Th Cells Act Via Two Synergistic Pathways To Promote Antiviral CD8⁺ T Cell Responses

Melanie Wiesel, Nicole Joller, Anna-Katharina Ehlert, Josh Crouse, Roman Spörri, Martin F. Bachmann and Annette Oxenius

*J Immunol* 2010; 185:5188-5197; Prepublished online 29 September 2010;
doi: 10.4049/jimmunol.1001990
http://www.jimmunol.org/content/185/9/5188
Th Cells Act Via Two Synergistic Pathways To Promote Antiviral CD8⁺ T Cell Responses

Melanie Wiesel,* Nicole Joller,* Anna-Katharina Ehlert,* Josh Crouse,* Roman Spörri,* Martin F. Bachmann,† and Annette Oxenius*

The mechanisms of how Th cells promote CD8⁺ T cell responses during viral infections are largely unknown. In this study, we unraveled the mechanisms of T cell help for CD8⁺ T cell responses during vaccinia virus infection. Our results demonstrate that Th cells promote vaccinia virus-specific CD8⁺ T cell responses via two interconnected synergistic pathways: First, CD40L expressed by activated CD4⁺ T cells instructs dendritic cells to produce bioactive IL-12p70, which is directly sensed by Ag-specific CD8⁺ T cells, resulting in increased IL-2Rα expression. Second, Th cells provide CD8⁺ T cells with IL-2, thereby enhancing their survival. Thus, Th cells are at the center of an important communication loop with a central role for IL-2/IL-2R and bioactive IL-12. The Journal of Immunology, 2010, 185: 5188–5197.

It is widely accepted that certain CD8⁺ T cell responses critically depend on the presence of CD4⁺ T cell help, in particular under noninflammatory conditions (e.g., minor Ags, dendritic cell immunization, or protein immunization). In these situations, CD8⁺ T cells contribute essentially to the activation and maturation of professional APCs predominately via CD40–CD40L interaction, thereby “licensing” the APCs to become potent inducers of CD8⁺ T cell responses (1–3). In contrast, many pathogenic infections, such as influenza, lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus, or other pathogens, induce strong primary CD8⁺ T cell responses in the absence of CD4⁺ T cell help (4–6). The ability of these infectious agents to directly activate APCs via pattern recognition receptors is thought to circumvent the need for CD4⁺ T cell help (7, 8). However, there is growing evidence that even in the setting of infections, CD8⁺ T cells play a crucial role in shaping the CD8⁺ T cell response: Although priming of CD8⁺ T cells occurs in many instances normally in the absence of CD4⁺ T cell help, memory CD8⁺ T cell differentiation, maintenance, and the ability to undergo secondary expansions critically depend on the presence of CD4⁺ T cell help in many (but not all) immunization protocols or infection models (9–11).

There are a number of mechanisms by which CD4⁺ T cells can mediate help for CD8⁺ T cell responses. These include 1) direct cell–cell interactions predominately between CD4⁺ T cells and APCs thereby licensing the APC to become more potent in activating CD8⁺ T cells; 2) direct interactions between CD4⁺ T cells and CD8⁺ T cells either by interaction of membrane molecules, such as CD40–CD40L (12), or via soluble mediators, such as IL-2 (13, 14) or IL-15 (15–17); and 3) indirect interactions via the Th cell-assisted production of isotype-switched Abs, which contribute to control of an infection and hence favor the development of fully functional effector and memory CD8⁺ T cells.

Collectively, these studies indicate that besides the requirement of T cell help, the mechanism of T cell help clearly differs between different infections or immunizations. Moreover, reports describing attempts to replace T cell help (4, 18) suggest that CD4⁺ T cell help for CD8⁺ T cell responses might be more complex than initially believed. As T cell help seems to influence various aspects of CD8⁺ T cell responses, there is a general need to identify and characterize the molecular mechanisms by which this help is conferred in different experimental settings.

In this work we studied the CD8⁺ T cell response to vaccinia virus (VV) infection in vivo and conducted a comprehensive analysis to obtain detailed knowledge about the relevant mechanisms of how Th cells support CD8⁺ T cell responses in this particular infection. Our results demonstrate that Th cells promote VV-specific CD8⁺ T cell responses via two interconnected synergistic pathways: First, CD40L expressed by activated CD4⁺ T cells instructs dendritic cells (DCs) to produce biologically active IL-12p70, which acts directly on Ag-specific CD8⁺ T cells to increase their expression of CD25, thereby rendering them more responsive to IL-2. Second, activated Th cells provide Ag-specific CD8⁺ T cells with IL-2, thereby enhancing their survival. Exogenous provision of this communication loop by agonistic CD40 Abs in combination with IL-2 completely substituted for T cell help. Thus, CD4⁺ T cell help for CD8⁺ T cells is mediated in vivo via two separate pathways, both acting in concert.

