Intrinsic IL-21 Signaling Is Critical for CD8 T Cell Survival and Memory Formation in Response to Vaccinia Viral Infection

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Intrinsic IL-21 Signaling Is Critical for CD8 T Cell Survival and Memory Formation in Response to Vaccinia Viral Infection

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CD4 T cell help plays an important role in promoting CD8 T cell immunity to pathogens. In models of infection with vaccinia virus (VV) and Listeria monocytogenes, CD4 T cell help is critical for the survival of activated CD8 T cells during both the primary and memory recall responses. Still unclear, however, is how CD4 T cell help promotes CD8 T cell survival. In this study, we first showed that CD4 T cell help for the CD8 T cell response to VV infection was mediated by IL-21, a cytokine produced predominantly by activated CD4 T cells, and that direct action of IL-21 on CD8 T cells was critical for the VV-specific CD8 T cell response in vivo. We next demonstrated that this intrinsic IL-21 signaling was essential for the survival of activated CD8 T cells and the generation of long-lived memory cells. We further revealed that IL-21 promoted CD8 T cell survival in a mechanism dependent on activation of the STAT1 and STAT3 pathways and subsequent upregulation of the prosurvival molecules Bcl-2 and Bcl-xL. These results identify a critical role for intrinsic IL-21 signaling in CD8 T cell responses to an acute viral infection in vivo and may help design effective vaccine strategies.

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IL-21 is the most recently identified member of the common γ-chain family of cytokines that includes IL-2, IL-4, IL-7, IL-9, and IL-15 (14, 15). This cytokine is mainly produced by activated CD4 T cells (14), including follicular helper T cells (16), as well as NKT cells (17), and it plays a critical role in the control of both innate and adaptive immune responses (15). IL-21 can regulate NK cell activation and expansion (18, 14) and promote terminal B cell differentiation into plasma cells, which is critical for Ab production (19, 20). IL-21 can also enhance resting T cell proliferation in vitro in combination with IL-7 or IL-15 and promote Ag-specific CD8 T cell expansion in vivo (21). Additionally, it is critical for the development of follicular helper T cells (16) and the inflammatory Th17 lineage (22, 23) and also contributes to autoimmunity (15). More recently, IL-21 has been shown to be an essential component of CD4 T cell help required to sustain the CD8 T cell response during chronic, but not acute, lymphocytic choriomeningitis virus (LCMV) infections (24–26). This is achieved by direct action of IL-21 on CD8 T cells to avoid deletion and maintain immunity. However, two major issues remain. First, the role of IL-21 in the CD8 T cell response to CD4 T cell help-dependent acute infections such as VV infection is still unknown. Second, the mechanism(s) underlying the cell-intrinsic, IL-21–dependent enhancement of CD8 T cell immunity is yet to be defined.

In this study, we first provided direct evidence that CD4 T cell help for CD8 T cell survival was mediated by IL-21. We then demonstrated that direct IL-21 signaling on CD8 T cells was required for the priming of VV-specific CD8 T cell response in vivo. Using clonotypic influenza hemagglutinin (HA)-specific transgenic T cells, we found that the activation, proliferation, or effector differentiation of CD8 T cells in response to VV infection in vivo was not affected by lack of IL-21 signaling. However, the survival of effector CD8 T cells was critically dependent on intrinsic IL-21 signaling. We further showed that CD4 T cell help for CD8 T cell survival was also critically dependent on IL-21 signaling in vivo. Additionally, CD8 T cells deficient in IL-21 signaling failed to develop into long-lived memory cells. We further observed that IL-21 promoted CD8 T cell survival by activating the STAT1 and
STAT3 signaling pathways and subsequent upregulation of the prosurvival molecules, Bcl-2 and Bcl-xL. In vivo, CD8 T cells defective for IL-21 signaling had reduced levels of STAT1 and STAT3 activation and Bcl-xL upregulation in response to VV infection. Collectively, our study indicates that intrinsic IL-21 signaling is required for the survival of activated CD8 T cells and the formation of long-lived memory cells in response to VV infection and may have important implications in the design of effective vaccine strategies.

