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RESEARCH ARTICLE

$CD4^+$ T-cell dependence of primary $CD8^+$ T-cell response against vaccinia virus depends upon route of infection and viral dose

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$CD4^+$ T-cell help (CD4 help) plays a pivotal role in $CD8^+$ T-cell responses against viral infections. However, the role in primary $CD8^+$ T-cell responses remains controversial. We evaluated the effects of infection route and viral dose on primary $CD8^+$ T-cell responses to vaccinia virus (VACV) in MHC class $II^{-/-}$ mice. CD4 help deficiency diminished the generation of VACV-specific $CD8^+$ T cells after intraperitoneal (i.p.) but not after intranasal (i.n.) infection. A large viral dose could not restore normal expansion of VACV-specific $CD8^+$ T cells in i.p. infected MHC $II^{-/-}$ mice. In contrast, dependence on CD4 help was observed in i.n. infected MHC $II^{-/-}$ mice when a small viral dose was used. These data suggested that primary $CD8^+$ T-cell responses are less dependent on CD4 help in i.n. infection compared to i.p. infection. Activated $CD8^+$ T cells produced more IFN-γ, TNF-α and granzyme B in i.n. infected mice than those in i.p. infected mice, regardless of CD4 help. IL-2 signaling *via* CD25 was not necessary to drive expansion of VACV-specific $CD8^+$ T cells in i.n. infection, but it was crucial in i.p. infection. VACV-specific $CD8^+$ T cells underwent increased apoptosis in the absence of CD4 help, but proliferated normally and had cytotoxic potential, regardless of infection route. Our results indicate that route of infection and viral dose are two determinants for CD4 help dependence, and intranasal infection induces more potent effector $CD8^+$ T cells than i.p. infection.
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INTRODUCTION

$CD8^+$ T cells are one of the key effectors in adaptive immunity and they play a critical role in protection from various pathogens. Viruses entering the body *via* various routes will face different microenvironments where different cells reside and interact. T-cell responses to a typical acute viral infection can be characterized into three major phases: effector T-cell expansion and differentiation, contraction, and memory T-cell formation. These phases are precisely driven and controlled by T-cell receptor engagement, costimulation and inflammatory cytokines as well as $CD4^+$ T-cell help (CD4 help).[1]

T-cell receptors of naive $CD8^+$ T cells recognize a specific epitope presented by MHC class I (MHC I) on antigen-presenting cells (APCs), constituting 'signal 1', which initiates a primary response and starts clonal expansion and differentiation.[2] Once activated, the expansion of $CD8^+$ T cells is preprogrammed and does not require further contact with antigen (Ag).[3,4] Ag-independent expansion is supported by IL-2 and further augmented by IL-7 or IL-15.[3] Costimulation provided by APCs, acting as 'signal 2', is essential to induce full activation of T cells and prevents them from becoming refractory to Ag stimulation.[5] The most important costimulatory pathways include CD28/CD80–CD86,[6] CD40L/CD40,[7] CD27/CD70,[8] 4-1BB/4-1BBL[9] and OX-40/OX40L.[10] Furthermore, inflammatory cytokines, such as IL-12 and type I IFNs, provide 'signal 3' at distinct stages of the response for optimal generation of effector and memory populations.[11]

In addition to these three signals, CD4 help plays a pivotal role in $CD8^+$ T-cell responses.[12] A number of studies have confirmed that CD4 help is required for development of $CD8^+$ T-cell memory and secondary expansion of $CD8^+$ T cells.[13–16] However, the role of CD4 help in the primary $CD8^+$ T-cell response remains controversial, since differing and even contradictory results are frequently observed. CD4

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help is necessary in priming $CD8^+$ T cells with non-infectious agents (such as minor histocompatibility Ags, tumor Ags, Ag-loaded splenocytes, grafts, alloantigens and soluble protein Ags), but is variably required for $CD8^+$ T-cell responses to infectious agents.[17] CD4 help is required for the primary $CD8^+$ T-cell response to herpes simplex virus,[18] but not to vesicular stomatitis virus infection.[19] It is required for sustaining cytotoxic T lymphocyte (CTL) responses during chronic infection with lymphocytic choriomeningitis virus variants, but not for resolving acute lymphocytic choriomeningitis virus infection.[20] These data indicate that the identity of the pathogen is an important variable. Furthermore, divergent results regarding the role for CD4 help have been reported with the same pathogen.[17] In vaccinia virus (VACV) infection, primary $CD8^+$ T-cell responses have been shown to be dependent on $CD4^+$ T cells in some reports,[21–23] while other studies report CD4 help independence.[14,24] Different experimental conditions were used in these studies, including the VACV strain, inoculum dose and route of infection as well as the mouse model.

VACV, a dsDNA virus, belongs to the family Poxviridae and the genus *Orthopoxvirus* and shares high homology with other orthopoxviruses, such as variola virus (the smallpox in humans), ectromelia virus (mousepox) and monkeypox.[25] The natural reservoir of VACV is not known, but it can replicate in mice. VACV vaccination was one of the most important medical practices in human history, resulting in the eradication of smallpox. Attenuated VACV has been used as a vaccine vector against infectious agents and cancers, and as a gene delivery system to study biological functions of foreign genes.[26] VACV-infected mouse models have been used extensively in the study of acute virus infection. Depending on experimental settings, VACV is administered by various routes, e.g., intranasal,[24] intraperitoneal,[14,21–23,27] intradermal,[28] intracranial[29] and intravenous.[30]

Despite numerous previous studies, there lacks a side-by-side comparison of the CD4 dependency of the $CD8^+$ T-cell response when either the viral dose or route of VACV infection is varied. Here we used MHC class II knockout (MHC $II^{-/-}$) mice to determine the CD4 help dependence of primary $CD8^+$ T-cell response to VACV Western Reserve strain infection by comparing the response in intranasal and intraperitoneal infection. Our data demonstrate that the requirement for CD4 help in primary $CD8^+$ T-cell response against VACV infection varies depending on the route of infection and viral dose, and intranasal infection induces more potent effector $CD8^+$ T cells than intraperitoneal infection.

MATERIALS AND METHODS

Mice and generation of mixed bone marrow chimeric mice

C57BL/6 (B6) and congenic B6-Ly5.2/Cr mice were purchased from the National Cancer Institute (Frederick, MD, USA). MHC class II knockout (MHC $II^{-/-}$) mice that lack $CD4^+$ lymphocytes[31] were obtained from Taconic Biosciences (Albany, NY, USA). $CD25^{-/-}$mice (B6.129S4-Il2ra^{tm1Dw}/J) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). For generating mixed bone marrow (BM) chimeric mice, B6-Ly5.2 recipient mice were lethally irradiated with 1000 rads (500 rads twice given 24 h apart), and subsequently injected intravenously (i.v.) with a total of 4.5×10^6 BM cells containing a mixture of B6-Ly5.2 ($CD45.1^+$) and $CD25^{-/-}$ ($CD45.2^+$) cells at a 1 : 2 ratio. Reconstitution of the BM was confirmed at 50 days post-BM transfer by staining blood cells for the congenic markers. All mice were bred or housed in the Dartmouth-Hitchcock Medical Center mouse facility. The Animal Care and Use Program of Dartmouth College approved all animal experiments.