Materials and Methods

Mice

The following mouse strains were kept and bred in a specific pathogen-free facility at the Institute for Microbiology, ETH Zürich: C57BL/6 (wild type; WT), MHCII−/− (19), CD4⁺−/− (20), CD40−/− (21), CD40L−/− (22), CD25−/− (23), and IL-2−/− (24). P14 transgenic mice expressing a TCR specific for LCMV peptide gp33–41 were described previously (25). P14 mice were maintained in a specific pathogen-free facility at The Journal of Immunology, 2010, 185: 5188–5197.
mice were crossed with IL-12Rβ2−/− mice (26) to yield IL-12Rβ2-deficient P14 cells. All animals were used at 6–12 wk of age.

Mixed bone marrow chimeras were generated as previously described (27).

Animal experiments were conducted in accordance with protocols approved by the Cantonal Veterinary Office (Zürich, Switzerland).

**Viruses and peptides**

Recombinant VV expressing the LCMV glycoprotein (VVG2) was originally obtained from Dr. D.H.L. Bishop (Oxford University, Oxford, U.K.) and was grown on BSC40 cells at low multiplicity of infection; quantification was performed as previously described (28).

The LCMV isolate WE was provided by Dr. R.M. Zinkernagel (University Hospital, Zürich, Switzerland) and was propagated at a low multiplicity of infection on L929 fibroblast cells.

LCMV-derived peptides (pDB14, KAVYNFATM, H-2Db) and VV-derived peptide BBR20.27 (TSYKFESV, H-2Kk) were purchased from NeoMPS (Strasbourg, France).

**Infections, immunizations, and Ab treatment**

Mice were infected i.p. with 5 × 10^6 PFU VVG2 or i.p. with 1 × 10^6 PFU LCMV.

When indicated, mice were cooinjected i.p. daily with a mix of 10 μg anti-IL-2 (clone S4B6) and 0.3 μg recombinant mouse IL-2 (BD Biosciences, Basel, Switzerland) in combination with 50 μg agonistic anti-CD40 mAb (clone FGGK4/5) 6 h and 24 h postinfection.

Mice were in vivo depleted of CD4+ T cells by injecting i.p. 0.2 mg purified anti-mouse CD4 YTS 191.1 mAb (29) at days 3 and 1 prior to infection.

**Adoptive transfer**

For adoptive transfer experiments, 10^6 purified CD8+ T cells isolated from naive Ly5.1+ P14 mice were adoptively transferred into naive recipient mice. To study the functionality of memory P14 cells, lymphocytes were isolated 36 d after VV infection from the spleen of WT or CD4−/− mice. CD8+ T cells were purified by MACS according to the instructions of the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany) and pooled within the group. Prior to transfer, the frequency of memory Ly5.1+ P14 cells was determined and adjusted to 2.5 × 10^5 per recipient mouse.

**Abs and peptide-MHC class I tetramers**

APC-conjugated peptide–MHC class I tetrameric complexes were generated as previously described (30). All Abs used were purchased from Biolegend (LuenenChem, Luzern, Switzerland); except annexin V and anti-phospho-AKT (pS475) purchased from BD Biosciences (Basel, Switzerland), anti-BCL-XL from SouthernBiotec (Birmingham, AL), and anti-IL-15Rα from R&D Systems (Abingdon, U.K.).

**CFSE labeling**

Cells were washed two times with PBS, followed by incubation with 2 μM CFSE (Invitrogen, Basel, Switzerland) for 5 min at 37°C, quenching with 20% FCS, and washing with RPMI 1640 containing 10% FCS.

**Immunofluorescence staining and analysis**

For staining, surface whole blood or single-cell suspensions from spleens were used: surface and intracellular cytokine stainings were performed as described previously (31). For intracellular BCL-XL staining, cells were permeabilized using Fix/Perm solution (BD Biosciences, Basel, Switzerland). Anti-phospho-AKT and annexin V staining was performed according to the instructions of the manufacturer (BD Biosciences, Basel, Switzerland). Data were recorded using a LSRII flow cytometer (BD Biosciences, Basel, Switzerland) and analyzed using FlowJo software (Tree Star, San Carlos, CA).

**In vitro stimulation of CD8+ T cells**

Purified CD8+ T cells (1 × 10^5) were stimulated with plate-bound anti-CD3 (10 μg/ml) and anti-CD28 (10 μg/ml) (both BD Biosciences, Basel, Switzerland) in RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, 50 μM 2-ME, and cocultured with recombinant mouse IL-12 (100 ng/ml; Biosource, Camarillo, CA), IFN-α (10^4 U/ml; PBL InterferonSource, Piscataway, NJ), and IL-15 (100 ng/ml; R&D Systems, Abingdon, U.K.) as indicated.