Materials and Methods

Mice
B10.D2, Thy1.1. B10.D2 mice were purchased from The Jackson Laboratory; clone 4 HA-TCR mice (CD4-/-) mice on the C57BL/6 background were purchased from The Jackson Laboratory and backcrossed onto the B10.D2 genetic background for nine generations. 129/Sv mice were obtained from Charles River Laboratories. STAT1-/- mice on the 129/Sv background were purchased from Taconic. IL-21 R-/- mice were used as described (19). All mice used for experiments were naive. CD8+ T cells were purified using anti-CD8 microbeads (Miltenyi Biotec). Naive T cells (2 × 10^6) were cultured with either CFSE-labeled or unlabeled naive clone 4 HA-specific CD4 T cells were purified using positive selection using anti-CD4 beads (Miltenyi Biotec). Naive WT 6.5 HA-specific CD4 T cells were purified by positive selection using anti-CD4 beads (Miltenyi Biotec). Naive T cells (2 × 10^6) were cultured with anti-CD4 mAb, GK1.5 (150 μg/ml), and may have important implications in the design of effective vaccine strategies.

Real-time PCR
Total RNA was isolated from purified cells using TRIzol reagent (Invitrogen), and cDNA was generated using a reverse transcription kit (Promega). Real-time PCR was performed using an iCycler (Bio-Rad) to measure SYBR Green incorporation. The following primer sets were used: IL-2, 5'-CCACCTCAGGCTCCACTTC-3', 5'-ATCCTGGGGAGTTTCAAGGT-3'; IL-7, 5'-ATCCTGTTCCTGGTCCTTG-3'; 5'-ACCGATTGTGTTGGCTTG-3'; IL-15, 5'-GAGGGCGGATCTATGTTCT-3'; 5'-GCAATTCGAGGAAAGACAG-3'; IL-21, 5'-CCCTTGTGCTGTTGAGTCATC-3'; 5'-TGTTTTCTTTCTCCCTCCTC-3'; Bcl-2, 5'-GAATGCAGACGAGAGAG-3'; 5'-AAGCCTGTCACAGGCGCTTA-3'; Bcl-xL, 5'-TGCTGCGACTTCTTCCTCC-3'; 5'-CTCACATTCCCGAGAGGCTGC-3'; and Bim, 5'-GAATGCACCCATCTACGACG-3'; 5'-AAGAATGCAGACTCGTGGTTC-3'. Amounts of mRNA were normalized to β-actin mRNA levels within each sample.

Adoptive transfer of T cells
Naive polyclonal CD8 T cells (Thy1.2+) were prepared from either WT or IL-21 R-/- mice. Briefly, single-cell suspensions were prepared from spleen and lymph nodes, and CD8+ T cells were positively selected using anti-CD8 microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. Naive clonotypic HA-specific CD8 T cells (Thy1.2+) were prepared from both WT and IL-21 R-/- clone 4 HA-TCR transgenic mice. Briefly, single-cell suspensions were prepared from spleen and lymph nodes of clone 4 mice, and clonotypic percentage was determined by flow cytometry analysis of CD8+V48.2+ cells as described (29, 30). The activation marker CD69 was checked to ensure that these clonotypic cells were naive. CD8+ T cells were positively selected and CD8+CD4- cells were purified using anti-CD8 microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. A total of 10^5 polyclonal CD8 T cells, or 10^4 or 10^6 clonotypic CD8 T cells, were adoptively transferred to naive recipients via tail vein in 200 μl HBSS. In some experiments cells were labeled with CFSE before transfer.

Isolation of lymphocytes from nonlymphoid tissues
Lymphocytes were isolated from nonlymphoid tissues as described previously (31). Briefly, liver or lung tissue was homogenized and passed through a 70-μm cell strainer. The single-cell suspension was resuspended in 10 ml HBSS and centrifuged on a 5 ml Ficoll gradient (Amersham Biosciences). Cells were harvested from the Ficoll gradient and washed twice with HBSS prior to analysis.

Intracellular staining
To measure intracellular levels of Bcl-xL, STAT1, STAT3, pSTAT1, and pSTAT3, cells were fixed with 3.7% formaldehyde, permeabilized with 90% methanol, blocked with 3% FBS, and subsequently stained with anti-Bcl-xL, anti-STAT1, and anti-STAT3 microbeads (Miltenyi Biotec), according to the manufacturer’s instructions. A total of 10^5 polyclonal CD8 T cells, or 10^4 or 10^6 clonotypic CD8 T cells, were adoptively transferred to naive recipients via tail vein in 200 μl HBSS. In some experiments cells were labeled with CFSE before transfer.