Virus, viral infection and plaque assay

Vaccinia virus Western Reserve strain (VACV-WR) was obtained from Dr William R Green (Geisel School of Medicine at Dartmouth, Lebanon, NH, USA), and was propagated and titered in the 143B cell line. Mice were infected with 10^3 PFU of VACV in 30 μl of PBS for intranasal inoculation (i.n.) or in 300 μl of PBS for intraperitoneal inoculation (i.p.) unless otherwise noted. For i.n. infection, mice were anesthetized with isoflurane. Virus titers in the lungs and ovaries were measured by plaque-forming assay as previously described.[32] Plaques were counted microscopically.

Tissue and cell preparations

To obtain single cell suspensions, spleens and livers were homogenized by passing through cell strainers, and red blood cells were lysed using Gey's solution. Lungs and livers were digested with collagenase (2.33 mg/ml) (Sigma-Aldrich, Milwaukee, WI, USA) and DNase (0.2 mg/ml) (Roche Diagnostics, Indianapolis, IN, USA) for 30 min.

Antibodies (Abs) and flow cytometry

Abs for flow cytometric analysis were purchased from eBioscience or BioLegend (San Diego, CA, USA) unless otherwise noted: CD8α PerCP-eFluor 710 (53-6.7), CD127 FITC (SB/199), KLRG1 PE (2F1 KLRG1), IFN-γ APC (XMG1.2), TNF-α FITC (MP6-XT22), granzyme B (GzmB) PE (GB12; Invitrogen, Carlsbad, CA, USA). Samples were analyzed using Accuri flow cytometers or MacsQuant flow cytometers in the Dartlab core facility at Geisel School of Medicine at Dartmouth. Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA) or Accuri software (BD Biosciences).

MHC/peptide tetramer

The VACV-specific epitope $B8R_{20–27}$, TSYKFESV, was made as a synthetic peptide based on the original MVA (modified *vaccinia virus* Ankara) sequence.[33] Peptide MHC I tetramers consisting of $B8R_{20–27}/K^b$ conjugated to allophycocyanin were obtained from the NIH Tetramer Core Facility (Emory University, Atlanta, GA, USA). Cells were stained with the tetramer together with F*c* block (2.4G2) for 1 h at room temperature in the dark in PBS with 2% bovine growth serum, followed by surface staining at 4 °C for 20 min in 96-well U-bottom plates and were analyzed by flow cytometry.

Staining of surface markers and intracellular cytokines/ effector molecules

Surface markers were stained with Abs in PBS with 2% bovine growth serum for 20 min at 4 °C. For intracellular cytokine/ effector molecule detection, splenocytes from infected mice were restimulated *ex vivo* with 1 μg/ml B8R peptide for 5 h at 37 °C in complete medium with 10 U/ml rIL-2 and 10 μg/ml brefeldin A (Sigma-Aldrich). Subsequently, cells were stained with Abs against surface markers, followed by fixation with 1% formaldehyde for 20 min at 4 °C and then stained with Abs against IFN-γ, TNF-α or GzmB in 0.5% saponin solution for 30 min at 4 °C.

Depletion of $CD4^+$ T cells

Mice were administered i.p. 500 μg of anti-CD4 Ab (GK1.5), at days −1 and 0 of infection, followed by 250 μg twice weekly thereafter until the mice were sacrificed. Control mice were either untreated or given rat IgG (Jackson ImmunoResearch Laboratories, West Groove, PA, USA). No differences in T-cell responses were observed in the two groups of control mice (data not shown).

Blockade of CD40L

CD40L blocking Ab MR-1 (500 μg) (BioXcell, West Lebanon, NH, USA) was administrated i.p., starting at days −1 and 0 of infection, followed by every 2 days throughout the duration of the experiments. Control mice were either untreated or given rat IgG.

Measurement of cell proliferation

To measure turnover of B8R-specific $CD8^+$ T cells *in vivo*, cell proliferation was measured by 5-bromo-2′-deoxyuridine (BrdU) staining. Mice were injected 1 mg BrdU (i.p.) twice at 12 h apart, starting at day 8 post infection (pi). Splenocytes were prepared 18 h later, and cells were stained with anti-CD8α Ab and B8R tetramer, and then stained with the anti-BrdU Ab according to the protocol provided (BD Pharmingen G, San Jose, CA, USA).

Apoptosis assay by Annexin V staining

Splenocytes were prepared from infected mice at day 10 pi. Cells were stained with B8R tetramer, anti-CD8α Ab and Annexin V using an Apoptosis Detection Kit (BD Biosciences). The Annexin V staining was performed in conjunction with 7-aminoactinomycin D (7-AAD), since viable cells with intact membranes can exclude 7-AAD.

Cytotoxicity assays *in vivo* and *ex vivo*

For *in vivo* cytotoxicity assay, splenocytes were prepared from B6 mice and were incubated with 1 μg/ml B8R peptide in complete medium at 37 °C for 1 h. The B8R-pulsed and -unpulsed cells were labeled with 2.5 μM and 0.25 μM CFSE, respectively, in HBSS at room temperature for 10 min. The B8R-pulsed ($CFSE^{hi}$) and -unpulsed ($CFSE^{lo}$) cells were mixed at a 1 : 1 ratio and used as target cells. 2×10^7 target cells were injected i.v. into i.n. infected wild-type (WT) or MHC $II^{-/-}$ mice at day 9 pi, or naive WT mice. Six hours later, mice were killed and splenocytes were stained with 10 μM 7-AAD at room temperature for 15 min. CFSE-positive and 7AAD-negative cells were analyzed by flow cytometry. Specific lysis was evaluated with percent specific killing, calculated according to the formulas: % specific lysis=(1−[ratio of infected recipients/ ratio of naive recipients])×100%, where ratio=number of $CFSE^{hi}$/number of $CFSE^{lo}$.

For *ex vivo* cytotoxicity assay, effector $CD8^+$ T cells were prepared from splenocytes of i.p. infected WT or MHC $II^{-/-}$ mice at day 10 pi by staining with anti-CD8α Ab and B8R tetramer. Target cells were prepared as described in *in vivo* cytotoxicity assay. Effector B8R-specific $CD8^+$ T cells were cultured with target cells at a ratio of 5 : 1 (10^5:2×10^4, normalized to the same total number of B8R-specific $CD8^+$ T cells) in complete media in 96 well plates at 37 °C for 6 h. Cells were then stained with 10 μM 7-AAD at room temperature for 15 min and CFSE-positive and 7AAD-negative cells were analyzed by flow cytometry. Specific lysis was calculated based on the formulas: % specific lysis=[1−(ratio of WT or MHC $II^{-/-}$ $B8R^+CD8^+$ group/ratio of target alone group)]×100%, where ratio=number of $CFSE^{hi}$/number of $CFSE^{lo}$.

Statistical analysis

Student's *t*-tests (two-tailed, unpaired) were performed using GraphPad Prism 5 (GraphPad, La Jolla, CA, USA). Values of $P<0.05$ were considered statistically significant.

