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

Patricia Novy,* Xiaopei Huang, † Warren J. Leonard, ‡ and Yiping Yang*,†

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 NK 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 (14, 18) and promote terminal B cell differentiation into plasma cells, which is critical for Aβ 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 between 6 and 8 wk of age. All experimental procedures involving the use of HA-TCR mice used in experiments. All mice used for experiments were purchased from The Jackson Laboratory and backcrossed onto the B10.D2 background. We intercrossed clone 4 BOEHMER (Harvard University, Boston, MA) (28). These transgenic strains were backcrossed onto the B10.D2 background for nine generations. The clone 4 HA-TCR transgenic mice that express a TCR recognizing a K d-restricted HA epitope (110SFERFEIFPKE120) were provided by Dr. H. von Boehmer (Harvard University, Boston, MA) (28). These transgenic strains were backcrossed onto the B10.D2 background. We intercrossed clone 4 HA-TCR mice with IL-21R 1/2 mice to generate the IL-21R 2/2 clone 4 HA-TCR mice. These mice were backcrossed for experiment between 6 and 8 wk of age. All experimental procedures involving the use of mice were done in accordance with protocols approved by the Animal Care and Use Committee of the Duke University Medical Center.

Materials and Methods

Mice

B10.D2, Thy1.1, B10.D2 mice were purchased from The Jackson Laboratory. CD8-deficient (CD8 1/2) mice were purchased from Jackson Laboratory. STATA 1/2 mice on the C57/B6 background were purchased from Jackson Laboratory and backcrossed onto the B10.D2 genetic background for nine generations. 129/Sv mice were obtained from Charles River Laboratories. STATA 1/2 mice on the C57/B6 background were purchased from Jackson Laboratory and backcrossed onto the B10.D2 genetic background for nine generations. The clone 4 HA-TCR transgenic mice that express a TCR recognizing a K d-restricted HA epitope (110SFERFEIFPKE120) were provided by Dr. L. Sherman (The Scripps Research Institute, La Jolla, CA) (27). The 6.5 TCR-HA transgenic mice that express a TCR recognizing an I E-restricted HA epitope (110SFERFEIFPKE120) were provided by Dr. H. von Boehmer (Harvard University, Boston, MA) (28). These transgenic strains were backcrossed onto the B10.D2 background. We intercrossed clone 4 HA-TCR mice with IL-21R 1/2 mice to generate the IL-21R 2/2 clone 4 HA-TCR mice. These mice were backcrossed for experiment between 6 and 8 wk of age. All experimental procedures involving the use of mice were done in accordance with protocols approved by the Animal Care and Use Committee of the Duke University Medical Center.

Immunizations and Ab treatment

rVV encoding HA (rVV-HA) and recombinant E1-deleted adenovirus encoding Ad (Ad-HA) were previously described (29). rVV-HA was grown in TK-143B cells, purified by sucrose banding, and titered by plaque-forming assay on TK-143B cells. Mice were infected with 1 10 PFU i.v. or 5 10 or 5 10 PFU i.p. Ad-HA was grown in 293 cells (American Type Culture Collection), purified by two rounds of CsCl density centrifugation and desalted by gel filtration through a Sephadex G-25 column (PD-10 column; Amersham Biosciences). The titer was determined by plaque-forming assay on 293 cells. Mice were infected with 2 10 PFU i.p. In vivo CD4 T cell depletion was performed by i.p. injection of the anti-CD4 mAb, GK1.5 (150 g), 4 d before rVV-HA infection as described (5).

