WSN-OVA. DT treatment was done on days 4 and 6. The OVA257-264-specific CD8+ T cell response was tested on day 10.
CD28 signals are required during the effector phase of the antiviral CD8+ T cell response. A, The scheme for blocking CD80, CD86, and CD28 late during the CD8+ T cell response after initial priming event shown. B, Blocking CD28 costimulation 6 d postinfection at the effector phase of the CD8+ T cell response reduces NP(366–374)-specific CD8+ T cells. Representative FACS plots showing NP(366–374)-specific CD8+ T cells responding in the lung to influenza A virus-infected animals after treatment with combinations of anti-CD28, anti-CD80, and anti-CD86 mAb or no treatment day 10 postinfection. Percentage shown represents fraction of CD8+ T cells binding NP(366–374) tetramers. C, Pooled percentages and absolute numbers of day 10 NP(366–374)-specific CD8+ T cells from untreated animals or days 6 and 8 Ab-treated mice shown. Lung lymphocytes were stained using NP(366–374) tetramers. Bars show mean and SEM (untreated and anti-CD28 Ab-treated, \( n = 14–19 \); isotype control, \( n = 9 \); all others, \( n = 3 \)). NP(366–374)-specific CD8+ T cells in spleen (D) and lung draining MLNs (E) on day 10 postinfection are shown. Bars depict the mean and SEM (\( n = 8–9 \)).

FIGURE 2. CD28 signals are required during the effector phase of the antiviral CD8+ T cell response. A, The scheme for blocking CD80, CD86, and CD28 late during the CD8+ T cell response after initial priming event shown. B, Blocking CD28 costimulation 6 d postinfection at the effector phase of the CD8+ T cell response reduces NP(366–374)-specific CD8+ T cells. Representative FACS plots showing NP(366–374)-specific CD8+ T cells responding in the lung to influenza A virus-infected animals after treatment with combinations of anti-CD28, anti-CD80, and anti-CD86 mAb or no treatment day 10 postinfection. Percentage shown represents fraction of CD8+ T cells binding NP(366–374) tetramers. C, Pooled percentages and absolute numbers of day 10 NP(366–374)-specific CD8+ T cells from untreated animals or days 6 and 8 Ab-treated mice shown. Lung lymphocytes were stained using NP(366–374) tetramers. Bars show mean and SEM (untreated and anti-CD28 Ab-treated, \( n = 14–19 \); isotype control, \( n = 9 \); all others, \( n = 3 \)). NP(366–374)-specific CD8+ T cells in spleen (D) and lung draining MLNs (E) on day 10 postinfection are shown. Bars depict the mean and SEM (\( n = 8–9 \)).

FIGURE 3. Day 8 effector OT-I CD8+ T cells have early effector phenotype. Representative FACS plots showing the expression of CD44, CD62L, KLRG1, and CD127 on naive OT-I cells and day 8 lung effector OT-I cells. Percentages indicate positive cells in each quadrant. The MFI of CD127 in the KLRG1hiCD127lo quadrant is shown. The data are representative from two experiments with \( n = 14 \) animals.
was much greater ($12.7 \pm 4.2 \times 10^5$) than in animals transferred with naive OT-I cells ($2.6 \pm 1.1 \times 10^4$) (Fig. 4B).

Because the proliferation of effector CD8$^+$ T cells described above could be driven in a non-Ag-specific manner by proinflammatory cytokines, we sought to determine whether Ag is required for this late-phase expansion of effector CD8$^+$ T cells. We generated day 8 lung OT-I effector CD8$^+$ T cells as above and transferred these into mice infected for 5 d with either WSN-OVA virus or PR8 virus. The numbers of donor OT-I cells were determined on day 10 postinfection. The absolute numbers of lung day 10 donor OT-I cells were greatly reduced in the absence of cognate Ag ($0.15 \pm 0.06 \times 10^5$ cells) compared with when Ag was present ($WSN$-OVA infections) ($9.5 \pm 2.7 \times 10^5$ cells) (Fig. 4C). Naive donor cells failed again to significantly expand (Fig. 4C).

