IL-21R on T Cells Is Critical for Sustained Functionality and Control of Chronic Viral Infection
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tial phase of chronic viral infection, suggesting that this could be a general feature of infections that elicit nonprotective adaptive immune responses, such as hepatitis B virus, HCV, and HIV (8, 30). This poor induction of IL-21 may result from the failure to initiate robust CD4+ T cell responses as well as the exhaustion or the viral-induced depletion of these cells that may occur during certain chronic infections. Therefore, we anticipate that the cautious development of approaches to modulate the levels of IL-21, or regulate the induction of cellular subsets that generate IL-21, will provide new therapeutic opportunities to improve immunity to diseases that require CD8+ T cell responses to be controlled, such as chronic viral infection and tumors.

References and Notes
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IL-21R on T Cells Is Critical for Sustained Functionality and Control of Chronic Viral Infection
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Chronic viral infection is often associated with the dysfunction of virus-specific T cells. Our studies using Ii21r-deficient (Ii21r−/−) mice now suggest that interleukin-21 (IL-21) is critical for the long-term maintenance and functionality of CD8+ T cells and the control of chronic lymphocytic choriomeningitis virus infection in mice. Cell-autonomous IL-21 receptor (IL-21R)-dependent signaling by CD8+ T cells was required for sustained cell proliferation and cytokine production during chronic infection. Ii21r−/− mice showed normal CD8+ T cell expansion, effector function, memory homeostasis, and recall responses during acute and after resolved infection with several other nonpersistent viruses. These data suggest that IL-21R signaling is required for the maintenance of polyfunctional T cells during chronic viral infections and have implications for understanding the immune response to other persisting antigens, such as tumors.

Chronic viral infections, including HIV and hepatitis B and C viruses (HBV and HCV), afflict >0.5 billion people worldwide. Although the reasons for ineffective antiviral immunity remain poorly defined, studies of chronic infection with lymphocytic choriomeningitis virus (LCMV) in mice and HIV, HCV, and HBV in humans show that the loss of T cell functionality, termed exhaustion, is a hallmark of chronic infection (1). Long-term maintenance of potent virus-specific CD8+ T cell responses requires help from CD4+ T cells and cytokines produced by T and non-T cells (2, 3). Specifically, members of a cytokine subfamily with receptors sharing the common gamma (γc) chain such as interleukin-2 (IL-2), IL-7, and IL-15 have distinct activities on the development and maintenance of antiviral effector and memory T cells (4–8). IL-21, an additional family member, has pleiotropic activities on CD4+ T cells (9), although its role in antiviral CD8+ T cell responses is unknown.

LCMV can cause acute or persistent infection, depending on the viral isolate and the dose of infection. High-dose infection with the fast-replicating strain LCMV-Docile (or strain Clone 13) results in virus persistence and exhausted virus-specific CD8+ T cells. In contrast, low-dose infection is efficiently cleared in immunocompetent mice.

To study the role of IL-21 in acute and chronic viral infection, we infected control and Ii21r−/− mice with low-[200 plaque-forming units (PFU)], intermediate- (2000 PFU), or high-dose (2 × 106 PFU) LCMV-Docile. Comparable expansion, cytokine production, and killing of virus-infected targets by viral epitope gp33−41-specific CD8+ T cells were observed 8 days after infection, indicating that the IL-21 receptor (IL-21R) was not required for priming and differentiation of virus-specific CD8+ T cells during the acute response (Fig. 1, A to C, and fig. S1, A to G). As expected, control mice infected with low or intermediate doses mounted efficient long-term antiviral CD8+ T cell responses and controlled the infection (Fig. 1, A to F), whereas infection with high-dose LCMV-Docile resulted in reduced frequencies of and interferon-γ (IFN-γ) production by virus-specific T cells (Fig. 1, A to C), in addition to viral persistence (Fig. 1F). We detected exhausted Ii21r−/− LCMV-specific CD8+ T cell responses in response to low and intermediate virus doses starting at day 15 after infection (Fig. 1, A to D). After 5 weeks, we observed reduced frequencies and total numbers of gp33−41-specific Ii21r−/−/CD8+ T cells in the blood and spleen. Moreover, the remaining gp33−41-specific CD8+ T cells failed to produce IFN-γ, tumor necrosis factor-α, and IL-2 and did not proliferate upon stimulation, all of which are characteristics of exhausted T cells (Fig. 1C and fig. S1, H and I). Consequently, Ii21r−/− mice developed chronic viremia after exposure to low or intermediate doses of virus (Fig. 1E and fig. S1K). Even at high-dose exposure, when both control and Ii21r−/− mice developed a chronic infection, viral titer were increased in the latter (Fig. 1F). The fre-