Retrovirus preparation and infection
pMSCV constructs encoding GFP alone and GFP with dominant-negative STAT3 (STAT3-D), provided by D. Link (Washington University, St. Louis, MO), were used to produce recombinant retroviruses using BOSC 23 cells as described previously (32). Ten micrograms of the plasmid was transfected into BOSC 23 cells using calcium chloride-mediated transfection.
Freshly prepared viral supernatants were added in the presence of 8 μg/ml polybrene to cultures 24 h after polyonal CD8 T cells were stimulated with 1 μg/ml anti-CD5 and 5 μg/ml anti-CD28. After 24 h at 37°C, the retroviral supernatants were removed and replaced with T cell medium with or without murine IL-21 (10 ng/ml) and cells were cultured for an additional 72 h. For RNA isolation, GFP+ cells were sorted by FACS using a MoFlo sorter (Beckman Coulter).

Statistical analysis

Results were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student t test.

Results

CD4 T cell help for CD8 T cell survival is mediated by IL-21 in vitro

To study the mechanism(s) by which CD4 T cell help promotes CD8 T cell survival, we first looked at the role of CD4 T cell licensing of DCs in CD8 T cell immunity since this has been shown to be important in HSV-1 infection (7). We used a previously described model where conventional CD8+ DCs from pathogen-infected mice can be used to prime naive CD8 T cells in vitro (33). WT or CD4−/− mice were infected with rVV-HA, and 24 h later, CD8+CD11c+B220− DCs were purified and used to stimulate naive HA-specific CD8 T cells derived from the clone 4 HA-TCR transgenic mice that express a TCR recognizing a Kd-restricted HA epitope in vitro. Four days later, similar extents of CD8 T cell proliferation measured by CFSE dilution (Fig. 1B) were observed with DCs derived from either rVV-HA–infected WT or CD4−/− mice, suggesting that CD4 T cell licensing of DCs during VV infection is not important in promoting CD8 T cell proliferation or survival.

We then examined whether CD4 T cell help is contact-dependent, as recent data have suggested that direct cell-to-cell contact between CD4 and CD8 T cells may protect CD8 T cells from activation-induced cell death (34). To address this issue, we used a culture system where naive polyclonal CD8 T cells are either mixed with naive CD4 T cells or separated from them in a transwell, followed by stimulation with soluble anti-CD3 and anti-CD28 mAbs for 4 d. Our data showed that while CD4 T cells did not enhance CD8 T cell proliferation (Fig. 1C), they markedly enhanced CD8 T cell survival whether mixed together or separated by a transwell (Fig. 1D), indicating that CD4 T cell help for CD8 T cell survival is mediated by a soluble factor in vitro.

We next determined what soluble factor from CD4 T cells enhanced CD8 T cell survival. Because the common γ-chain cytokines IL-2, IL-7, IL-15, and IL-21 have been shown to be the key regulators of CD8 T cell responses (15, 35), we examined which of these cytokines were upregulated in the activated CD4 T cell population by real-time PCR. Both IL-2 and IL-21 mRNA levels were increased in activated CD4 T cells (Fig. 2A). Because IL-2 has been shown to be dispensable for CD8 T cell survival in vivo (36), we tested whether IL-21 could play a role in the survival of activated CD8 T cells. The addition of recombinant IL-21 to polyclonal CD8 T cells that were stimulated with anti-CD3 and anti-CD28 enhanced their survival in vitro, an effect that was dependent on the presence of the IL-21R on the CD8 T cells (Fig. 2B).

To determine whether CD4 T cell–derived IL-21 enhances CD8 T cell survival, we intercrossed the IL-21R−/− (IL-21R−/−) mice with the clone 4 HA-TCR mice to generate IL-21R−/− clone 4 T cells. We then cultured either WT or IL-21R−/− clone 4 CD8 T cells with DCs derived from rVV-HA–infected WT mice in the presence or absence of HA-specific CD4 T cells from the 6.5 TCR-HA mice that express a TCR recognizing an Ld-restricted HA epitope. We found that the enhancement of CD8 T cell survival by CD4 T cells was abolished in the absence of IL-21 signaling (Fig. 2C). Taken together, these results suggest that CD4 T cell help for the survival of activated CD8 T cells is mediated by direct IL-21 signaling on CD8 T cells in vitro.