Although the numbers of cells were much smaller, these reductions were also found in MLN and spleens (data not shown). Thus, the expansion and survival of effector CD8$^+$ T cells late in the response requires the presence of Ag. To confirm that CD28 co-stimulation was directly required by effector CD8$^+$ T cells, we generated day 8 lung effector OT-I cells as above and transferred equal numbers of these into WT and CD80$^{-/-}$CD86$^{-/-}$ mice infected for 5 d with WSN-OVA virus (Fig. 4D). As above on day 10 of infection, we examined the donor-derived $OVA_{257-264}$-specific CD8$^+$ T cell response. Transfer of effector OT-I cells into CD80$^{-/-}$CD86$^{-/-}$ mice resulted in significantly lower donor lung $OVA_{257-264}$-specific CD8$^+$ T cells in day 8 lung effector or naive OT-I recipients are shown. Recipients are either PR8 or WSN-OVA infected. Absolute numbers of day 10 postinfection donor lung $OVA_{257-264}$-specific CD8$^+$ T cells in day 8 lung effector or naive OT-I recipients are shown. Recipients are either PR8 or WSN-OVA infected. Representative FACS plots (D) and absolute number (E) of day 10 postinfection donor lung $OVA_{257-264}$-specific CD8$^+$ T cells in day 8 lung effector or naive OT-I recipient CD80$^{-/-}$CD86$^{-/-}$ animals. Statistically significant differences between groups are indicated by horizontal lines and asterisks. F. Absolute number of donor effector efector CD8$^+$ T cells in IL-2 treated and untreated WT and B7$^{-/-}$ animals adoptively transferred on day 5 postinfection with day 8 effector OT-I cells. Bars indicate the mean and SEM for $n = 3$ to 4 per group. G. The absolute number of CFSE-labeled donor effector OT-I cells in the lungs and MLNs of mice on days 7, 8, and 10 after WSN-OVA infection. Mean and SEM shown ($n = 6$ per group). H. Representative histograms showing CFSE dilution in donor effector OT-I cells in the lungs on days 7 and 8 and MLN on day 8.


IL-2 due to autocrine secretion. To test this, we treated our B7-/- animals with rIL-2. We observed that we were able to restore the expansion of the donor effector CD8+ T cells in the lungs in the IL-2–treated CD80+/CD86-/- animals (5.5 × 10^4 ± 1.5) compared with the untreated CD80+/CD86-/- (0.99 × 10^4 ± 0.35) (Fig. 4F).

To determine the site of expansion of the effector CD8+ T cells, we CFSE labeled the day 8 effector CD8+ T cells and transferred them into day 5 WSN-OVA–infected recipient mice and harvested the lungs and LNs of the mice on days 7, 8, and 10 postinfection. The absolute number of donor OVA(257–264)-specific CD8+ T cells was higher at all of these time points in the lungs compared with LN as shown in Fig. 4G. Donor effector OVA(257–264)-specific CD8+ T cells in the lungs increased in number from 6.1 ± 2.2 × 10^4 on day 7 to 19.5 ± 11.7 on day 8 and to 118.2 ± 39.1 × 10^3 on day 10. Within the LN, the virus-specific CD8+ T cells were undetectable on day 7, very low in number on day 8 (0.2 ± 0.08 × 10^3), and increased on day 10 (21.5 ± 8.9 × 10^2). In lungs on day 7, donor OVA(257–264)-specific CD8+ T cells were undivided as determined by CFSE dilution (Fig. 4H). On day 8, both lung and LN cells showed a similar level of CFSE dilution (Fig. 4H), whereas on day 10, both sites at both sites had fully diluted CFSE (data not shown). Given that on day 10, there was a 100-fold higher number of dividing donor OVA(257–264)-specific CD8+ T cells in the lungs compared with LN, this would suggest that the primary site of cell division of the donor effector OVA(257–264)-specific CD8+ T cells is the lung.