RESULTS

CD4 help deficiency impairs primary VACV-specific $CD8^+$ T-cell responses in intraperitoneally infected mice but not in intranasally infected mice

Clonal expansion of Ag-specific $CD8^+$ T cells is essential for adaptive immunity to viral infection. Upon infection, Ag-specific $CD8^+$ T cells can undergo 10^4- to 10^5-fold expansion in a week.[34] It is estimated that there are approximately 1070 naive $CD8^+$ T cells/spleen specific for $B8R_{20–27}$, a dominant epitope of VACV in mice.[35] We first studied the kinetics of the VACV-specific $CD8^+$ T-cell response by tetramer staining of B8R-specific $CD8^+$ T cells. WT B6 mice and MHC $II^{-/-}$mice were infected i.n. or i.p. with 10^3 PFU of VACV, a dose less than the lethal dose of 10^5 PFU *via* i.n. infection.[32] WT and MHC $II^{-/-}$ mice infected i.n. mounted a robust primary $CD8^+$ T-cell response, and expansion of B8R-specific $CD8^+$ T cells reached a peak at d10 pi in the spleen (Figure 1a and b) and the lung (Supplementary Figure 1). Over time, B8R-specific $CD8^+$ T-cell populations contracted, and the population in MHC $II^{-/-}$ mice had a faster contraction than that had in the WT cohort by d21 pi. In contrast, the i.p. infected MHC $II^{-/-}$ mice had an attenuated primary $CD8^+$ T-cell response, and the number of B8R-specific $CD8^+$ T cells was significantly reduced, compared with infected WT mice in the spleen (Figure 1a and b) and the lung (Supplementary Figure 1). Similar results were also observed in mice that were depleted of $CD4^+$ T cells using anti-CD4 Ab (GK1.5) (data not shown).

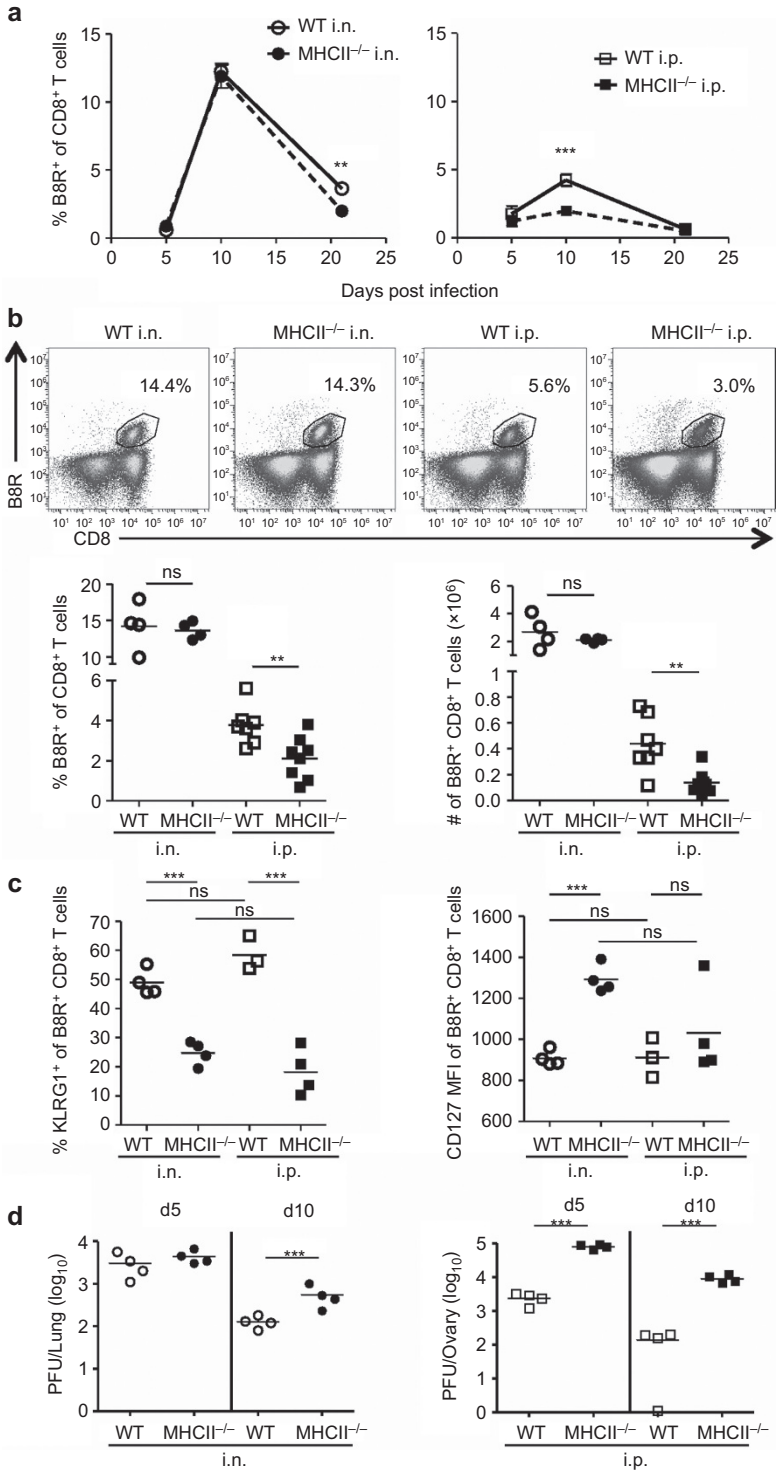

Figure 1 Primary CD8$^+$ T-cell responses in intranasally or intraperitoneally infected mice in the absence of CD4 help. C57BL/6 (WT) and MHC class II knockout (MHC II$^{-/-}$) mice were infected with 10^3 PFU of VACV-WR i.n. or i.p.. B8R-specific CD8$^+$ T cells were identified by staining with B8R tetramer and anti-CD8α Ab at the indicated times. (**a**) Kinetics of B8R-specific CD8$^+$ T-cell response. Frequencies of B8R-specific cells within the CD8$^+$ T-cell population in the spleens of WT and MHC II$^{-/-}$ mice infected i.n. or i.p. are shown. (**b**) B8R-specific CD8$^+$ T-cell populations in the spleens at day 10 post infection (pi). Representative FACS plots (top panel); frequency and total numbers (bottom panel). (**c**) Expression of KLRG1 and CD127 on B8R-specific CD8$^+$ T cells at d9 pi. (**d**) Viral titers in lungs and ovaries, determined by plaque-forming assays. Each point on the graph represents a single mouse, and horizontal bars indicate the means. Data are representative of two to three independent experiments with three to eight mice per group. $^{**}P<0.01$; $^{***}P<0.001$. Ab, antibody; i.n., intranasally; i.p., intraperitoneally; ns, not significant; pi, post infection; VACV-WR, vaccinia virus Western Reserve; WT, wild-type.

Upon first Ag encounter, naive $CD8^+$ T cells can expand and develop into short-lived effector cells (SLECs), which confer immediate protection, but decline following Ag clearance. Alternatively, $CD8^+$ T cells can become memory precursor effector cells.[36,37] $KLRG1^{hi}CD127^{lo}$ and $KLRG1^{lo}CD127^{hi}$ $CD8^+$ T cells are considered SLECs and memory precursor effector cells, respectively.[38] In this study, the frequency of cells expressing KLRG1 was reduced and CD127 expression was at a higher level on B8R-specific $CD8^+$ T cells in MHC $II^{-/-}$ mice compared to WT mice, and there were no significant differences between the i.p. and i.n. infections (Figure 1c). These data indicate that SLECs are diminished in the absence of CD4 help.