The priming of VV-specific CD8 T cells in vivo is dependent on intrinsic IL-21 signaling

We next asked whether this intrinsic IL-21 signaling is required for the CD8 T cell response to VV infection. To address this issue, we transferred polyclonal CD8 T cells from WT or IL-21R−/− mice (Thy1.2+) into congenic B10.D2 recipients (Thy1.1+) that were subsequently vaccinated with rVV-HA. Seven days later, splenocytes were analyzed for VV-specific CD8 T cell response by intracellular IFN-γ staining upon restimulation in vitro with the Ld-restricted VV F2L epitope immunodominant peptide (37). We found that the percentage and the absolute cell number of the F2L-specific CD8 T cells were significantly (p < 0.001) reduced in the transferred IL-21R−/− CD8 T cells, compared with the transferred WT controls (Fig. 3A, 3B). However, the endogenous CD8 T cell response to VV F2L is comparable in both recipients (Fig. 3A). When the transferred CD62Llow (activated) CD8 T cells were stained for annexin V, IL-21R−/− cells displayed a significant (p < 0.001) increase in annexin V positivity (43.8%) compared with WT cells (18.7%; Fig. 3C, 3D). These data suggest that...
the priming and survival of VV-specific CD8 T cells is dependent on IL-21 signaling.

Intrinsic IL-21 signaling is critical for the survival of activated CD8 T cells in vivo

Defective generation of VV-specific CD8 T cells in the absence of IL-21 signaling could be due to insufficient activation, proliferation, or effector differentiation, in addition to poor survival. To address these possibilities, naive WT or IL-21R−/− clone 4 HA-specific CD8 T cells (Thy1.2+) were transferred into congenic B10.D2 (Thy1.1+) mice that were subsequently infected with rVV-HA (5 × 10⁶ PFU, i.p.). Seven days later, splenocytes were harvested for analysis. A and B, Splenocytes were stained with anti-CD8, anti-Thy1.2, and anti–IFN-γ intracellularly. A, Percentages of IFN-γ–producing CD8+ T cells among each respective group are indicated. Ploids are gated on CD8+ T cells. B, The mean absolute numbers ± SD of IFN-γ–producing cells per spleen are indicated (n = 4/group). C and D, Splenocytes were stained with anti-CD8, anti-Thy1.2, anti-CD62L, and annexin V. C, Percentage of annexin V+ cells among transferred CD62Llow cells are indicated. Naive plot is gated on CD62Lhigh cells. WT and IL-21R−/− plots are gated on Thy1.2+CD8+CD62Llow cells. D, The mean absolute numbers ± SD of annexin V+CD62Llow cells among those transferred are indicated (n = 4/group). Data are representative of three independent experiments.

To address what contributes to the defective clonal expansion of IL-21R−/− clone 4 CD8 T cells into naive mice and subsequently infected the hosts with rVV-HA. Twenty-four hours postinfection, both WT and IL-21R−/− clone 4 CD8 T cells displayed a similarly activated CD44highCD69high phenotype compared with the naive CD44lowCD69low phenotype (Fig. 5A), suggesting that early activation of CD8 T cells is not affected by lack of IL-21 signaling. Three days postinfection, CFSE-labeled WT and IL-21R−/− clone 4 CD8 T cells underwent similar levels of proliferation as measured by CFSE dilution (Fig. 5B), suggesting that lack of IL-21 signaling does not affect CD8 T cell proliferation in vivo. Additionally, despite having a reduced clonal population, effector differentiation of IL-21R−/− clone 4 CD8 T cells is not affected, since the IFN-γ production on a per cell basis is not affected based on the mean fluorescence intensity (MFI) of staining (Fig. 4A). Similarly, the production of TNF-α appears to be similar in the absence of IL-21 signaling (Fig. 5C). The phenotype of effector CD8 T cells as measured by CD62L downregulation and CD122
factor differentiation of CD8 T cells in response to VV infection results suggest that although the activation, proliferation, and effector differentiation of CD8 T cells is critically dependent on intrinsic IL-21 signaling in vivo.

**CD4 T cell help for CD8 T cell survival is also critically dependent on IL-21 signaling in vivo**

We next determined whether CD4 T cell help for CD8 T cell survival was also dependent on IL-21 signaling in vivo. We used a model where either WT or IL-21R−/− clone 4 CD8 T cells were transferred into naive mice that had been treated with the CD4-depleting Ab GK1.5 four days prior, followed by reconstitution of HA-specific CD4 T cells from the 6.5 HA-TCR transgenic mice. Seven days after rVV-HA infection, lymphocytes were harvested for analysis. Consistent with our previous findings (13), clone 4 CD8 T cells primed in the absence of CD4 T cell help failed to efficiently expand (Fig. 6A, 6B). When CD4 T cells from the 6.5 HA-TCR transgenic mice were added, WT clone 4 CD8 T cells more efficiently expanded to form an effector pool; however, this CD4 help for expansion was not observed with IL-21R−/− clone 4 CD8 T cells. The addition of HA-specific CD4 T cells also led to an improved survival of WT, but not IL-21R−/−, clone 4 CD8 T cells (Fig. 6C). These results indicate that CD4 T cell help for CD8 T cell clonal expansion and survival to vaccinia infection in vivo is also critically dependent on IL-21 signaling.