**CD82 signaling promotes the survival of effector CD8+ T cells**

The decrease in lung virus-specific CD8+ T cell populations in the absence of DCs and CD28 costimulation may be the result of several mechanisms such as reduced expansion, survival, or migration to the lung. When CD28 costimulation was blocked or absent, the reduced numbers of NP(366–374)-specific CD8+ T cells were not limited to the lung tissue and but also observed in LN and spleens (Fig. 2D, 2E, Supplemental Fig. 1). This universal reduction argues against altered migration of virus-specific CD8+ T cells to the lung when CD28 signaling is blocked late in the response. Examination of cell cycle progression marker Ki-67 showed little difference in the number of actively dividing NP(366–374)-specific CD8+ T cells with regard to late-phase anti-CD28 treatment (Fig. 5A). Blocking CD28 after day 6, however, did affect the number of virus-specific CD8+ T cells undergoing apoptosis. The percentage of NP(366–374)-specific CD8+ T cells staining positive for activated caspase 3 were increased in the lungs of animals treated with anti-CD28 (Fig. 5B, 5C). This increase was apparent by day 9 and extended through the peak of the CD8+ T cell response on day 10 (7.36 ± 1.4% versus 17.33 ± 4.0% for untreated and anti-CD28 Ab-treated animals, respectively; p < 0.001) (Fig. 5C). When effector CD8+ T cells were transferred into CD11c-DTR mice, donor effector CD8+ T cells underwent apoptosis at a high frequency, when DCs were depleted (Fig. 5D) as indicated by the increased frequency of Annexin V expressing activated effector CD8+ T cells in the lungs on day 10. Together with the above findings, this suggests that

**FIGURE 5.** Lack of CD28 signaling increases apoptosis of NP(366–374)-specific CD8+ T cells. A, Blocking CD28 during effector phase does not affect proliferation of NP(366–374)-specific CD8+ T cells. The frequency of intracellular Ki-67–positive NP(366–374)-specific CD8+ T cells at different time points postinfection after anti-CD28 Ab treatment (dashed line, open symbol) or no treatment (solid square and symbol) is shown. Mean ± SEM shown (n = 3 animals per group per time point). B, Blocking CD28 after day 6 postinfection increases apoptotic NP(366–374)-specific CD8+ T cells. Representative FACS plot showing the percentage of day 10 NP(366–374)-specific CD8+ T cells staining positive for activated caspase 3 in the lungs of untreated or animals treated with anti-CD28 (Fig. 5A). Blocking CD28 after day 6, however, did affect the number of virus-specific CD8+ T cells undergoing apoptosis. The percentage of NP(366–374)-specific CD8+ T cells staining positive for activated caspase 3 were increased in the lungs of animals treated with anti-CD28 (Fig. 5B, 5C). This increase was apparent by day 9 and extended through the peak of the CD8+ T cell response on day 10 (7.36 ± 1.4% versus 17.33 ± 4.0% for untreated and anti-CD28 Ab-treated animals, respectively; p < 0.001) (Fig. 5C). When effector CD8+ T cells were transferred into CD11c-DTR mice, donor effector CD8+ T cells underwent apoptosis at a high frequency, when DCs were depleted (Fig. 5D) as indicated by the increased frequency of Annexin V expressing activated effector CD8+ T cells in the lungs on day 10. Together with the above findings, this suggests that
CD28 costimulation and DCs are both required for the survival of effector CD8⁺ T cells.

To further investigate possible mechanisms of effector CD8⁺ T cell apoptosis when CD28 costimulation is absent late in the response, we examined the expression of anti- and proapoptotic molecules in Ag-specific CD8⁺ T cells responding in the lung. Bcl-xL induced survival of activated T cells is attributed to CD28 costimulation (29). We therefore examined whether decreased Bcl-xL levels were responsible for the increase in apoptosis we saw in the absence of CD28 costimulation late in the response. Anti-CD28 Ab treatment did not affect the levels of Bcl-xL in NP(366–374)-specific CD8⁺ T cells on day 8 (MFI ± SEM: 510 ± 49 and 666 ± 36 in untreated and anti-CD28–treated animals, respectively; n = 3–6 per group) or day 10 (83.3 ± 5.44 in untreated mice and 85.3 ± 9.82 in anti-CD28 treated mice; n = 3–6 per group). Bcl-2 levels were slightly increased to 108 ± 3.18 (MFI ± SEM) in NP(366–374)-specific CD8⁺ T cells from anti-CD28–treated mice as compared with 87.8 ± 3.83 (p < 0.015) in untreated mice. Thus, decreases of prosurvival Bcl-2 family members were not responsible for the increased apoptosis seen when CD28 costimulation was blocked. When pro- and antiapoptotic molecules were examined in effector cells adoptively transferred into CD80⁻/⁻CD86⁻/⁻ mice, we found no difference in Bcl-2, Bcl-xL, and Noxa levels in donor effector CD8⁺ T cells (data not shown). Bim levels, however, were again increased in donor effector CD8⁺ T cells when transferred into CD80⁻/⁻CD86⁻/⁻ hosts (Fig. 5E).