To study the effect of the diminished primary $CD8^+$ T-cell response on viral control, we detected viral load by plaque-forming assay. Lungs and ovaries are the major organs for VACV replication in the i.n. and i.p. infection, respectively.[27,39] In this experiment, infection led to high viral titers in both WT and MHC $II^{-/-}$ mice at d5 pi, regardless of infection route. The viral titers declined by d10 pi, but higher viral burdens remained in the organs of MHC $II^{-/-}$ mice in both i.n. and i.p. infected groups (Figure 1d). This indicates that the absence of CD4 help impairs viral control, but does not strictly correlate with the size of the B8R-specific $CD8^+$ T-cell population. The high affinity Ab response is also impaired in MHC $II^{-/-}$ mice,[27] and this likely contributes to the reduction in virus control observed in these mice.

Viral dose determines the CD4 help dependence of VACV-specific $CD8^+$ T-cell expansion in intranasally infected mice

We wished to explore factors contributing to the difference in the requirement for CD4 help in clonal expansion between i.n. infection and i.p. infection. First, we analyzed the effect of viral load by administering different doses of virus. In previous experiments (Figure 1), our standard inoculating dose of virus was 10^3 PFU/mouse in both i.n. and i.p. infections. For i.p. infection, both 10^4 PFU and 2×10^6 PFU doses manifested the same CD4 help dependence as was seen with the standard 10^3PFU dose (Figure 2a). The data indicate that an increased initial viral load does not preclude the requirement of CD4 help for primary $CD8^+$ T-cell responses in i.p. infection.

Unexpectedly, when reducing inoculated dose (10^2 PFU of VACV) in i.n. infected MHC $II^{-/-}$ mice, generation of B8R-specific $CD8^+$ T cells did need CD4 help. The population of B8R-specific $CD8^+$ T cells in MHC $II^{-/-}$ mice was smaller compared to WT mice (Figure 2b). These data suggest that while the requirement for CD4 help in the i.p. infection cannot be compensated by a high virus load, CD4 help in i.n. infected mice is required when a low virus dose is administered. Accordingly, inoculated viral dose is another factor determining dependence on CD4 help for primary $CD8^+$ T-cell responses.

Enhanced effector capacity in VACV-specific $CD8^+$ T cells following intranasal infection, compared with intraperitoneal infection

To test the influence of infection route and CD4 help on effector molecule production, we measured production of

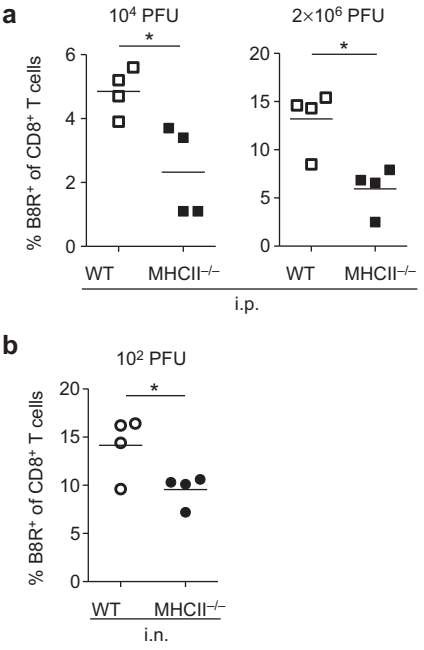

Figure 2 Influence of amount of viral inoculum on generation of VACV-specific $CD8^+$ T cells. B8R-specific $CD8^+$ T cells were prepared from the spleens of infected mice at d10 pi. (**a**) Frequency of B8R-specific cells within the $CD8^+$ T-cell population in mice infected i.p. with 10^4 or 2×10^6 PFU of VACV. (**b**) Frequency of B8R-specific cells within the $CD8^+$ T-cell population in mice infected i.n. with 1×10^2 PFU of VACV. Data are representative of two independent experiments with four mice per group. $*P<0.05$. pi, post infection; VACV, vaccinia virus.

cytokines IFN-γ and TNF-α as well as effector molecule GzmB by activated $CD8^+$ T cells. IFN-γ^+CD8^+ T cells from i.n. infected mice produced more IFN-γ on a per cell basis, as determined by mean fluorescence intensity (MFI), compared with i.p. infected mice, regardless of CD4 help (Figure 3a). The frequency of TNF-α^+ cells (Figure 3b, top), and production of TNF-α per cell (Figure 3b, bottom) were significantly higher in the IFN-γ^+CD8^+ T cells from i.n. infected mice than those from i.p. infected mice. Interestingly, regardless of infection route, mice lacking CD4 help contained a higher frequency of cells producing both IFN-γ and TNF-α than WT mice (Figure 3b). Production of GzmB was higher on a per cell basis in the IFN-γ^+CD8^+ T cells from i.n. infected mice than those from i.p. infected mice (Figure 3c). These results indicate that i.n. infection can induce enhanced production of IFN-γ, TNF-α and GzmB from IFN-γ^+CD8^+ T cells, compared to i.p. infection. In addition, the absence of CD4 help did not reduce the effector cytokine/molecule production by B8R-specific $CD8^+$ T cells.

CD4 help-deficiency does not affect cytotoxic activity of VACV-specific $CD8^+$ T cells

We performed an *in vivo* cytotoxicity assay to assess effect of CD4 help on the activity of VACV-specific $CD8^+$ T cells in i.n infected WT and MHC $II^{-/-}$ mice. As these mice had the same

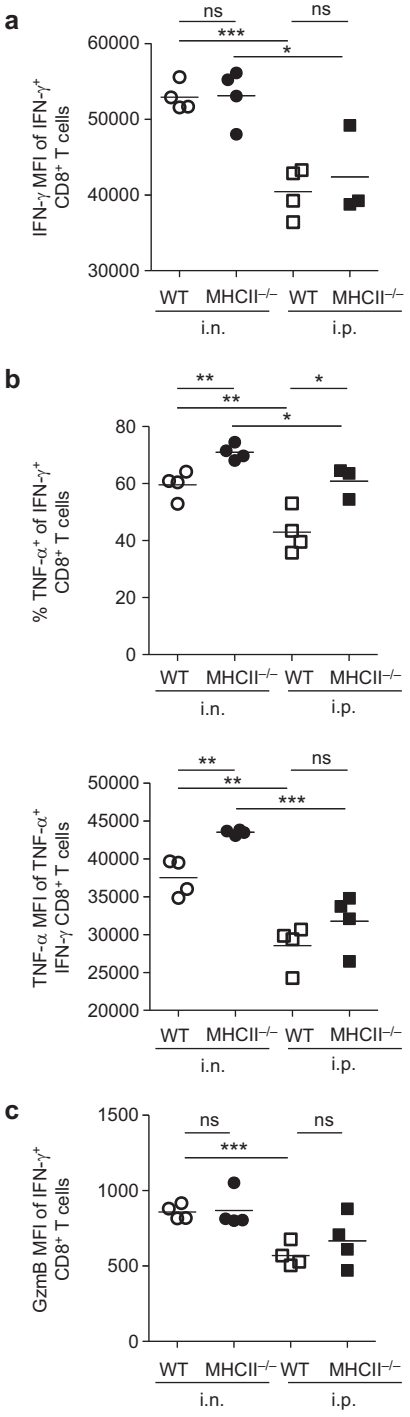

Figure 3 Production of IFN-γ, TNF-α and GzmB by VACV-specific $CD8^+$ T cells in the presence or absence of CD4 help. Splenocytes were prepared at d9 pi from mice which were infected with 10^3 PFU of VACV. The cells were restimulated *ex vivo* with B8R peptide and stained with anti-CD8α, -IFN-γ, -TNF-α and -GzmB Abs. (**a**) IFN-γ MFI of IFN-γ^+CD8^+ T cells. (**b**) Frequency of TNF-α-producing cells within IFN-γ^+CD8^+ T cells (top panel) and TNF-α MFI of TNF-α^+IFN-γ^+CD8^+ T cells (bottom panel). (**c**) GzmB MFI of IFN-γ^+CD8^+ T cells. Data are representative of two independent experiments with three to four mice per group. *$P<0.05$; **$P<0.01$; ***$P<0.001$. Ab, antibody; GzmB, granzyme B; MFI, mean fluorescence intensity; ns, not significant; pi, post infection; VACV, vaccinia virus.