**Intrinsic IL-21 signaling is required for the formation of memory CD8 T cells in vivo**

The observation that the activated IL-21R−/− CD8 T cells survived poorly prompted us to study their ability to develop into memory cells. Forty-two days postinfection, mice were harvested for analysis of memory cell formation in lymphoid and nonlymphoid organs. Consistent with our previous studies utilizing rVV-HA infection (13, 38–40), WT clone 4 effector CD8 T cells had undergone contraction to form a memory pool, whereas IL-21R−/− clone 4 effector CD8 T cells could not survive the contraction phase to develop into memory cells (Figs. 4, 7A, 7B). To ensure that the lack of memory formation from IL-21R−/− clone 4 T cells was not due to a low level below the threshold of detection in our system, we boosted mice at day 42 with Ad-HA to assess the recall response. We observed a robust recall expansion of WT clone 4 memory CD8 T cells (Fig. 7C, 7D). However, there were still no detectable IL-21R−/− clone 4 CD8 T cells (Fig. 7C, 7D). Collectively, our data indicate that intrinsic IL-21 signaling in CD8 T cells is required for the formation of long-lived memory cells in response to VV infection.

**IL-21 enhances the survival of activated CD8 T cells via the STAT1 and STAT3 pathways**

How does IL-21 promote CD8 T cell survival? It has been shown that IL-21 can activate the STAT1, STAT3, and STAT5 pathways (41, 42). Furthermore, previous studies in our laboratory have described a requirement for STAT1 signaling in the survival of activated CD8 T cells in vivo (39). To study whether these STAT pathways play a role in IL-21–mediated CD8 T cell survival, naive polyclonal CD8 T cells were cultured in the presence of recombinant IL-21 and then examined for the activation of STAT1, STAT3, and STAT5 by intracellular staining for pSTAT1, pSTAT3, and pSTAT5. Marked activation of both STAT1 and STAT3, but not STAT5, was observed in cultures supplemented with IL-21, compared with the medium only control (Fig. 8A). To further determine whether the STAT1 and STAT3 pathways were required for IL-21–dependent CD8 T cell survival, naive WT or STAT1−/− polyclonal CD8 T cells were retrovirally transduced with either empty vector or a STAT3 dominant-negative (STAT3-D) vector.

**The Journal of Immunology**

**FIGURE 4.** CD8 T cell clonal expansion in response to VV infection is compromised in the absence of IL-21 signaling. Purified naive clone 4 CD8 T cells (1 × 10^5) (Thy1.2+) from either WT or IL-21R−/− mice were adoptively transferred into congenic B10.D2 mice (Thy1.1+) that were subsequently infected with rVV-HA (5 × 10^5 PFU, i.p.). Seven days later, spleen and other lymphoid and nonlymphoid organs were harvested for analysis of expansion and function of clonotypic CD8 T cells. A, Splenocytes were stained with anti-CD8 and anti-Thy-1.2 and with anti-IFN-γ intracellularly. The percentage of total clonotypic CD8 T cells among total lymphocytes is indicated (left panels); the percentage of IFN-γ-producing clonotypic cells among total CD8 T cells is indicated, with the numbers in parentheses showing the MFI (×10^2) of IFN-γ–producing clonotypic CD8 T cells (right panels). B, The mean absolute numbers ± SD of clonotypic T cells per spleen, combined six PLNs, whole liver, or whole lung are indicated (n = 4/group). Data shown are representative of four independent experiments.

upregulation is also unchanged in IL-21R−/− clone 4 CD8 T cells as compared with WT (Fig. 5C).