Next, we sought to determine the role of CD4⁺ T cells in this model. Much work has shown the importance of CD4⁺ T cells in promoting the survival of CD8⁺ T cells (30, 31), and therefore, the proapoptotic effect of blocking CD28 may be mediated indirectly via CD4⁺ T cells. CD4⁺ T cells can interact directly or indirectly to help CD8⁺ T cell responses (32, 33). To determine whether blocking CD28 directly affects NP(366–374)-specific CD8⁺ T cells, or is acting indirectly by modulating signals produced by CD4⁺ T cells, we blocked CD28 in class II MHC-deficient animals that lack CD4⁺ T cells and WT animals. Blocking CD28 after day 6 decreased the frequency and absolute numbers of virus-specific CD8⁺ T cells in both WT and class II-deficient animals (Fig. 6). Thus, CD4⁺ T cells are not involved in the inhibition of the CD8⁺ T cell response when CD28 is blocked, and this suggests a direct effect on CD8⁺ T cells.

CD95/Fas and TRAIL are critical mediators of apoptosis of CD8⁺ T cells during the contraction of the CD8⁺ T cell response (34–37). Therefore, we tested whether the decrease in NP(366–374)-specific CD8⁺ T cells observed when CD28 is blocked was mediated by Fas or TRAIL-induced apoptosis by using Fas signaling-deficient (LPR) and TRAIL-deficient mice. Blocking CD28 after day 6 in LPR animals decreased NP(366–374)-specific CD8⁺ T cells similar to WT animals (Fig. 6). Reduced NP(366–374)-specific CD8⁺ T cells was also observed when CD28 signaling was blocked post day 6 in the TRAIL-deficient and WT mice (data not shown). Therefore, TRAIL and Fas do not mediate the increased apoptosis seen when CD28 is blocked at the effector phase of the CD8⁺ T cell response to influenza infection.

**Discussion**

DCs are believed to initiate the CD8⁺ T cell response in the draining LN by providing signals through the TCR and CD28. Beyond this initiation phase, however, the role of DCs and CD28 costimulation during later phases and at the effector site is largely unexplored. Previous studies have suggested that CD8⁺ T cells responses are programmed during priming and do not require Ag beyond initiation (1–3); however, these conclusions were based on short 3–6 d in vivo expansion or survival of CD8⁺ T cells (1–3). In these studies, in vitro-activated T cells were transferred into uninfected mice, and it was showed that the cells undergo divisions in the absence of Ag for up to 6 d after transfer into uninfected mice. Our studies show that in the context of a viral infection such as influenza, effector CD8⁺ T cells fail to fully expand when transferred into uninfected recipients or animals infected with an influenza virus that does not express cognate peptide (Fig. 4B, 4C). Based on the above, it appears that initial divisions of CD8⁺ T cells after encounter with Ag-presenting DCs in draining LNs is Ag independent, but that effector cells need to re-encounter Ag to continue expansion. This Ag re-encounter is most likely occurring in the lung, where DCs also provide costimulation and cytokines to maintain the primary CD8⁺ T cell response. The formation of effector lymphoid tissues at effector sites of infection has raised questions as to their function and the interactions occurring outside of the draining LNs during a primary immune response (18). During influenza infection, lymphoid structures such as iBALT are formed in the lung and contribute to immune responses (17). Whether the DCs are presenting both the Ag and costimulation to CD8⁺ T cells or other cells present Ag in the lung has yet to be determined. Our data suggest that the expansion of the virus-specific CD8⁺ T cells during the effector phase occurs in the lungs. This was based the finding that the numbers of virus-specific CD8⁺ T cells were always higher in the lungs compared with the LN after adoptive transfer of effector cells. CFSE labeling suggested that divisions are occurring in both sites, but the majority of cells dividing are present in the lung. These types of experiments cannot, however, exclude that cells receiving stimulation as they circulate through the MLN then migrate to the lungs, where they continue to divide. Reciprocally, they cannot exclude that some cells activated in the lungs do not recirculate back to the LN. Based on the location of dividing CD8⁺ T cells, which were >100-fold in the lung on day 7, our data suggest that effectors are expanding in the lungs.