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Fig. 1. IL-21R is essential for sustained antiviral CD8+ T cell responses and control of viral infection. Control and Il21r−/− mice were infected with the indicated doses of LCMV-Docile. Blood samples (A) and splenocytes (B to D) were taken to monitor the presence of CD8+ T cells specific for the gp33-41 epitope [(A), (B), and (D)] by flow cytometry. wt, wild-type control. Splenocytes were stimulated with gp33-41 peptide 4 hours before intracellular staining with antibodies to the indicated cytokines (C). Graphs show frequencies of gp33-41+ cells [(A) and (B)] and IFN-γ+ cells (C) of CD8+ T cells or total number of gp33-41+ CD8+ T cells (D). Values indicate averages ± SEM of four mice per group (A) or individual mice [(B) to (D)]. Viral titers in indicated organs at day 35 after infection with 2000 PFU (E) or 2 × 10^6 PFU (F) of LCMV-Docile are shown. Symbols represent values of individual mice. *P < 0.05. Representative data are shown from three independent experiments.
We observed no differences in gp33-41-specific cytokine production of gp33-41-specific memory CD8+ T cells in the blood between IL-21R-deficient and control mice up to 70 days after infection, whereas we observed nonneutralizing LCMV-specific immunoglobulin G antibodies at day 19 in control and IL-21R–/– mice (fig. S1L). LCMV-specific neutralizing antibodies appeared late after infection (10) and were undetectable in both control and IL-21R–/– mice up to 70 days after infection, whereas we observed nonneutralizing LCMV-specific immunoglobulin G antibodies at day 19 in control mice and reduced by a factor of 3 in IL-21R–/– mice infected with LCMV-Docile or LCMV-WE (fig. S1, M and N). IL-21R–/– mice showed normal acute CD8+ T cell responses to and viral clearance of infection with both influenza virus and vaccinia virus (fig. S2). Together, these results suggest that IL-21R was dispensable for acute antiviral CD8+ T cell responses, but was essential for maintenance of virus-specific CD8+ T cells, sustained effector responses, and eventual control of chronic infection.

To address whether IL-21R was required for memory T cell responses, we studied infection with LCMV strain WE, which is less virulent than LCMV-Docile and is cleared by CD8+ T cells by day 10 after infection. Antigen-experienced LCMV-specific CD8+ T cells constitute a stable pool of memory cells over long periods of time (11–13). We observed no differences in gp33-41-specific CD8+ T cell frequencies in the blood between IL-21R–/– and control mice up to 70 days after infection (Fig. 2A). At day 35, maintenance, cytokine production, and proliferation of memory gp33-41-specific CD8+ T cells were unaffected in IL-21R–/– mice (fig. S3). Hence, virus was effectively controlled and undetectable in the blood from day 11 up to day 75 after infection (fig. S1O). Memory cells were then challenged with a high dose of LCMV-WE, which triggered rapid expansion and cytokine production of gp33-41-specific memory CD8+ T cells in both IL-21R–/– and control mice, indicating efficient and protective recall responses in the absence of IL-21R (Fig. 2, B and C). In addition, we observed efficient recall responses of IL-21R–/– mice after immunization with replication-incompetent virus-like particles and challenge with vaccinia virus (fig. S4). Taken together, these data clearly demonstrate that IL-21R is dispensable for efficient CD8+ T cell effector and memory responses during acute and resolved infection with nonpersistent viruses.

We next generated mixed bone-marrow chimeras in which the marrow of irradiated C57BL/6 (B6) mice (CD45.1) was reconstituted with a 1:1 mixture of bone marrow from IL-21R–/– (CD45.2) and control (CD45.1) mice, and 1:1 chimerism was confirmed in both CD4+ and CD8+ T cells (fig. S5A). After infection with LCMV-Docile, both control and IL-21R–/–-deficient CD8+ T cells expanded comparably during the acute phase (Fig. 3A), whereas we saw dramatic differences in CD8+ T cell maintenance starting at 2 weeks after infection (Fig. 3, A and B). At day 35, the frequency of gp33-41-specific IL-21R–/– CD8+ T cells and IFN-γ-producing CD8+ T cells was reduced as compared with that in controls (1.3 ± 0.5% versus 6.7 ± 2.2% and 1.22 ± 1% versus 4.9 ±1.8%, respectively) in both control and IL-21R–/– deficient CD8+ T cells at day 35 after infection (Fig. 3B). At day 50, frequencies of gp33-41-specific CD8+ T cells in the blood at indicated days (B) and IFN-γ–producing gp33-41-specific T cells in the spleen at day 7 after challenge (C) are shown. Values indicate percent frequencies and represent averages of groups of mice (n = 4 or 5 per group) ± SD (A). Symbols represent individual mice ([B] and [C]). Representative data are shown from two independent experiments.