We next sought to determine whether the observed reduction in clonal population size seen in IL-21R−/− CD8 T cells was due to enhanced apoptosis in the absence of IL-21 signaling. We transferred naive WT or IL-21R−/− clone 4 CD8 T cells into naive hosts that were subsequently infected with rVV-HA. Seven days later, splenocytes were stained with annexin V to detect cells undergoing apoptosis. Similar to our finding with the polyclonal CD8 T cells (Fig. 3C, 3D), IL-21R−/− clone 4 CD8 T cells had a significant increase in annexin V positivity (41.3%) compared with WT clone 4 cells (17.9%) (Fig. 5C). Taken together, these results suggest that although the activation, proliferation, and effector differentiation of CD8 T cells in response to VV infection are unaffected by a lack of IL-21 signaling, the survival of activated CD8 T cells is critically dependent on intrinsic IL-21 signaling in vivo.
and cultured in the presence of IL-21. We observed that CD8 T cell apoptosis was markedly reduced when WT cells with empty vector were cultured in the presence of IL-21, compared with WT cells with empty vector alone (33.4% from 81.4%) (Fig. 8B). However, this inhibition of apoptosis by IL-21 was abrogated in the absence of STAT1 (Fig. 8B), suggesting that STAT1 is essential for IL-21–dependent CD8 T cell survival. Additionally, WT cells transduced with STAT3-D also showed a reduction in IL-21–induced inhibition of apoptosis (Fig. 8B). The partial effect observed with STAT3-D could be a result of incomplete inhibition

**FIGURE 5.** IL-21 signaling is required for the survival, but not the activation or proliferation, of CD8 T cells. A and B, Purified naive clone 4 CD8 T cells (1 × 10^5) from WT or IL-21R^−/− mice were transferred to congenic recipients that were subsequently infected with rVV-HA (5 × 10^5 PFU, i.p.). Some mice were left uninfected (Naive). A, Twenty-four hours postinfection, splenocytes were harvested and stained with Abs to CD8, Thy1.2, and the activation markers CD44 or CD69. Percentages of CD44^high and CD69^high are indicated. B, Three days postinfection, in vivo division of CFSE-labeled clonotypic cells in the spleen was analyzed. C, Purified naive clone 4 CD8 T cells (1 × 10^5) from either WT or IL-21R^−/− mice were transferred into recipients that were subsequently infected with rVV-HA (5 × 10^5 PFU, i.p.). Seven days later, splenocytes were stained with anti-CD8 and anti-Thy1.2 and analyzed for the expression of surface markers and the production of the effector molecules. The percentages of TNF-α–producing CD62L^low, CD122^high, and annexin V^+ clonotypic CD8 T cells are indicated. All plots are gated on CD8^+Thy1.2^ cells. Data shown are representative of four independent experiments.

**FIGURE 6.** CD4 T help for CD8 T cell clonal expansion and survival in vivo is critically dependent on IL-21 signaling. Thy1.1^+ B10.D2 mice were treated with the CD4-depleting Ab, GK1.5, on day −4. On day 0, 1 × 10^4 naive clone 4 CD8 T cells (Thy1.2^+) from either WT or IL-21R^−/− mice were transferred into these mice (CD8). In some mice, 1 × 10^5 naive 6.5 CD4 T cells were also transferred (CD8 + CD4). After cell transfer, mice were subsequently infected with rVV-HA (5 × 10^5 PFU, i.p.). Seven days later, spleen and other lymphoid and nonlymphoid organs were harvested for analysis of expansion of clonotypic CD8 T cells. A, Splenocytes were stained with anti-CD8 and anti-Thy1.2. The percentage of total clonotypic CD8 T cells among total lymphocytes is indicated. B, The mean absolute numbers ± SD of clonotypic T cells per spleen, combined six PLNs, whole liver, or whole lung, are indicated (n = 3/group). C, Splenocytes were stained with anti-CD8 and annexin V. Percentage of annexin V^+ clonotypic CD8 T cells is indicated.
by the STAT3-D due to the level of expression, but nevertheless indicates that STAT3 also contributes to the survival of CD8 T cells by IL-21. We further observed that increased expression of the prosurvival factors Bcl-2 and Bcl-xL observed with addition of IL-21 to the culture was also reduced when STAT1 was abrogated, or STAT3 was inhibited, or both were suppressed (Fig. 8C). Expression of the proapoptotic factor Bim was unchanged in these conditions (data not shown). Collectively, these data suggest that IL-21–dependent enhancement of CD8 T cell survival is mediated by both the STAT1 and STAT3 pathways and subsequent upregulation of Bcl-2 and Bcl-xL.