frequency of B8R-specific $CD8^+$ T cells (Figure 1b), a direct comparison between the two groups could be made using this assay. B8R-pulsed ($CFSE^{hi}$) and -unpulsed ($CFSE^{lo}$) splenocytes from B6 mice were mixed as target cells, and 2×10^7 of the target cells were injected to WT and MHC $II^{-/-}$ mice at d10 pi. Six hours later, spleens were harvested and target cells were detected by flow cytometry. B8R-pulsed target cells were completely cleared from VACV-infected mice, and there was no difference between WT and MHC $II^{-/-}$ mice (Figure 4a). This indicated highly effective cytotoxic function in both WT and CD4 help-deficient groups.

Since B8R-specific $CD8^+$ T cells in i.p. infected MHC $II^{-/-}$mice were present at a much lower level than that in i.p. infected WT mice (Figure 1b), the *in vivo* cytotoxicity assay could not be used to compare these groups directly. Thus, we performed an *ex vivo* cytotoxicity assay to assess effect of CD4 help on CTL activity in i.p. infected WT and MHC $II^{-/-}$ mice. Target cells were prepared in the same way as those in the *in vivo* cytotoxicity assay. Effector B8R-specific $CD8^+$ T cells were prepared from the splenocytes of i.p. infected WT and MHC $II^{-/-}$ mice at d10 pi. Effector cells were cocultured with target cells at a ratio of 5 : 1 for 6 h. The specific lysis was approximately 20% and there was no difference in CTL activity between the two groups (Figure 4b). This result is in agreement with previous work with ^{51}Cr release assays in which cytotoxicity toward VACV-infected target cells is not diminished in i.p. infected $CD4^+$ T cell-depleted mice.[27] These data suggest that cytotoxic activity of VACV-specific $CD8^+$ T cells is not affected by the absence of CD4 help.

Differential requirement for IL-2 signaling through CD25 in i.n. *versus* i.p. infection

$CD4^+$ T cell-derived IL-2 is essential for help-dependent primary $CD8^+$ T-cell responses,[40] and CD25 (IL-2 receptor α) is necessary to form the high-affinity IL-2 receptor. We investigated whether there were the same requirements for the high-affinity IL-2 receptor in $CD8^+$ T-cell responses in mice infected by either the i.n. or i.p. route. Mixed BM chimeric mice were used, that contained both WT $CD25^{+/+}$ ($CD45.1^+$) and $CD25^{-/-}$ ($CD45.2^+$) cells. $CD25^{-/-}$ mice cannot be used directly due to severe autoimmune disease in these mice because they lack regulatory T cells.[41] Spleens, lungs, peripheral blood lymphocytes (PBLs), BM and livers were collected from the chimeric mice at d10 pi. Cells were stained with B8R tetramer and anti-CD8α Ab. The expansion of $CD25^{-/-}$ B8R-specific $CD8^+$ T cells was significantly diminished compared to WT cells in i.p. infected chimeric mice, which is consistent with our previous studies.[22] In contrast, i.n. infected chimeric mice mounted strong $CD8^+$ T-cell responses from both the WT and $CD25^{-/-}$ compartments and no statistical differences could be detected in the spleens, lungs, BM or livers (Figure 5b). A small but statistically significant difference was detected in the PBL; however, this difference was smaller than that observed in the PBL of i.p. infected chimeric mice. The data demonstrate that CD25-deficiency significantly

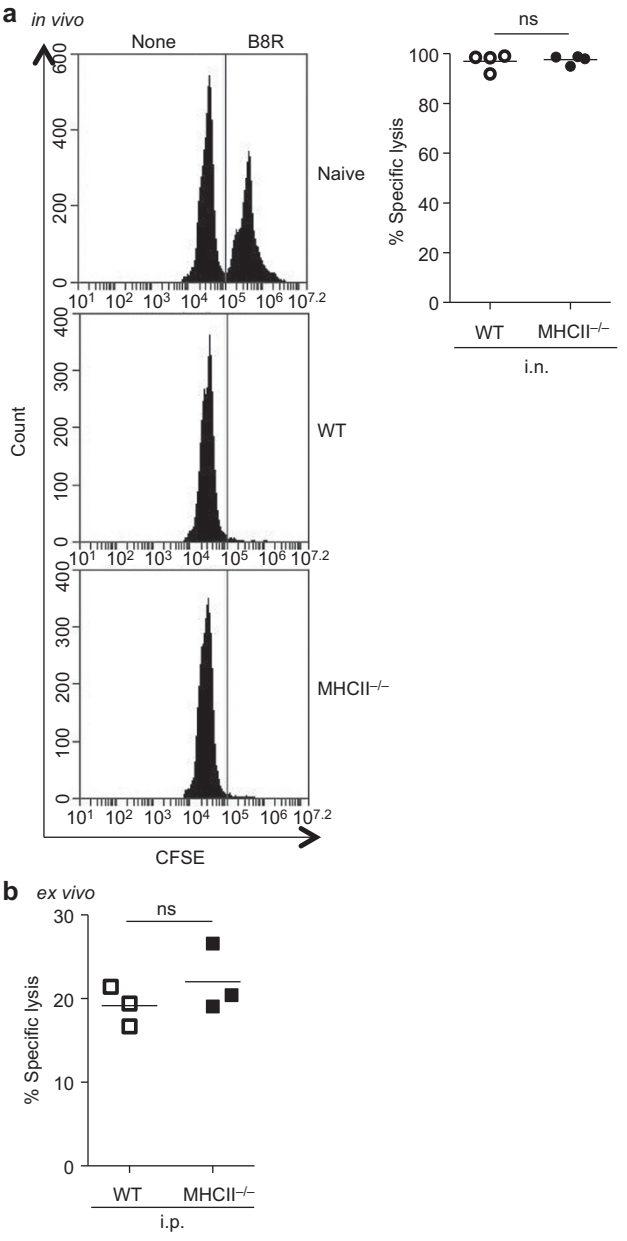

Figure 4 Cytotoxic activity of VACV-specific CD8$^+$ T cells is not affected by the absence of CD4 help. (**a**) *In vivo* cytotoxic activity. Target cells, B8R-pulsed (CFSE^{hi}) and-unpulsed (CFSE^{lo}) splenocytes (1 : 1), were transferred to i.n. infected mice (10^3 PFU) at d10 pi. Six hours later, spleens were harvested and CFSE-labeled cells were analyzed by flow cytometry. Representative FACS histograms of live target cells and percent specific lysis are shown. (**b**) *Ex vivo* cytotoxic activity. Effector cells (B8R-specific CD8$^+$ T cells) were prepared from splenocytes of i.p. infected mice (10^3 PFU) at d10 pi. Effector cells were cultured with target cells at 5 : 1 ratio (normalized to same total number of B8R-specific CD8$^+$ T cells) for 6 h, and CFSE-labeled target cells were analyzed. Percent specific lysis is shown. Data are representative of two independent experiments with three to four mice per group. i.n., intranasally; i.p., intraperitoneally; ns, not significant; pi, post infection; VACV, vaccinia virus.

diminishes the expansion of the VACV-specific CD8$^+$ T-cell pool in mice infected i.p. but not i.n., suggesting that there are compensatory mechanisms in play during lung infection.