IL-21R signaling is required for sustained in vivo proliferation of antiviral CD8+ T cells. IL-21R+/+ (wt) (CD45.1)/IL-21R–/– (CD45.2) mixed bone-marrow chimeras were infected with 2000 PFU of LCMV-Docile, and antiviral CD8+ T cell responses were measured in the blood (A) and in the spleen (B to E). Shown are frequencies of wt or IL-21R–/–-deficient gp33-41-specific CD8+ T cells at indicated days [A] and [B]) and gp33-41-specific T cells producing IFN-γ at day 35 (C), as measured by intracellular staining. (D) At day 10 after infection, bone-marrow chimeras received daily injections of BrdU for 4 days, and BrdU incorporation by wt or IL-21R–/–-deficient CD8+ T cells was determined by BrdU staining and flow cytometry 1 and 5 days later (days 14 and 19). (E) Flow cytometric analysis of PD-1 expression by wt and IL-21R–/–-deficient CD8+ T cells at day 35 after infection. Graphs represent histograms of PD-1 expression by control (CD45.1) or IL-21R–/– (CD45.2) cells gated on gp33-41-specific CD8+ T cells (top) or total CD8+ T cells (bottom). MFI, mean fluorescence intensity. Graphs show a representative sample of a group of mice (n = 4). Values indicate average ± SD. Representative data are shown from two independent experiments.
Fig. 4. IL-21 is mainly produced by virus-specific CD4+ T cells. Groups of mice were infected with indicated doses LCMV-Docile. (A and B) At indicated days, splenocytes were cultured with gp33-41 or gp61-80 (100 nM) in the presence of monensin for 5 hours before staining of CD4, CD8, CD11c, and CD11b cells by specific monoclonal antibodies and intracellular IL-10 or IL-21, and then analyzed by flow cytometry. (A) Frequency of IL-10–producing cells gated on CD11b+ cells. (B) Histograms show IL-21 production stained with an Fc fusion protein by gp33-41–specific CD4+ IFN-γ+ cells (top) and by gp33-41–specific CD8+ IFN-γ+ cells (bottom) at indicated days after infection with 2000 PFU of LCMV-Docile. Shaded curves represent staining with a control Fc fusion protein. IL-21+ IFN-γ–neg cells were undetectable. PMA, phorbol 12-myristate 13-acetate; iono, calcium ionophore. (C) IL-21 expression by CD4+ and CD8+ T cells (purity >90%) from infected mice as measured by quantitative polymerase chain reaction. Representative data are shown from two independent experiments [(A) and (B)].

respectively) (Fig. 3, B and C), demonstrating that CD8+ T cells required cell-autonomous IL-21R signaling for sustained responses during chronic LCMV infection. The virus was not cleared by day 35 in mixed bone-marrow chimeras (fig. S5B). Reduced frequencies of LCMV-specific IL-21R–deficient CD8+ T cells may result from impaired proliferation or from increased cell death. To address this question, LCMV-Docile–infected mixed bone-marrow chimeras were injected with bromodeoxyuridine (BrdU) at 24-hour intervals from days 10 to 13 after infection. IL-21R–deficient CD8+ T cells showed strikingly reduced proliferation as compared with control CD8+ T cells (50% versus 25%) at day 14 after infection (Fig. 3D). Six days after the last pulse, BrdU+ populations of control and II21r−/− CD8+ T cells were reduced by half (~20% versus 10%) (Fig. 3D) as compared with the respective populations at day 14, which indicated a similar contraction of control and IL-21R–deficient CD8+ T cells. These data suggest that IL-21R–deficient signaling in CD8+ T cells was required for their continuous proliferation during chronic infection and not for their survival.

Chronic viral infection and CD8+ T cell dysfunction have been strongly associated with sustained expression of the inhibitory receptor programmed death 1 (PD-1) (14, 15). We found that PD-1 was strongly expressed by the vast majority of both gp33-41–specific control and II21r−/− CD8+ T cells in mixed bone-marrow chimeras 5 weeks after infection. (Fig. 3E). Similar results were obtained by comparing PD-1 expression on CD8+ T cells in control and II21r−/− mice (fig. S6).

Like PD-1, the inhibitory cytokine IL-10 can interfere with antiviral T cell responses and the clearance of LCMV (16, 17). The frequency of IL-10–producing cells peaked in the acute phase of LCMV infection. Surprisingly, the frequency of IL-10–producing cells was decreased in II21r−/− mice (Fig. 4A). Most IL-10+ cells were macrophages (fig. S7). Thus, CD8+ T cell exhaustion in II21r−/− mice is not associated with up-regulation of IL-10.