**Real-time quantitative polymerase chain reaction**

Total RNA was isolated from MACS-purified splenic DCs using TRIzol reagent (Invitrogen, Basel, Switzerland), and cDNA was generated using M-MLV Reverse Transcriptase RNa, H Minus (Promega, Dübendorf, Switzerland). Real-time PCR was performed using a Rotorgene 3000 (Corbett Research, Eight Miles Plains, Australia) to measure SYBR green (Sensi Mix, R&D Systems) incorporation. The following primer sets were used: IL-12p35: 5'-AGACTT-GGGCGAGGCTC3'-3', 5'-TTTTTG-GGCGCAGGCTC3'-3'; IL-12p40: 5'-GGAACAGCCGCAAGCAGAAT-3', 5'-AACCTTGAAGGAAAG TAGAGATGG-3'; TRAIL: 5'-GACAGAGCTAGAACAG3'-3', 5'-GGCCCTAGGCTTTCATCAC-3'; β-actin: 5'-CCCTGAAGTACCCCATTGAAC-3', 5'-CTTTCCTACGGTGCGCCTT-3'. The amount of mRNA was normalized to β-actin RNA levels for each sample. The fold induction was calculated according to the relative expression in naive mice.

**Statistical analysis**

Statistical significance was determined by a two-tailed unpaired t test using GraphPad Prism (GraphPad Software, La Jolla, CA).

**Supplementary methods**

Mice. Ox-40−/− mice were kindly provided by Dr. Manfred Kopf (ETH Zürich, Zürich, Switzerland).

**Induction of IL-15 Abs.** Mouse IL-15 was covalently conjugated to Qβ-virus-like particles (VLPs) (Qβ-IL-15) by a two-step procedure, as recently described (32).

Mice were immunized three times at 14-d intervals with Qβ-IL-15 (50 μg) or Qβ-VLP alone (Qβ-control) (50 μg) diluted in PBS to 200 μl by s.c. injection in the absence of adjuvants.

ELISA. Sera from immunized mice were collected 50 d after the first immunization and serially diluted in PBS containing 0.05% Tween 20 and 2% BSA and applied to ELISA plates (Nunc, Langenselbold, Germany) coated with 2 μg/ml recombinant mouse IL-15 (R&D Systems) or 2 μg/ml Qβ protein. Reactivity of serum Ab with the target protein was determined using HRP-conjugated goat anti-mouse IgG secondary Ab (Jackson ImmunoResearch Laboratories, Suffolk, U.K.) 1:1000 diluted in PBS, 0.05% Tween 20, 2% BSA. After development with 1,2-phenylenediamine dihydrochloride (0.4 mg/ml in 0.066 M Na2HPO4, 0.035 M citric acid, 0.01% H2O2 [pH 5]), OD450 was determined using an ELISA reader (Bio-Rad, Biel, Switzerland). Titers were expressed as the serum dilution that leads to half-maximal OD450 (OD50%).

**Results**

**Th cells are critically involved in vaccinia virus-specific CD8+ T cell priming**

The requirement for CD4+ T cell help and the mechanisms by which CD4+ T cells affect primary and secondary CD8+ T cell responses differs between various pathogens. In this study, we investigated whether T cell help is important for CD8+ T cell priming upon VV infection as well as for the maintenance and functionality of memory CD8+ T cells. To this end, a low number of Ly5.1+ TCR transgenic CD8+ T cells with specificity for the LCMV gp33–41 epitope (P14 cells) were transferred into WT (Ly5.2+) or CD4−/− (Ly5.2−) mice, which were subsequently infected with a recombinant VV expressing the LCMV glycoprotein (VVG2). Of note, we carefully titrated the number of P14 cells and assessed the T cell help dependence of the response after VVG2 infection, as it was demonstrated that the precursor frequency of CD8+ T cells may overcame the requirement of CD4+ T cell help (data not shown and Ref. 33). Using adoptive transfer of 10^5 P14 cells resulted in comparable T cell help dependence as observed for endogenous CD8+ T cells. Notably, the peak response at day 6 postinfection (p.i.) was significantly reduced in frequency and total number in CD4−/− mice (helpless) compared with that in WT mice (helped; Fig. 1A, 1B), but the relative maintenance of helpless memory P14 cells was comparable with that of helped memory P14 cells. On a functional level, equivalent ratios of helped and helpless P14 cells were capable of IFN-γ production upon restimulation (Fig. 1C). This was also true for endogenous CD8+ T cells with specificity for...
the gp33 and the immunodominant VV epitope B8R (34): endogenous gp33- or B8R-specific CD8+ T cell response were 3-fold reduced in the absence of T cell help (gp33: 1.0% in WT versus 0.4% in CD4-/- mice; B8R: 1.9% in WT versus 0.7% in CD4-/- mice). Of note, VV titers in the ovaries day 6 p.i. were comparable in WT and CD4-/- mice (data not shown), indicating that reduced frequencies of VV-specific CD8+ T cells in the absence of T cell help were not due to differences in Ag load.