Abs and flow cytometry

mAbs (all from BD Biosciences unless indicated) used for staining were PE-Cy5-conjugated anti-CD8; FITC-conjugated anti-B220, -CD8, -CD4, -CD69, -CD62L, -CD122, -IFN-g, -TNF-a, -Thy1.2, -pSTAT1, -pSTAT3, and -pSTAT5; PE-conjugated anti-Thy1.2, -CD11c, -Bcl-xL (Santa Cruz Biotechnology), -mouse IgG (SouthernBiotech), and annexin V; biotin-conjugated anti-CD11c, and FITC-conjugated anti-B220 and subjected to flow cytometry analysis. For ex vivo detection of pSTAT1 and pSTAT3, splenocytes were stimulated for 4 h in 200 ml CML medium (RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin, and 50 mM 2-ME) at a concentration of 10 6 cells/ml in the presence of 50 mg/ml PMA and 100 mg/ml ionomycin before fixation. To assess production of IFN-g, supernatants were cultured in 200 ml CML medium in the presence of 5 mg/ml brefeldin A and either 2 mg/ml L- restricted VV F2L 26–34 peptide or the K d-restricted HA518–526 peptide for 6 h at 37˚C. After incubation, cells were washed and stained with anti-CD8 and anti-Thy1.2. Cells were then permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer’s instructions and subsequently stained with anti-IFN-g or anti-TNF-a.

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 naïve 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 survival is mediated by a soluble factor in vitro. 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 left uninfected (Naive). Twenty-four hours later, CD8+CD11c+B220− DCs were purified by FACS and cultured with purified naive clone 4 HA-specific CD8 T cells for 4 d. A, Division of CFSE-labeled clonotypic CD8 T cells. B, Annexin V staining. Percentage of annexin V+ clonotypic CD8 T cells is indicated. C and D, Polyclonal CD8 T cells were stimulated with soluble anti-CD3 and anti-CD28 or left unstimulated for 4 d. In some wells, polyclonal CD4 T cells were added by either mixing with the CD8 T cells or placing in a transwell support. C, Division of CFSE-labeled CD8 T cells. D, Annexin V staining. Percentage of annexin V+ CD8 cells is indicated. All plots are gated on CD8+ T cells. Data are representative of three independent experiments.

We next determined what soluble factor from CD4 T cells enhances 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−/− mice with the clone 4 HA-TCR 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 IEd-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 (<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 mice (Thy1.1+) that were subsequently infected with rVV-HA (5 × 10^6 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. Plots are gated on CD8+ T cells. B, The 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 CD62L+ cells are indicated. Naïve plot is gated on CD62L+ cells. WT and IL-21R−/− plots are gated on Thy1.2+CD8+CD62L+ cells. D, The mean absolute numbers ± SD of annexin V+CD62L+ 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, we transferred naive WT or 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 CD44^highCD69^high phenotype compared with the naïve CD44^lowCD69^low 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

**Figure 2.** CD4 T cell help for the survival of activated CD8 T cells in vitro is mediated by IL-21. A, Polyclonal CD4 T cells were stimulated with soluble anti-CD3 and anti-CD28 or left unstimulated. Thirty-six hours later, RNA was extracted and real-time quantitative PCR was used to measure the expression of IL-2-7, IL-15, and IL-21. mRNA abundance was normalized to β-actin. Data are presented as the fold increase of the corresponding cytokine mRNA relative to that of unstimulated cells. B, Polyclonal WT or IL-21R−/− CD8 T cells were stimulated with soluble anti-CD3 and anti-CD28 or left unstimulated as a control for 4 d. Recombinant murine IL-21 was added where indicated. Cells were stained with anti-CD8 and annexin V. Percentages of annexin V+CD8+ T cells are indicated. C, CD8+CD11c+B220+ DCs were purified from uninfected (Naive) and rVV-HA–infected (rVV-HA) spleens and cultured for 4 d with purified naive clone 4 HA-specific CD8 T cells from WT or IL-21R−/− clone 4 HA-TCR mice. Purified naive 6.5 HA-specific CD4 T cells were transferred into congenic B10.D2 mice (Thy1.1+) that were subsequently infected with rVV-HA. Seven days later, splenocytes were analyzed for clonal expansion of CD8 T cells in vivo. Purified polyclonal naive CD8 T cells (1 × 10^7) (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 × 10^6 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. Plots are gated on CD8+ T cells. B, The 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 CD62L+ cells are indicated. Naïve plot is gated on CD62L+ cells. WT and IL-21R−/− plots are gated on Thy1.2+CD8+CD62L+ cells. D, The mean absolute numbers ± SD of annexin V+CD62L+ cells among those transferred are indicated (n = 4/group). Data are representative of three independent experiments.