Our studies show that CD11c⁺ cells are required for the effector phase of the primary CD8⁺ T cell response. Such CD11c⁺ cells could be DCs or alveolar macrophages. We believe that the lung DCs are presenting Ag beyond day 6 in our studies, as alveolar
macrophages are known to play an inhibitory role and dampen APC function of lung DCs (38). Alveolar macrophages do not express CD86 after day 2 post influenza virus infection, whereas lung DCs exhibit rapid and sustained CD86 expression during the course of infection (6) (Fig. 1A). Depletion of CD11c+ cells at the initiation of influenza virus infection and during priming of CD8+ T cells reduces the primary CD8+ T cell response, and these can be restored with intratracheal DC administration but not alveolar macrophages (6); this argues against an immunostimulatory role for alveolar macrophages. Our CD11c+ cell depletion and CD28 costimulation blockade studies 6 d postinfection strongly suggests that DCs expressing CD80/CD86 are required for the effector CD8+ T cell response. A recent study (39) showed that antigenic signals from DCs may be required for a second time by virus-specific CD8+ T cells in the lungs. These studies were done by treating mice with clodronate liposomes 48 h post influenza A virus infection to deplete lung DCs and macrophages and examined CD8+ T cells very early in the effector phase on day 6 of infection, when effector CD8+ T cells are still very low (39). To determine specifically the antigenic and CD28 costimulation requirements for effector CD8+ T cells, we generated effector CD8+ T cells and did an adoptive transfer of the virus-specific CD8+ T cells into infected mice that lacked either cognate Ag, DCs, or CD28 costimulation at the effector phase. Our experimental results suggest that DCs, CD28, and Ag are required at the later stages (post day 6) of the effector phase of the response when a dramatic accumulation of effector CD8+ T cells occurs. DC and CD28 during this late phase promote the survival of effector cells.

During a CD8+ T cell response, effector CD8+ T cells gradually acquired SLEC or MPEC phenotypes (27, 28). Such SLEC and MPEC during LCMV or Listeria infections express different levels of KLRG1 and CD127 (27, 28, 40–42). SLEC or MPEC may require different signals to expand or be reactivated. To understand what effector cell type was requiring DC, Ag, and CD28 costimulation during the effector phase, we determined the phenotype of day 8 effector OVA257–264-specific CD8+ T cells we performed adoptive transfers with. We found that these day 8 effector cells could not be classified as SLEC or MPEC and were CD44+CD62L- KLRG1+CD127lo, and this phenotype is similar to early day 4 effector LCMV-specific CD8+ T cells (27, 28). This would suggest that lung day 8 effector OVA257–264-specific CD8+ T cells are early effector cells and before SLEC or MPEC development (27, 28). Currently, we do not know how the development of SLEC and MPEC is affected when these early effector CD8+ T cells we transfer fail to interact with DCs or receive costimulation and Ag. Based on the findings that CpG oligodeoxynucleotides and IL-12 can promote SLEC formation (27, 28), it is tempting to speculate that SLEC formation is impaired when effector CD8+ T cells do not interact with DC, Ag, or CD28 costimulation; however, this and the impact on MPEC formation remain to be determined. These studies raise the question of whether CD28 costimulation signals at the effector phase can affect the differentiation, memory precursor, quality, and quantity of memory of virus-specific CD8+ T cells.

Although our studies examine the primary CD8+ T cell response, the expansion of memory Ag-specific CD4+ T cells upon rechallenge may also occur in the lung instead of LNs (43), and therefore, our findings may be applicable to effector CD4+ T cell responses also.

Our study shows that CD28 costimulation after day 6 post-infection promotes the survival of effector virus-specific CD8+ T cells. This CD28 costimulation most likely is provided by DCs, which may also provide additional survival signals late in the primary CD8+ T cell response such as CD27 signaling, which is required to prevent apoptosis (44, 45). The mechanisms of death, however, induced by the absence of CD27 or CD28 signaling is different. The absence of CD27 signaling leads to FAS and CD4+ T cell-dependent apoptosis of effector CD8+ T cells (44). Blocking CD28 signaling decreased CD8+ T cells independently of CD4+ T cell help, FAS, and TRAIL signaling. This also suggests that direct ligation of CD28 on effector CD8+ T cells is required for their survival and maintains reduced levels of the proapoptotic molecule BIM. Our preliminary data shows that CD28 signaling is also required for higher expression of CD122 (data not shown), and we believe that increased expression of this receptor subunit on CD8+ T cells may be important for both IL-2 and IL-15 signaling. Others have shown that DCs may also provide prosurvival signals to virus-specific CD8+ T cells via trans-presentation of IL-15 (46).