Next we addressed the question of whether helpless memory CD8+ T cells are competent to undergo secondary expansion. We transferred equal numbers of helped and helpless memory P14 CD8+ T cells into naive WT mice, which were subsequently challenged i.p. with 10^5 PFU LCMV-WE. Six days after challenge, the functionality of the transferred helped versus helpless P14 cells was assessed by restimulation with gp33 peptide, followed by intracellular cytokine staining for IFN-γ. Plots are gated on CD8+ T cells; numbers indicate the frequency of P14 cells among CD8+ T cells (upper row) and the percentage of IFN-γ–producing P14 versus endogenous CD8+ T cells (lower row). E, Total numbers of memory P14 cells in the spleen at day 6 after LCMV challenge are shown (n = 3). Data are representative of two independent experiments.

FIGURE 1. Th cells are critically involved in VV-specific CD8+ T cell priming. A, WT (circles) and CD4-/- (squares) mice were adoptively transferred with 10^5 Ly5.1+ P14 cells and infected with VVG2. Frequency of P14 cells among CD8+ T cells was assessed in blood. Error bars indicate the SD within a group (n = 3). B and C, Six days after infection, total number of P14 cells was determined in the spleen. Splenocytes were stimulated in vitro with gp33- or B8R-peptide (n = 4). Plots are gated on CD8+ T cells; frequencies of P14 cells are indicated in the upper row on the left side. The percentage among lymphocytes of IFN-γ–producing endogenous (Ly5.1+) and transferred P14 cells (Ly5.1+) specific for either gp33 (middle row) or B8R (lower row) is shown; in brackets, the frequency of IFN-γ–producing cells among P14 cells is indicated. D and E, Memory P14 cells generated upon VVG2 infection either in the presence (helped P14) or absence (helpless P14) of T cell help were isolated at day 36. Equal amounts of memory P14 cells (2.5 × 10^5) were transferred into naïve WT mice, which were subsequently challenged i.p. with 10^5 PFU LCMV-WE. Six days after challenge, the functionality of the transferred helped versus helpless P14 cells was assessed by restimulation with gp33 peptide, followed by intracellular cytokine staining for IFN-γ. Plots are gated on CD8+ T cells; numbers indicate the frequency of P14 cells among CD8+ T cells (upper row) and the percentage of IFN-γ–producing P14 versus endogenous CD8+ T cells (lower row). E, Total numbers of memory P14 cells in the spleen at day 6 after LCMV challenge are shown (n = 3). Data are representative of two independent experiments.

F–H, P14 cells (10^6) were transferred into WT and CD4-/- mice prior to VVG2 infection. F, Three days after infection, in vivo division of CFSE-labeled P14 cells in the spleen was analyzed in WT (upper row) and CD4-/- (lower row) mice; uninfected controls are shown in gray. G, Seven days after infection, P14 cells from WT (upper row) and CD4-/- (lower row) mice were harvested and stained with annexin V and for BCL-XL. Histograms are gated on P14 cells from infected mice (solid black line) compared with naive WT controls (filled gray area). The geometric MFI of P14 cells is indicated. Seven days after VVG2 infection, P14 cells were FACS sorted from the spleen, and the relative expression of TRAIL mRNA (relative to β-actin) was determined by quantitative PCR (n = 3). Error bars indicate the SD within a group. Each analysis was performed at least twice, and one representative result is shown. n.s., not significant. ***p < 0.001.
over, both helped and helpless memory CD8\(^+\) T cell subsets were able to clear LCMV by day 7 postchallenge (data not shown). Overall, we could not detect any differences in the functionality of memory CD8\(^+\) T cells generated in the presence or absence of T cell help.

The comparatively poor overall expansion of gp33- and B8R-specific CD8\(^+\) T cells in CD4\(^{-/-}\) mice could result from either reduced proliferation or impaired survival. To distinguish between these possibilities, we transferred CFSE-labeled P14 cells into WT and CD4\(^{-/-}\) mice and infected them with VV-G2. As indicated in Fig. 1F, the CFSE dilution profile was equivalent in the presence or absence of T cell help. We therefore speculated that T cell help is required for the survival of activated CD8\(^+\) T cells rather than for their activation and proliferation. To test this hypothesis, we measured apoptosis in CD8\(^+\) T cell by annexin V staining and analyzed the expression of the prosurvival molecule BCL-XL as well as the apoptosis-related molecule TRAIL. Help-facilitates the survival of activated CD8\(^+\) T cells, we investigated the CD40–CD40L axis is required for VV-specific CD8\(^+\) T cell priming, we transferred P14 cells into CD40\(^{-/-}\) and CD40L\(^{-/-}\) as well as WT and CD4\(^{-/-}\) mice as controls and infected them with VVG2. At day 6 p.i., the frequency and total number of P14 cells was significantly reduced (more than 3-fold) in mice lacking either T cell help or the CD40–CD40L interaction compared with that in WT mice (Fig. 2A, 2B). These results demonstrate that the CD40–CD40L pathway does play an important role in CD8\(^+\) T cell priming upon VV infection. Moreover, the results indicate that CD40 expression on CD8\(^+\) T cells (12) is not relevant in this setting as the transferred P14 cells were CD40 sufficient.