**Discussion**

In this article, we demonstrate that CD4 T cell help for CD8 T cell response is mediated by IL-21 and that direct action of IL-21 on
CD8 T cells is critical for VV-specific CD8 T cell response in vivo. We further reveal that this cell-intrinsic IL-21 signaling is critical for the survival of activated CD8 T cells, and CD8 T cells deficient in IL-21 signaling fail to develop into long-lived memory cells in response to VV infection in vivo. We further demonstrate that IL-21 induces the prosurvival molecules Bcl-2 and Bcl-xL in a STAT1- and STAT3-dependent manner in vitro and that CD8 T cells defective for IL-21 signaling had reduced levels of STAT1 and STAT3 activation and Bcl-xL upregulation in response to VV infection in vivo.

Recent studies have revealed that IL-21 is a key component of CD4 T cell help that is required for maintaining the CD8 T cell response during chronic LCMV infections (24–26). This is achieved by direct action of IL-21 on virus-specific CD8 T cells to avoid deletion and, thus, sustain immunity. Interestingly, IL-21 signaling is not required for the CD8 T cell response to acute LCMV infections (24–26). This could be attributed to the observation that the primary CD8 T cell response during acute LCMV infection is independent of CD4 T cell help (9, 11). In a model of CD4 T cell help-dependent CD8 T cell response to acute VV infection, IL-21 activates the STAT1 and STAT3 signaling pathway, leading to the enhanced survival of CD8 T cells in vitro. A. Purified polyclonal CD8 cells were cultured for 1 h in medium alone (Media) or medium supplemented with recombinant murine IL-21 (+ IL-21). Cells were stained with anti-CD8 and anti-pSTAT1, anti-pSTAT3, or anti-pSTAT5 intracellularly. B and C. Purified polyclonal CD8 cells from WT or STAT1<sup>−/−</sup> mice were retrovirally infected with empty vector (Control) or STAT3-D vector (STAT3-D) and stimulated with soluble anti-CD3 and anti-CD28 in medium alone (Media) or medium supplemented with IL-21 (+ IL-21). B. Four days later, cells were stained with anti-CD8 and annexin V. Percentage of annexin V<sup>+</sup> CD8 cells is indicated. Plots are gated on GFP<sup>+</sup> cells. C. GFP<sup>+</sup> cells were sorted and mRNA was extracted and quantitative PCR was used to measure the expression of Bcl-2 and Bcl-xL mRNA abundance was normalized to β-actin. Data are representative of two independent experiments. *p ≤ 0.001.

FIGURE 9. IL-21 signaling promotes activation of the STAT1 and STAT3 pathways and upregulation of Bcl-xL in vivo. Purified naive clone 4 CD8 T cells (1 × 10<sup>5</sup>) from either WT or IL-21R<sup>−/−</sup> were adoptively transferred into congenic B10.D2 mice (Thy1.1<sup>+</sup>) that were subsequently infected with rVV-HA (5 × 10<sup>5</sup> PFU, i.p.) or left uninfected (Naive). A. Five days postinfection, splenocytes were stimulated with PMA and ionomycin and then stained with anti-CD8, anti-Thy1.2, as well as anti-pSTAT1, anti-pSTAT3, anti-STAT1, anti-STAT3, or an isotype control intracellularly. The percentage of pSTAT1<sup>+</sup> and pSTAT3<sup>+</sup> among clonotypic CD8<sup>+</sup> T cells is indicated. B. Seven days postinfection, splenocytes were stained with anti-CD8, anti-Thy1.2, and anti-Bcl-xL intracellularly. MFI of clonotypic CD8<sup>+</sup> T cells is indicated. Plots are gated on CD8<sup>+</sup>Thy1.2<sup>+</sup> cells. Data are representative of three independent experiments.
infection (13), in this study we show that IL-21 signaling is also essential for the primary CD8 T cell responses in vivo. Furthermore, we also provide direct evidence that CD4 T cell help for the CD8 T cell response is mediated IL-21. The mechanisms underlying differential requirements for IL-21 in the CD8 T cell response to different pathogens remain elusive. It might be that some acute infections could induce factors that may compensate for the loss of IL-21 signaling. Thus, future studies are needed to address this question.

A previous study has shown that IL-21 in combination with IL-7 or IL-15 augments the proliferation of resting CD8 T cells in vitro in the absence of TCR signals and promotes Ag-specific CD8 T cell expansion and their function in vivo in response to VV-encoding HIV gp160 Ag (21). Our data that direct IL-21 signaling on CD8 T cells is critical for the expansion of VV-specific CD8 T cells in vivo are in line with this observation. Furthermore, to our knowledge, our results with clonotypic HA-specific T cells demonstrate for the first time that the defective clonal expansion and function of Ag-specific CD8 T cells in the absence of IL-21 signaling is due to poor survival, but not initial activation, proliferation, or effector differentiation, of the activated Ag-specific CD8 T cells. Although IL-21 promotes resting CD8 T cell proliferation in vitro in the absence of TCR signals, CD8 T cell proliferation in vivo in response to VV infection is not affected by lack of IL-21 signaling, which could be due to TCR ligation and CD28 costimulation also inducing CD8 T cell proliferation in vivo.