However, our studies indicate that IL-15 alone may not be sufficient for survival, as the virus-specific effector CD8+ T cells transferred into CD80−/−CD86−/−-deficient animals did not expand optimally. This suggests that the virus-specific CD8+ T cells during the effector phase need both IL-15 and costimulation signals as prosurvival factors. Our current study reveals an unappreciated role of CD28 costimulation during the CD8+ T cell effector phase and may explain the need for CD28 costimulation recently described by us and others for the generation of optimal secondary responses from memory CD4+ and CD8+ T cells (25, 47, 48). In addition to this, we show that the absence of CD28 costimulation and antigenic signals at the effector phase has an impact on viral clearance, as our studies show that there is decreased viral clearance in the absence of DCs at the effector stage most likely due to the reduced numbers of virus-specific CD8+ T cells. We show that IL-2 can restore the effector CD8+ T cell response in the absence of CD28 costimulation and supports the hypothesis that CD28 costimulation induced γc cytokine production or responsiveness may regulate the survival of effector virus-specific CD8+ T cells in the presence of an inflammatory environment where anti-inflammatory cytokines such as TGF-β suppress the effector CD8+ T cell response (49).

Our studies reveal a novel role for DCs, CD28 signaling, and Ag during the effector phase of influenza virus infection and at the effector site. This requirement by effector CD8+ T cell responses for DC, CD28, and cognate Ag suggests that they are not subject to the Ag-independent program naive cells are submitted to during priming (1–3). We show that DCs and CD28 signaling late in the response are required for the survival of effector virus-specific CD8+ T cells. Our results support the idea that CD11c+ DCs entering the lung sustain effector virus-specific CD8+ T cells by increasing their expansion in the lung (50) and survival capability. This study also highlights the fact that in the absence of CD28 costimulation at the effector phase, the Ag-specific CD8+ T cells undergo BIM-mediated apoptosis that is CD95/Fas independent, whereas in the absence of CD27 signaling at the effector phase, cell death is CD95/Fas-mediated as shown by previous studies (44). These data indicate that multiple costimulation signals regulate the cell death of effector virus-specific effector CD8+ T cells.

Overall, our studies highlight that the concept of programming of CD8+ T cells should not be applied to the entire CD8+ T cell response. The initial phases of activation and expansion of the CD8+ T cell responses may be programmed during priming in the draining LNs to expand in an Ag-independent manner (1–3). However, we show that programming of the activated virus-specific CD8+ T cells in the LN is not sufficient to maintain their expansion and survival at the periphery or the effector site, which is the lungs in the case of influenza A infections. The DC and CD28 requirement of effector CD8+ T cells has important roles in the development of virus-specific CD8+ T cells.
implications for antiviral and antitumor effector CD8+ T cell responses as one of the immune evasion mechanisms used by viruses (19–22) and tumors (23, 24) is the suppression of DC maturation and inhibition of expression of CD28 costimulatory ligands CD80 and CD86. This requirement for CD28 signaling by effector cells may also contribute to the immune dysfunction found in chronic infections such as HIV infection and aging, where CD28+ CD8+ T cell increases. Our studies demonstrate that DCs not only initiate CD8+ T cell responses in the LN but also are required together with Ag to expand the response at the effector site late in the response. Thus, the effector phase of the CD8+ T cell response is not subject to Ag-independent programming, which may only apply to early naive cell activation and proliferation.

Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure S1. Absolute numbers of donor CD8+ T cells are reduced in mediastinal lymph node (MLN) and spleen of B7−/− animals. Day 8 lung effector OT-I cells were transferred into day 5 WSN-OVA virus infected wild type and B7−/− animals and mice were harvested 5 days later on 10 post infection. Absolute number in A, MLN and B, spleen of donor OVA(257-264)-specific CD8+ T cells on day 10 post infection shown. Data are from 2 independent experiments and n=5-6 per group.