To identify the cell type required to express CD40L in VV infection, considering that a recent report indicated that DCs are capable of expressing CD40L upon activation (36), we generated mixed bone marrow (BM) chimeras, harboring CD4\(^+\) T cells capable (control chimeras) or incapable (test chimeras) of expressing CD40L. Prior to infection with VVG2, P14 cells were transferred into control and test chimeras. On day 6 p.i., the frequency of P14 cells assessed in blood as well as the total counts in the spleen were more than 3-fold decreased in the test chimeras compared with that in the control chimeras (Fig. 2D–F). We therefore concluded that CD40L expression by CD4\(^+\) T cells is a crucial component of T cell help for CD8\(^+\) T cells during VV infection. It is conceivable that CD40L-mediated licensing of DCs by CD4\(^+\) T cells leads to upregulation of costimulatory molecules or secretion of soluble mediators, which might increase the activation and survival of CD8\(^+\) T cells. However, we could not detect any difference in the expression of activation/costimulatory molecule
expression in DCs in the presence or absence of T cell help (Supplemental Fig. 1).

We also tested a possible involvement of OX-40/OX-40L or IL-15, as both were recently described as potential mediators of T cell help (16, 37). However, CD8+ T cell priming was not altered in OX-40−/− mice compared with that in WT mice (Supplemental Fig. 2), and in vivo IL-15 neutralization (Supplemental Fig. 3) did not influence the level of VV-specific CD8+ T cell responses.

**Th cells regulate the expression of CD25 on CD8+ T cells**

As shown in Fig. 1G, helpless CD8+ T cells are prone to undergo apoptosis within the first days after VVG2 infection, suggesting that certain survival signals are missing that rescue the CD8+ T cells from activation-induced apoptosis in presence of T cell help. Among the factors that have been implied in the survival and maintenance of activated CD8+ T cells is the cytokine IL-2. We therefore analyzed the expression of the IL-2R α-chain (CD25) on P14 cells at day 3 after VVG2 infection in the spleens of WT, CD4−/−, MHCIIC−/−, and CD40L−/− mice. The level of CD25 expression on P14 cells in CD4−/− as well as MHCIIC−/− or CD40L−/− mice was significantly lower than that in WT control mice (Fig. 3A). As increased expression of CD25 is likely to lead to enhanced IL-2 signaling in CD8+ T cells, we measured the levels of phospho-AKT, a downstream mediator of IL-2. AKT is known to play a role in cell survival and might thus be responsible for the increased survival of activated VV-specific CD8+ T cells in the presence of T cell help. Notably, we could detect significantly increased phosphorylation of AKT (pS473) in P14 cells in the presence of T cell help (Fig. 3B), suggesting that the survival of activated CD8+ T cells is mediated by engagement of the IL-2R and downstream activation of the phosphoinositide 3-kinase (PI3K)–AKT pathway.

As our results highlight clear differences in the expression level of CD25, which correlate with the survival of activated CD8+ T cells, we raised the question whether specific factors could induce the upregulation of CD25 on CD8+ T cells. We therefore set