Role of CD40–CD40L pathway in the generation of VACV-specific CD8$^+$ T-cell population in i.n. infection

It has been reported that signaling *via* the CD40–CD40L pathway can replace CD4 help in priming the CTL response.[42,43] In this experiment, we measured generation of B8R-specfic CD8$^+$ T cells in mice that were administered CD40L blocking Ab (MR-1) and infected i.n. with 10^2 PFU of VACV. This viral dose was used as it induces a CD4 help-dependent response after i.n. infection (Figure 2b). Blockade of CD40L resulted in populations of B8R-specific CD8$^+$ T cells that were significantly smaller than in WT mice, and similar to MHC II$^{-/-}$ mice (Figure 6a). The data indicate that CD40–CD40L signaling is required for expansion of B8R-specific CD8$^+$ T cells in i.n. infection with as mall viral dose. In contrast, for i.n. infection with 10^3 PFU of VACV, blockade of CD40–CD40L signaling did not significantly affect the expansion of the B8R-specific CD8$^+$ T-cell population in both WT and MHC II$^{-/-}$ mice (Figure 6b). This is consistent with the CD4 help-independent status of the response after i.n. infection with 10^3 PFU of VACV (Figure 1a and b). Thus, whether CD40–CD40L signaling is required for expansion of B8R-specific CD8$^+$ T cells depends upon viral dose after i.n. infection.

CD4 help deficiency results in increased apoptosis of VACV-specific CD8$^+$ T cells

We measured the proliferation of B8R-specific CD8$^+$ T cells *in vivo* by BrdU incorporation. Mice were administered BrdU at d8 pi, and 18 h later, splenocytes were prepared and stained with anti-CD8α Ab and B8R tetramer, and then with anti-BrdU Ab. B8R-specific CD8$^+$ T cells from WT and MHC II$^{-/-}$ mice had similar rates of BrdU incorporation, regardless of infection route, indicating that Ag-specific CD8$^+$ T cells were able to proliferate equivalently in CD4 help-deficient mice and WT mice (Figure 7a).

Next, we determined the proportion of B8R-specific CD8$^+$ T cells undergoing apoptosis in the absence of CD4 help, using Annexin V staining in conjunction with 7-AAD, since viable cells with intact membranes can exclude 7-AAD. Splenocytes were prepared from WT and MHC II$^{-/-}$ mice at d9 pi. The proportions of Annexin V$^+$ cells in B8R-specific CD8$^+$7-AAD$^-$ T cells were higher in cells from MHC II$^{-/-}$ mice than those from WT mice in both i.n. and i.p. infection (Figure 7b). This indicates that VACV-specific CD8$^+$ T cells undergo more apoptosis in the absence of CD4 help.

We also determined the proportion of cells undergoing apoptosis in the absence of CD40L signaling in i.n. infected mice with different viral doses. Blockade of CD40L resulted in more apoptosis in mice infected with 10^3 PFU (Figure 7c, left) and 10^2 PFU (Figure 7c, right) compared to the WT cohort. These data suggest that CD40–CD40L pathway is responsible for preventing apoptosis of VACV-specific CD8$^+$ T cells.

DISCUSSION

Several studies have indicated that the entry route of pathogens influences immune responses. Intravenous and intranasal infections with *Listeria monocytogenes* induce Th1 and Th17

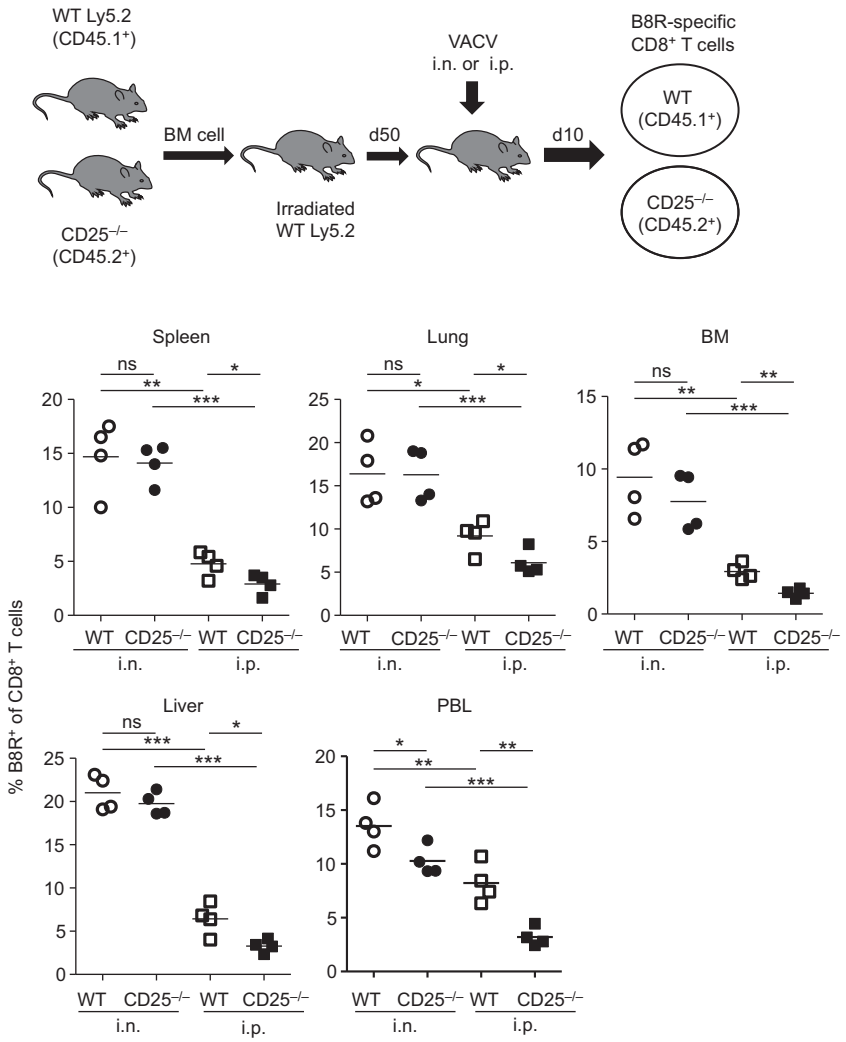

Figure 5 Signaling through CD25 is required to drive CD8$^+$ T-cell responses in i.p. but not i.n infected mice. (**a**) Design of CD25$^{-/-}$ chimeric mice generation and infection with 10^3 PFU of VACV. (**b**) Frequency of B8R-specific CD8$^+$ T cells in WT and CD25$^{-/-}$chimeric mice at d10 pi. B8R-specific CD8$^+$ T cells were detected in various organ/tissues, including the spleens, lungs, BM, livers and PBLs. *P<0.05; **P<0.01; ***P<0.001. BM, bone marrow; i.n., intranasally; i.p., intraperitoneally; ns, not significant; PBL, peripheral blood lymphocyte; pi, post infection; VACV, vaccinia virus; WT, wild-type.