**Figure 3.** Intrinsic IL-21 signaling is required for the priming of VV-specific CD8 T cells in vivo. Purified polyclonal naive CD8 T cells (1 × 10^7) (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 × 10^6 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. Plots are gated on CD8+ T cells. B, The 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 CD62L+ cells are indicated. Naïve plot is gated on CD62L+ cells. WT and IL-21R−/− plots are gated on Thy1.2+CD8+CD62L+ cells. D, The mean absolute numbers ± SD of annexin V+CD62L+ cells among those transferred are indicated (n = 4/group). Data are representative of three independent experiments.
four independent experiments.

The clonal population size seen in IL-21R−/− as compared with WT (Fig. 5B). Taken together, 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 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.

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

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 × 10⁶) from WT or IL-21R⁻/⁻ mice were transferred to congenic recipients that were subsequently infected with rVV-HA (5 × 10⁵ 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 CD4₄high and CD6₉high 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 × 10⁶) from either WT or IL-21R⁻/⁻ mice were transferred into recipients that were subsequently infected with rVV-HA (5 × 10⁵ 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 CD6₂Lₗow, CD₁₂₂ₗ₉₁, 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.
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.

IL-21 signaling on CD8 T cells promotes activation of STAT1 and STAT3 and induction of Bcl-xL in response to VV infection in vivo

We next examined whether IL-21 signaling on CD8 T cells promotes activation of STAT1 and STAT3 and expression of Bcl-xL upon VV infection in vivo. Naive WT or IL-21R−/− clone 4 CD8 T cells were transferred into congenic B10.D2 mice that were subsequently infected with rVV-HA. Five days postinfection, splenocytes were harvested and stained intracellularly for pSTAT1 and pSTAT3. Significant activation of both STAT1 and STAT3 was observed in WT clone 4 CD8 T cells upon VV infection in vivo, compared with the naive control (Fig. 9A). However, the extent of STAT1 and STAT3 activation was reduced in IL-21R−/− clone 4 T cells, compared with their WT counterparts (Fig. 9A). This reduction was not due to a decreased level of total STAT1 or STAT3 in IL-21R−/− cells (Fig. 9A). Similar to our in vitro results, this decrease in pSTAT1 and pSTAT3 activity was correlated with a decrease in the Bcl-xL level as measured by MFI in IL-21R−/− clone 4 T cells (Fig. 9B). These results indicate that intrinsic IL-21 signaling in CD8 T cells is required for efficient activation of STAT1 and STAT3 as well as upregulation of Bcl-xL upon VV infection in vivo.

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-x\textsubscript{L} 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-x\textsubscript{L} 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

**FIGURE 8.** 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\textsuperscript{-/-} 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\textsuperscript{+} CD8 cells is indicated. Plots are gated on GFP\textsuperscript{+} cells. C, GFP\textsuperscript{+} cells were sorted and mRNA was extracted and quantitative PCR was used to measure the expression of Bcl-2 and Bcl-x\textsubscript{L}. mRNA abundance was normalized to \textbeta-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-x\textsubscript{L} in vivo. Purified naive clone 4 CD8 T cells (1 × 10\textsuperscript{5}) (Thy1.2\textsuperscript{+}) from either WT or IL-21R\textsuperscript{2/-} were adoptively transferred into congenic B10.D2 mice (Thy1.1\textsuperscript{+}) that were subsequently infected with rVV-HA (5 × 10\textsuperscript{5} 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\textsuperscript{+} and pSTAT3\textsuperscript{+} among clonotypic CD8 T cells is indicated. B, Seven days postinfection, splenocytes were stained with anti-CD8, anti-Thy1.2, and anti-Bcl-x\textsubscript{L} intracellularly. MFI of clonotypic CD8 T cells is indicated. Plots are gated on CD8\textsuperscript{+}Thy1.2\textsuperscript{+} 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 evident 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 develop 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 NKT 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 IL-21. The other authors have no financial conflicts of interest.
IL-21 IN CD8 T CELL SURVIVAL AND MEMORY FORMATION