**FIGURE 3.** CD4+ T cells regulate the expression of CD25 on CD8+ T cells. Ly5.1+ P14 cells (10⁶) were transferred into WT, CD4−/−, MHCIIC−/−, and CD40L−/− mice prior to the infection with VVG2. A and B, Histograms, gated on P14 cells, show the expression of CD25 (A) and phospho-AKT (p-AKT; pS473) (B) in WT (upper row) and CD4−/− (lower row) mice at day 3 p.i.; the geometric MFI of P14 cells from infected mice (solid line) or naive WT CD8+ T cells (shaded histograms) is indicated. Each bar graph represents one independent experiment, with the bars indicating the average geometric MFI. Error bars indicate the SD within a group (n = 3–5). C, Purified CD8+ T cells, activated in vitro with anti-CD3 and anti-CD28, were cocultured with IL-12, IFN-α, IL-12 and IFN-α, or IL-15. Expression of CD25 on CD8+ T cells was assessed 48 h later. Histograms show the geometric MFI of naive CD8+ T cells (dashed line), activated CD8+ T cells (filled gray area), or activated CD8+ T cells cocultured with different cytokines (solid line). Data are representative of four independent experiments. D, A total of 10⁶ P14 WT or IL-12Rβ2−/− P14 CD8+ T cells were transferred into WT mice and infected with VVG2. Three days later, the expression of CD25 on both CD8+ T cell subsets was assessed in the spleen. Representative histograms, gated on P14 WT (upper row) or P14 IL-12Rβ2−/− (lower row) cells, show CD25 expression. The geometric MFI of P14 cells from infected mice (solid line) or naive WT CD8+ T cells as a control (shaded histograms) is indicated. The bar graph summarizes the geometric MFI of four mice per group; error bars indicate the SD within a group. E, A total of 10⁶ of either P14 WT or IL-12Rβ2−/− P14 were transferred into WT and CD4−/− mice and infected with VVG2. The frequency of P14 WT and P14 IL-12Rβ2−/− cells among total lymphocytes was determined in the blood of WT and CD4−/− mice 6 d p.i. Data are representative of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; n.s., not significant.
up an in vitro CD8⁺ T cell activation system in coculture with IL-12p70, IFN-α, IL-12p70 together with IFN-α or IL-15, and we assessed the expression of CD25 48 h poststimulation. Although CD25 expression obtained with α-CD3/CD28 stimulation is already substantial, incubation with IL-12p70 alone or in combination with IFN-α led to a further increase, whereas IFN-α or IL-15 alone had no influence (Fig. 3C), indicating that IL-12 can promote CD25 expression on CD8⁺ T cells.

To corroborate this result under physiological in vivo conditions, WT and IL-12Rβ2−/− deficient P14 cells were transferred into WT mice prior to infection with VVG2. The expression of CD25 on P14 cells at day 4 after VV infection was strongly reduced in the absence of IL-12 sensing, confirming the in vitro findings (Fig. 3D). In line with the role of CD25 for the survival of activated CD8⁺ T cells, the frequency of IL-12Rβ2−/− P14 was more than 3-fold lower compared with WT P14 CD8⁺ T cells (Fig. 3E). These data demonstrate that direct IL-12 signaling in CD8⁺ T cells is important during the course of CD8⁺ T cell priming in the context of VV infection.

Collectively, these results highlight an important role for IL-12 signaling in Ag-specific CD8⁺ T cells, which leads to the upregulation of CD25 and therefore promotes survival by the activation of the PI3K–AKT pathway.

T cell help regulates the expression of IL-12p35

The similar phenotype of helpless and IL-12Rβ2−/−–deficient CD8⁺ T cells in VV infection could indicate a role for T cell help in the regulation of IL-12p70. It is unlikely that CD8⁺ T cells themselves produce substantial amounts of IL-12p70 in VV-infected mice, but rather that the DCs are the principal producers of this cytokine (38, 39). In fact, cognate interaction of CD4⁺ T cells with microbially stimulated DCs has been reported to be crucial for the production of bioactive IL-12p70 in various systems (40–42). To investigate whether Th cells are involved in IL-12p70 production by DCs in VV infection, we purified splenic DCs from WT, CD4−/−, and CD40−/− mice 4 h after VV infection and quantified mRNA levels of the IL-12p40 and p35 subunits. Whereas the level of VV-induced mRNA for IL-12p40 in CD4−/− mice did not differ significantly between WT and CD4−/− mice, message for the p35 subunit was reduced 12-fold in CD4−/− mice (Fig. 4A, 4B). Importantly, the levels of p35 mRNA were also reduced in CD40−/− mice, lending further support to the role of CD40 engagement by CD40L-expressing CD4⁺ T cells for the induction of biologically active IL-12p70 production.

IL-2 signaling in CD8⁺ T cells is necessary for their expansion/survival

Reduced IL-12p70 expression in absence of CD4⁺ T cells resulted in impaired upregulation of CD25 on helpless CD8⁺ T cells, which was linked to their impaired survival. These results let us suspect a CD8⁺ T cell-intrinsic role for IL-2 signaling. To confirm this, we generated mixed BM chimeric mice with a CD8⁺ T cell compartment consisting of CD25⁺/⁻ and CD25⁻/⁻ cells, which could be distinguished by a congenic marker (Lyt5.1 and Lyt5.2, respectively). In addition, one group of chimeric mice was depleted of CD4⁺ T cells by i.p. injection of an anti-CD4 Ab prior to infection with VVG2 (anti-CD4). As shown in Fig. 5B and 5C, IL-2 signaling of sufficient CD8⁺ T cells expanded to 2-fold higher numbers than that in CD25-deficient CD8⁺ T cells after infection. Importantly, the frequencies and numbers of CD25⁻/⁻ gp33-specific CD8⁺ T cells were comparably reduced in chimeric mice that were additionally depleted of CD4⁺ T cells (Fig. 5D).