CD4$^+$ effector T cells, respectively.[44] Intravenous and intrarectal infections with simian immunodeficiency virus result in difference in time of virus appearance in the blood and size of the virus-specific immune response, and the natural mucosal barrier may delay viral spreading.[45] By examining intradermal, subcutaneous, i.p. or i.v. infection, studies have shown that the immunodominance hierarchy is also affected by the route of infection.[46] Additionally, the role of CD4 help in the CD8$^+$ T-cell responses does not follow a simple on–off rule. In herpes simplex virus 1 infection, the primary CD8$^+$ T-cell response is strictly CD4 help-dependent with subcutaneous (footpad) infection,[47,48] but is largely independent with ocular infection.[49] In this study with 1×10^3 PFU VACV-WR infection, B8R-specific CD8$^+$ T cells displayed a poor expansion (Figure 1a and b) in i.p. infected CD4 help deficient mice. In contrast, after i.n. infection, B8R-specific CD8$^+$ T cells expanded equivalently in either the presence or absence of CD4 help. These data indicate that the route of infection can markedly affect Ag-specific CD8$^+$ T-cell responses in the absence of CD4 help.

We studied the effect of Ag dose by inoculating various doses of VACV. An increased viral load could not restore expansion of B8R-specific CD8$^+$ T cells in i.p. infection, even when the dose was increased to 2000-fold more than the standard dose used in this study (Figure 2a). This suggested that for VACV-WR infection, dependency on CD4 help could not be overcome by providing more Ag stimulation. Consistent with our data, another group also demonstrated that primary CD8$^+$ T-cell expansion is dependent on CD4 help when mice were infected i.p with 5×10^6 PFU of VACV (VV-G2).[23] While another report found CD8$^+$ T-cell expansion was not dependent on CD4 help after i.p. infection with 10^7 PFU of VACV (rVV33),[14] this study

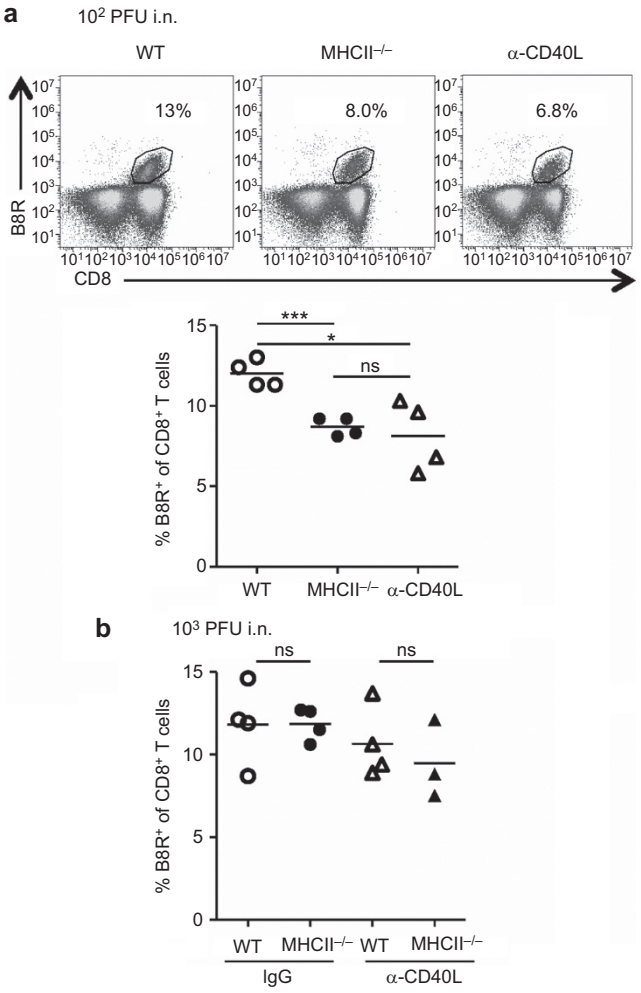

Figure 6 Effect of CD40L signaling deficiency on the generation of VACV-specific CD8$^+$ T-cell population in i.n. infection. Splenocytes were prepared from WT, MHC II$^{-/-}$, CD40L-blocked (α-CD40L) WT or α-CD40L MHC II$^{-/-}$mice at d9 pi. Blockade of CD40L signaling was performed with a CD40L-blocking Ab, MR-1. (**a**) Representative FACS plots and frequencies of B8R-specific cells within CD8$^+$ T cells in mice infected with 10^2 PFU of VACV. (**b**) Frequency of B8R-specific cells within CD8$^+$ T cells in mice infected with 10^3 PFU of VACV. Data are representative of two independent experiments with three to four mice per group. *P<0.05; ***P<0.001. i.n., intranasally; ns, not significant; pi, post infection; VACV, vaccinia virus; WT, wild-type.

used CD4$^{-/-}$ mice, which we now know contain an aberrant population of 'helper' CD8$^+$ T cells.[50] Our studies show that reduction in the viral load, from 10^3 to 10^2 PFU, did result in dependence on CD4 help following i.n. infection (Figure 2b). These data indicate that in addition to route of infection, viral dose is also an important factor determining the degree to which primary CD8$^+$ T-cell responses require CD4 help.

We analyzed the profiles of antiviral effector molecules or cytokines. B8R-specific CD8$^+$ T cells produced more IFN-γ, TNF-α and GzmB in i.n. infected mice than in i.p. infected mice, regardless of CD4 help (Figure 3). Although the underlying mechanism is not clear, higher expression of effector molecules by B8R-specific CD8$^+$ T cells in i.n. infection may be a result of the mucosal microenvironment in the lung. It would therefore be interesting to determine if the same is true for other mucosal routes of infection. Additionally, we analyzed cytotoxicity of CD8$^+$ T cells in the absence of CD4 help. The VACV-specific CD8$^+$ T cells showed similar cytotoxic activity in WT and MHC II$^{-/-}$ mice (Figure 4). Therefore