The signals that promote the generation and maintenance of the memory T cell population remain incompletely defined. Studies have shown that the nature and the strength of TCR signals (38, 43, 44), the costimulation (45), as well as the inflammatory milieu (46) can influence the formation of memory T cells. Additionally, the common γ-chain cytokines IL-7 and IL-15 play an important role in the maintenance of memory CD8 T cells by promoting homeostatic expansion (47, 48). Furthermore, CD4 T cell help is critical for the generation of long-lived, functional memory CD8 T cells (10–12). In this study, we provide evidence that CD4 help is mediated by another common γ-chain cytokine, IL-21, and that CD8 T cells defective for IL-21 signaling could not survive the contraction phase to differentiate into long-lived memory cells in response to VV infection in vivo. The observation that no memory CD8 T cells are detected in the absence of IL-21 signaling is somewhat different from our previous finding in CD4−/− mice that CD8 memory pool is detectable, albeit significantly reduced (13). This difference could be due to low levels of IL-21 produced by non-CD4 T cells such as NK T cells in CD4−/− mice (17).

How does IL-21 signaling in CD8 T cells promote their survival? Previous studies have shown that IL-21 can activate the STAT1, STAT3, and STAT5 pathways (41, 42). In CD8 T cells, IL-21 signaling can preferentially activate the STAT1 and STAT3 pathways, leading to enhanced T cell proliferation in combination with IL-15 in vitro in the absence of TCR signals (42). Additionally, it has been shown that STAT1 signaling in CD8 T cells is required for CD8 T cell survival and memory formation in vivo (39). In this study, we show that IL-21 signaling activates both STAT1 and STAT3 pathways in vitro and in vivo, and that IL-21 enhances the survival of anti–CD3-activated CD8 T cells in a STAT1- and STAT3-dependent manner. We further demonstrate that IL-21 signaling upregulates the expression of the prosurvival molecules Bcl-2 and Bcl-xL in activated CD8 T cells, which is mediated by both STAT1 and STAT3. Collectively, our data support a model that IL-21 signaling promotes the survival of activated CD8 T cells by inducing prosurvival molecules, such as Bcl-2 and Bcl-xL, in a STAT1- and STAT3-dependent fashion. Indeed, it has been shown in other cell types that activation of STAT1 or STAT3 can upregulate Bcl-2 and Bcl-xL, leading to cell survival (49–51).

Downregulation of TRAIL expression by CD4 T cell help has been shown to protect memory CD8 T cells from activation-induced cell death during a recall expansion (52). A more recent study has suggested that CD4 T cell help consists of both TRAIL-dependent and -independent mechanisms (53). Indeed, we have previously shown that in the absence of CD4 T cell help, TRAIL expression is upregulated in the activated CD8 T cells, whereas Bcl-xL expression is downregulated (13), suggesting that both the TRAIL pathway and the intrinsic apoptotic pathway may be involved in promoting the survival of activated CD8 T cells. Thus, we cannot rule out the possibility that IL-21-STAT1/STAT3 signaling may also result in downregulation of TRAIL, leading to reduced apoptosis of activated CD8 T cells. Thus, future studies will be needed to define whether TRAIL is involved in IL-21-dependent CD8 T cell survival.

In conclusion, we have shown that CD4 T cell help for CD8 T cell response to VV infection is mediated by IL-21. This is achieved by direct action of IL-21 on CD8 T cells to promote their survival via a mechanism dependent on activation of the STAT1 and STAT3 pathways and subsequent upregulation of Bcl-2 and Bcl-xL. Furthermore, effector CD8 T cells do not survive the contraction phase to differentiate into long-lived memory cells in the absence of intrinsic IL-21 signaling. These results identify a critical role for direct IL-21 signaling in CD8 T cell survival and memory formation following an acute viral infection in vivo and may have important implications for the design of effective strategies for treating infectious diseases and cancer.

Disclosures
W.J.L. is an inventor on patents and patent applications related to the present invention. The other authors have no financial conflicts of interest.

References
IL-21 IN CD8 T CELL SURVIVAL AND MEMORY FORMATION