Finally, the frequencies of IFN-γ-producing gp33- and B8R-specific CD8⁺ T cells were significantly reduced in the absence of CD25. CD4 depletion reduced in particular the frequencies of CD25⁺/⁻ IFN-γ-producing gp33- and B8R-specific CD8⁺ T cells (Fig. 5E). These results indicate, first, that IL-2 signaling in CD8⁺ T cells is crucial during priming and, second, an important role of CD4⁺ T cell help for CD8⁺ T cells in VV infection is the provision of IL-2.

Activated Th cells provide IL-2

The above results suggested Th cells to be a critical source of IL-2; however, Th cells as well as CD8⁺ T cells themselves and DCs (43) have been described to be capable of producing IL-2 upon activation. To unambiguously identify the cellular source of IL-2, we generated mixed BM chimeric mice harboring CD4⁺ T cells lacking the capacity to secrete IL-2 (test chimeras). Six days after VVG2 infection, Ag-specific CD8⁺ T cells from test and control chimeras were quantified and analyzed functionally for Ag-induced cytokine production upon ex vivo restimulation. In the test chimeras, the frequency of gp33-specific cells among CD8⁺ T cells was decreased more than 4-fold in the blood (Fig. 6B, 6C), and total numbers of gp33/Dβ tetramer-positive CD8⁺ T cells were decreased up to 6-fold in the spleen (Fig. 6D). Further, the frequency of IFN-γ-producing gp33- and B8R-specific cells in test chimeras was decreased 3-fold and 6-fold, respectively (Fig. 6E). Because the numbers of cells that are potential IL-2 producers besides CD4⁺ T cells (e.g., CD8⁺ T cells and DCs) differs in the test and control chimeras by 50%, we cannot formally exclude that the presence of these IL-2–deficient cells might contribute to the reduced VV-specific CD8⁺ T cell priming. Nevertheless, our results strongly suggest that IL-2 secretion by CD4⁺ T cells is a critical component of T cell help for CD8⁺ T cells during VV infection.

Agonistic CD40 Abs in combination with IL-2 can completely substitute for T cell help

Based on our results, we propose that during VV infection, helper CD4⁺ T cells mediate the survival of activated CD8⁺ T cells via two interconnected synergistic pathways. To support these findings further, we aimed to substitute T cell help by providing either exogenous agonistic CD40 Ab, IL-2 in form of superagonistic IL-2/anti–IL-2 complexes (44) or a combination thereof. To this end, WT and CD4⁻/⁻ mice were injected with anti-CD40 Ab, treated with IL-2/anti–IL-2 complexes, or received both treatments to-
gether. In agreement with our hypothesis, neither CD40 treatment nor daily injections of IL-2/anti–IL-2 complexes alone was sufficient to compensate for lack of T cell help (Fig. 7A). However, mice that received both anti-CD40 and IL-2/anti–IL-2 complexes during the priming phase showed a P14 expansion that was comparable in WT and CD42/2 mice (Fig. 7A). The total numbers of P14 cells in the spleen confirmed this finding (Fig. 7B). Therefore, CD40-mediated production of bioactive IL-12p70 by DCs and provision of IL-2 are sufficient to replace the need for T cell help in CD8+ T cell priming during VV infection (Fig. 8).

Discussion

In this study, we have elucidated the in vivo mechanisms of CD4+ T cell help during VV-specific CD8+ T cell priming with the following results (Fig. 8): First, T cell help is crucial for the survival of activated CD8+ T cells but dispensable for the
CD4+ T cells promote the production of biologically active IL-12p70 in DCs via CD40L interaction. Subsequently, IL-12p70 is directly sensed by CD8+ T cells upon VV infection and hence the production of bioactive IL-12p70 in DCs is crucially dependent on CD40-mediated signaling in DCs. These results are consistent with a recent report demonstrating that the production of high levels of bioactive IL-12p70 was dependent on two factors: microbial stimulis that primarily lead to the secretion of the biologically inactive IL-12p40 subunit and signals through CD40 that induce the production of the limiting subunit IL-12p35, which is essential for bioactive IL-12p70.

In line with our results, Obar et al. (49) described recently that the upregulation of CD25 on Ag-specific CD8+ T cells early after VV infection (day 4) is driven by CD4+ T cell help. With respect to the mechanisms, the authors speculate that this effect could be a direct result of CD28:CD80/86-mediated signals on CD8+ T cells. In contrast with this hypothesis, we could not detect significant differences in the expression of CD80/CD86 on DCs dependent on help, as discussed earlier. In fact, our results indicate CD40L-induced IL-12 production by DCs and subsequent IL-12R signaling on CD8+ T cells is responsible for the upregulation of CD25 on virus-specific CD8+ T cells upon VV infection. Moreover, we verified and extended our results by demonstrating that CD25-expressing CD8+ T cells exhibit increased survival, mediated by CD4+ T cell derived IL-2, which was associated with downstream activation of the PI3K–AKT pathway. These results are in agreement with an in vitro study by Agarwal et al. (50) showing that CD25 expression on activated CD8+ T cells could be regulated by IL-12.