The above results demonstrate that virus-specific CD8+ T cells require IL-21 to prevent exhaustion during chronic viral infection. We next wanted to define the cellular source of IL-21. Intracellular staining showed that a fraction of gp61-80 epitope–specific CD4+ T cells coproduced IFN-γ and IL-21 in response to LCMV-Docile infection, whereas substantial IL-21 production by virus-specific CD8+ T cells was detectable only by combined phorbol ester and calcium ionophore stimulation (Fig. 4B). Consistent with protein expression, IL-21 mRNA expression was considerably higher in CD4+ than in CD8+ T cells (Fig. 4C). Thus, CD4+ T cells are the likely source of IL-21 that helps to sustain CD8+ T cell functionality, although autocrine-produced IL-21, or IL-21 produced by another immune cell, may also play a role. Among CD4+ subsets, T helper cell 17 cells have been suggested to be the main producers of IL-21; however, we did not detect IL-17–producing gp61-80–specific CD4+ T cells at days 8 and 15 after infection (fig. S7C).

Our data show that IL-21–dependent signaling is critical for the prevention of T cell exhaustion and control of chronic viral infection. Comparison of IL-21R and IL-2R–dependent signaling in the regulation of antiviral T cell responses reveals interesting differences in the requirements for γc cytokines during viral infection. Although both IL-2Rα and IL-2Rγ are dispensable for acute CD8+ T cell expansion and effector function (4, 5), they are both essential for the maintenance of CD8+ T cells during chronic infection (4). In contrast, only IL-2Rα is required for recall responses. During the acute primary response, it is thought that IL-2 programs the long-term fate of CD8+ T cells to mount a secondary response (5). IL-2 therapy during the chronic phase has been shown to enhance antiviral CD8+ T cell responses and viral clearance (18). Our results imply that a combination therapy of IL-2 and IL-21 may be more beneficial for treatment of chronic viral infection.

References and Notes
Merkel Cells Are Essential for Light-Touch Responses

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The peripheral nervous system detects different somatosensory stimuli, including pain, temperature, and touch. Merkel cell-neurite complexes are touch receptors composed of sensory afferents and Merkel cells. The role that Merkel cells play in light-touch responses has been the center of controversy for over 100 years. We used Cre-loxP technology to conditionally delete the transcription factor Atoh1 from the body skin and foot pads of mice. Merkel cells are absent from these areas in Atoh1CKO animals. Experimental skin/nerve preparations from Atoh1CKO animals demonstrate complete loss of the characteristic neurophysiologic responses normally mediated by Merkel cell-neurite complexes. Merkel cells are, therefore, required for the proper encoding of Merkel receptor responses, suggesting that these cells form an indispensable part of the somatosensory system.

Different qualities of touch are encoded by discrete touch receptors, each with distinctive coding properties (1–3). One form of light touch important for tactile discrimination of shapes and textures is mediated by Merkel cell-neurite complexes, which exhibit a characteristic response to light skin indentation (4, 5). Merkel cell-neurite complexes are composed of nerve fibers associated with Merkel cells, an enigmatic skin cell population first described in 1875 (6). In mammalian skin, Merkel cells are normally found in whisker follicles of the face, specialized epithelial structures of the hairy skin called touch domes, and epidermal invaginations of the plantar foot surface called rete ridges (7). Merkel cells have been proposed to be the sensory receptor cells of the complexes because they form synaptic contacts with somatosensory afferents (8, 9); however, studies that indirectly tested this model have yielded conflicting results (10–16).

Atoh1 is a basic helix-loop-helix transcription factor expressed by Merkel cells in all areas of the skin (17). Atoh1 null mice die within minutes of birth, which prevents a detailed assessment of nonlethal phenotypes resulting from deletion of the gene. We used the Hoxb1Cre allele (18), which is expressed throughout the epidermis and dermis of body skin, but not head skin (Fig. 1, A to A′′), to delete a floxed allele of Atoh1 (Atoh1flox) in transgenic mice (20). Conditional knockout (Atoh1CKO) animals were born in the expected Mendelian ratio, but roughly 50% of these animals died within 24 to 36 hours of birth.

The overall structure of the touch dome, including the palisading epiderthium and location of the guard hair, was preserved in Atoh1CKO animals (Fig. 1, B and C, and Fig. 2, A and B). Atoh1 is a positive autoregulator of its own expression (21), so we analyzed β-galactosidase expression driven by the Atoh1lacZ knock-in allele (22) to demonstrate that Atoh1 was deleted from the skin of E16.5 Atoh1CKO mice (Fig. 1, B and C). X-gal staining was found in touch domes and foot pads of heterozygous Atoh1lacZ mice but not Atoh1CKO animals (Fig. 1, B′ and C′). To determine whether Merkel cells were present, we