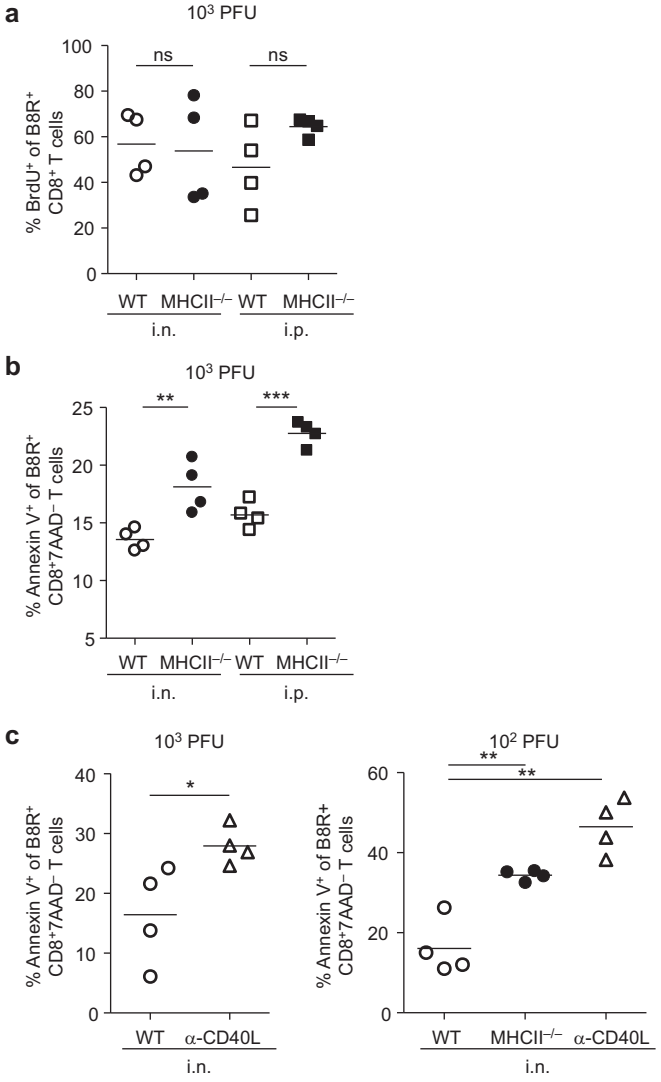

Figure 7 Proliferation and apoptosis of VACV-specific CD8$^+$ T cells. *In vivo* proliferation was measured by detecting incorporation of BrdU. Apoptosis was measured by Annexin V staining in conjunction with 7-AAD. B8R-specific CD8$^+$ T cells were prepared from the spleens of infected mice. (**a**) Frequency of BrdU$^+$ cells within B8R-specific CD8$^+$ T-cell population in WT or MHC II$^{-/-}$ mice at d9 pi. (**b**) Frequency of Annexin V$^+$ cells within B8R-specific CD8$^+$7AAD$^-$ T-cell population in WT or MHC II$^{-/-}$ mice at d10 pi. (**c**) Frequency of Annexin V$^+$ cells within B8R-specific CD8$^+$7AAD$^-$ T-cell population in WT, MHC II$^{-/-}$ or α-CD40L WT mice at d9 pi. Data are representative of two independent experiments with four mice per group. *P<0.05; **P<0.01; ***P<0.001. 7-ADD, 7-aminoactinomycin D; BrdU, 5-bromo-2′-deoxyuridine; ns, not significant; pi, post infection; VACV, vaccinia virus; WT, wild-type.

cytotoxic function is not impaired in the absence of CD4 help, regardless of the route of infection.

T helper cell-derived IL-2 is a central component of help for CD8$^+$ T-cell responses.[40] Our lab has previously reported that CD4$^+$ T cells upregulate CD25 expression on Ag-specific CD8$^+$ T cells, thereby promoting the expansion of the virus-specific CD8$^+$ T-cell population, specifically SLECs.[22] Other studies have shown that IL-2 signaling promotes expression of the transcription factor Blimp-1, which is necessary for differentiation of SLECs.[51] In this study, we confirmed that signaling through CD25 was essential for driving expansion of the population after i.p. infection, but not after i.n. infection (Figure 5). The data suggest that deficiency of IL-2/CD25 signaling may be partially responsible for the reduced primary B8R-specific CD8$^+$ T-cell response in the absence of CD4 help following i.p. infection. However, i.n. infected mice did not show dependence on the IL-2/CD25 pathway, suggesting that some pathways or signaling in lung infection can replace or compensate for CD4 help.

Dendritic cells (DCs) are potent professional APCs, bridging the innate and adaptive immune systems to induce primary immune responses.[52,53] A CD4 help signal is transmitted from DCs to CD8$^+$ T cells partly *via* CD40–CD40L interactions between DCs and CD4$^+$ T cells.[42] Immunization with non-infectious agents requires the CD4 help,[17] and CD40–CD40L signaling can replace CD4 help in priming of CTL responses to Ag-loaded splenocytes and alloantigen.[42,43] In addition, upregulating CD40L on DCs can promote optimal priming of CD8$^+$ T cells against influenza in the absence of CD4 help.[54] Work from our lab has shown that expansion of the VACV-specific CD8$^+$ T-cell population in CD40$^{-/-}$ mice is equivalent to WT mice after i.p. infection.[22] However, Wiesel *et al.*[23] found that expansion of VACV-specific CD8$^+$ T cells is impaired in the absence of CD40–CD40L in i.p. infection. Differences in the infectious dose, the viral strain and mouse model might partly explain the discrepancy. In this study, expansion of virus-specific CD8$^+$ T-cell population was dependent on CD4 help (Figure 2b) and the CD40–CD40L pathway (Figure 6a) in i.n. infected mice with 10^2 PFU of VACV. This suggests that CD4 help through CD40–CD40L signaling is required for optimal primary CD8$^+$ T-cell responses when mice are infected i.n. at a small viral dose. In contrast, after i.n. infection with 10^3 PFU of VACV, the expansion was independent of CD4 help (Figure 1a and b) and the CD40–CD40L pathway (Figure 6b). Recognition of microbial products by Toll-like receptors directly activate APCs and thus, bypass the need for CD4 help.[55] We are currently investigating whether higher viral doses result in better DC maturation, which may reduce the requirement for CD4 help.

To explore the mechanism by which the absence of CD4 help resulted in a smaller population of B8R-specific CD8$^+$ T cells, we measured proliferation and apoptosis of VACV-specific CD8$^+$ T cells. The B8R-specific CD8$^+$ T cells proliferated normally in the absence of CD4 help regardless of the route of infection (Figure 7a). However, CD4 help deficiency resulted in increased apoptosis of the B8R-specific CD8$^+$ T cells in mice infected either i.n. or i.p. (Figure 7b). This agrees with previous reports in which an i.p. infected mouse model was used.[21,23] Moreover, CD40L signaling-deficiency also resulted in increased apoptosis of the B8R-specific CD8$^+$ T cells in i.n. infected mice regardless of viral dose (Figure 7c). Increased apoptosis may contribute to the reduced clonal expansion observed in i.p. infected mice lacking CD4 help. However, a similar increase in apoptosis was observed following i.n. infection, but this was insufficient to reduce the clonal expansion. Currently, we do not know the mechanism underlying this discrepancy. Possibly B8R-specific cell death rates are not increased in organs other than the spleen, and there may be differential recruitment of these cells to the spleen in the presence or absence of CD4$^+$ T cells. Alternatively, there may be another, as yet undefined, mechanism that may compensate for reduced CD8$^+$ T-cell viability during respiratory infection in the absence of CD4 help.

As the front line exposed to the air, the lung is equipped with an elaborate network of DCs to sense entering pathogens.[56] Studies have demonstrated that mucosal vaccination is more effective than systemic vaccination in establishing protective mucosal immune responses.[57] This is because an optimum mucosal vaccine strategy can activate local innate immune responses for generating neutralizing sIgA Abs, polyfunctional CD8$^+$ CTLs and CD4$^+$ T-cell effector memory responses.[57] Our findings demonstrate that there is remarkable variability in the dependence of the CD8$^+$ T-cell response on CD4 help. The identical pathogen can have differing requirements for CD4 help owing to a different route of infection or viral dose. In addition, infection in the lung microenvironment may favor superior effector activity in virus-specific CD8$^+$ T cells. Natural infections with orthopox viruses occur through the respiratory tract; thus, the i.n. infection model may provide some important insights into the host response to VACV infection. This information is also very relevant for vaccine design, as it highlights the route of immunization and dose of immunogen must be selected with great care to achieve an optimal effector CD8$^+$ T-cell response.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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