In line with our results, it has recently been shown that IL-2 signals perceived during the priming period are important for the programming of effector and effector memory CD8+ T cells (51), and those IL-2 signals are decisive for the expression of the transcription factor Eomes, for upregulation of perforin transcription and suppression of BCL-6 and IL-7Rα (52). Furthermore,
very recent studies have shown that CD8+ T cells expressing high levels of CD25 during early phases of viral infection proliferate faster and exhibit a more pronounced effector phenotype (52, 53).

In contrast with our data, Obar et al. (49) found CD25 expression on endogenous VV-specific CD8+ T cells comparable in CD40-deficient and WT mice; however, we highlighted a crucial role for CD40–CD40L interaction during VV infection in several experiments. First, the primary response was impaired in the absence of CD40 or CD40L, and, second, CD40L expression on CD4+ T cells was found to be critical. Additionally, the fact that IL-12 levels are significantly reduced in CD40−/− mice (comparable with that in CD4−/− mice) further strengthens our hypothesis. Currently, we are unable to explain this discrepancy, but differences in the infectious dose (2 × 10⁶ PFU versus 5 × 10⁶ PFU in our study), the viral strain (WT versus recombinant VVg2), and potential differences in animal housing conditions that might influence the “steady-state” level of DC maturation might explain the different results.

With respect to the survival of CD8+ T cells, there is evidence in lymphocytes that AKT provides a survival signal by increasing the expression of BCL-2 and BCL-XL (54). As our results clearly indicate that helpless CD8+ T cells undergo increased cell death correlating with reduced levels of BCL-XL, we speculate that activation of AKT downstream of the IL-2R results in elevated levels of BCL-XL and prevents the cells from undergoing apoptosis. Our results demonstrating that IL-2 signaling in CD8+ T cells is crucial during priming with VV further support this hypothesis. Nevertheless, we cannot exclude that other pathways downstream of the IL-2R contribute to increased survival of CD8+ T cells, as the expression of TRAIL was also found to be increased in the absence of CD4+ T cells and hence IL-2 signaling.

Our data indicate a dual role of CD4+ T cells to support VV-specific CD8+ T cell responses; namely, 1) the promotion of IL-12p70 production by DCs, which induces expression of the high-affinity IL-2R on VV-specific CD8+ T cells and 2) the supply of IL-2. Thus, exogenous provision of these two signals should compensate for the lack of CD4+ T cells. This was indeed the case as concomitant administration of superagonistic IL-2/anti-IL-2 complexes and agonistic CD40 signaling promoted VV-specific CD8+ T cell expansion and survival to equivalent levels in Th cell-sufficient and -deficient hosts.

It is conceivable that in addition to CD25 upregulation, IL-12 signaling on CD8+ T cells supports the differentiation into effector T cells and initiates gene regulation programs required for survival and formation of long-lived memory cells (55). Such survival effects of IL-12p70 on CD8+ T cells could be mediated by BCL-3, a member of the Icb-family (56). Moreover, it was recently shown that differential epigenetic remodeling of genes involved in CD8+ T cell function occurs in helpless CD8+ T cells (50). Therefore, it might be possible that IL-12 signaling on CD8+ T cells initiates an altered histone acetylation program, which could be beneficial for their survival. Further studies are needed to address these possibilities. However, provision of high levels of IL-12 alone, either induced by injection of an agonistic anti-CD40 mAb (Ref. 41 and data not shown) or upon infection with a recombinant VV expressing IL-12p70 (data not shown) were not sufficient to compensate for a lack of T cell help. This further confirms the dual role of T cell help in the context of VV infection.

Collectively, this study clarifies the mechanistic pathway of T cell help for virus-specific CD8+ T cell responses during VV infection, highlighting an important communication network between CD4+ T cells, DCs, and CD8+ T cells with a central role for IL-2 and IL-12p70, both acting in close synergy.

Acknowledgments

We thank Nathalie Oetiker for assistance in cell sorting, Susanne Feedrich for excellent animal caretaking, Manfred Kopf (ETH Zürich) for providing OX-40−/− mice, Katrin Schwarz and Yu Zou (Cytos Biotechnology AG) for QB-IL-15 VLPs, the α-CD25 Ab, and the provision of various mouse strains. We are grateful to the members of the Oxenius group for helpful discussions and critical reading of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

References


