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Article

Cell-Intrinsic IL-27 and gp130 Cytokine Receptor Signaling Regulates Virus-Specific CD4$^+$ T Cell Responses and Viral Control during Chronic Infection

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SUMMARY

The outcome of chronic viral infections, which affect millions of people worldwide, is greatly dependent on CD4$^+$ T cells. Here we showed that T cell-specific ablation of the common interleukin-6 (IL-6) family receptor, gp130, profoundly compromised virus-specific CD4$^+$ T cell survival, T follicular helper responses, and IL-21 production at late stages of chronic lymphocytic choriomeningitis virus (LCMV) infection. These effects were cell intrinsic for CD4$^+$ T cells and were accompanied by a reduction of CD8$^+$ T cells, antibodies, and a severe failure in viral control. We identified IL-27 as a gp130 cytokine that promoted antiviral CD4$^+$ T cell accumulation in vivo and that rapidly induced IL-21 ex vivo. Furthermore, IL-27R was critical for control of persistent LCMV in vivo. These results reveal that gp130 cytokines (particularly IL-27) are key regulators of CD4$^+$ T cell responses during an established chronic viral infection, empowering both humoral and cytotoxic immunity.

INTRODUCTION

Chronic viral infections such as human immunodeficiency virus-type 1 (HIV-1), hepatitis C virus (HCV), and hepatitis B virus (HBV) affect hundreds of millions of people worldwide (Virgin et al., 2009). Viral persistence is associated with decreased numbers of both virus specific CD4$^+$ and CD8$^+$ T cells along with a progressive loss of their ability to kill infected targets and produce important effector molecules such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin-2 (IL-2) (Letvin and Walker, 2003; Rehermann and Nascimbeni, 2005). Indeed, upregulation of multiple inhibitory surface molecules including PD-1, LAG-3, and Tim3 (Barber et al., 2006; Blackburn et al., 2009; Wherry et al., 2007), along with IL-10 (Brooks et al., 2006; Ejrnaes et al., 2006), during chronic infection contributes to the hierarchical loss of function seen in virus-specific T cells. In addition, transforming growth factor-β (TGF-β) signaling limits the numbers of virus-specific T cells (Boettler et al., 2012; Garidou et al., 2012; Tinoco et al., 2009). These features appear commonly among actively replicating chronic viruses in humans and other animals (Clerici et al., 1994; Cumont et al., 2007; Day et al., 2006; Le Clerc et al., 2009; Rigopoulou et al., 2005; Urbani et al., 2006), and this highly immunosuppressive environment prevents viral eradication and makes the host extremely susceptible to secondary infections and cancers.

Individuals control persistent viruses to differing extents, ranging from elite controllers of HIV-1 and acutely resolving HCV patients to fast HIV and HCV progressors (Letvin and Walker, 2003; Rehermann and Nascimbeni, 2005). This varied degree of viral control associates with different quality and quantity of CD4$^+$ and CD8$^+$ T cell responses (Virgin et al., 2009). In particular, CD4$^+$ T cells are central for several aspects of antiviral responses. The outcome of HIV infection depends critically on the rate and the extent of virus-specific CD4$^+$ T cell expansion with decreased CD4$^+$ T cell numbers tightly correlating with progression into AIDS (Porichis and Kaufmann, 2011; Virgin and Walker, 2010). Similarly, spontaneous recovery from HCV and HBV infection associates with vigorous CD4$^+$ and CD8$^+$ T cell responses while CD4$^+$ T cell depleted chimpanzees fail to clear HBV or HCV and develop severe liver disease (Rehermann and Nascimbeni, 2005). CD4$^+$ T cells are also essential to control the persistent variant of lymphocytic choriomeningitis virus, LCMV Clone 13 (Cl13), in a mouse model of chronic viral infection (Battegay et al., 1994; Matloubian et al., 1994a). Recently, this model has been used to uncover CD4$^+$-derived IL-21 as vital for CD8$^+$ (but not CD4$^+$) T cell maintenance during late chronic infection (Elsaesser et al., 2009; Fröhlich et al., 2009; Yi et al., 2009). IL-21 has also been found to positively correlate with lower viral loads in HIV- and HCV-infected patients (Chevalier et al., 2011; Iannello et al., 2010; Yue et al., 2010). In addition, LCMV Cl13 infection results in the late accumulation of virus-specific T follicular helper (Tfh) cells (Fahey et al., 2011; Harker et al., 2011), a process subsequently observed during HBV, HCV, simian immunodeficiency virus (SIV), and HIV infection (Feng et al., 2011, 2012; Lindqvist et al., 2012; Petrovas et al., 2012). This is in part dependent on IL-6 signaling on CD4$^+$ T cells during the later stages of chronic infection and results in enhanced virus-specific antibody responses and viral control (Harker et al., 2011; Petrovas et al., 2012).

IL-6 is the archetypal member of the IL-6 cytokine family, which includes IL-11, IL-27, leukemia inhibitory factor (LIF), cardiotrophin-1, ciliary neurotrophic factor, and oncostatin M (OSM) (Silver and Hunter, 2010). The members of the IL-6 family can exert both unique and overlapping functions through their common use of the constitutively expressed transmembrane receptor gp130, which dimerizes with cytokine-specific receptors

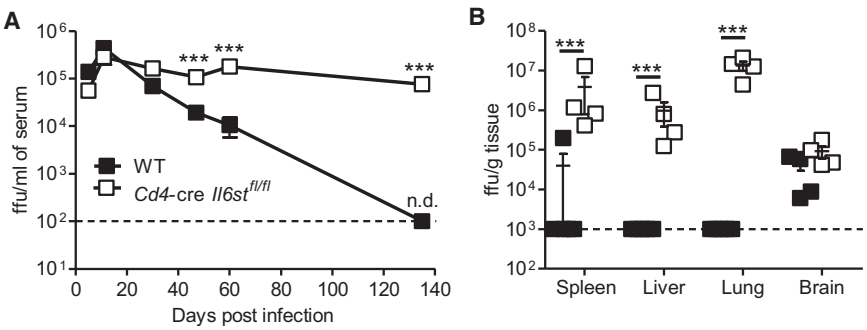


Figure 1. GP130 Signaling in T cells Is Crucial for Control of Chronic LCMV Infection

WT (C57B/6 or *Il6st*$^{fl/fl}$) and *Cd4*-cre *Il6st*$^{fl/fl}$ mice were infected with 2 × 10^6 pfu of LCMV Cl13 i.v. Viral load was monitored in the serum throughout infection (A) and the indicated tissues at day 135 p.i. by immunofocus assay (B). Data is representative of two experimental repeats; n ≥ 4 mice per group with mean ± SEM depicted. This figure is supported by Figure S1.

on cytokine binding. Given this conserved signaling pathway, we speculated that along with IL-6, other members of the IL-6 family of cytokines may have potent effects on T cell function during chronic infection. Supporting this, IL-6 deficiency does not affect in vivo IL-21 during chronic infection; however, ex vivo IL-6 stimulation of CD4$^+$ T cells from LCMV Cl13 infected mice causes rapid expression of *Il21* (Harker et al., 2011), suggesting the presence of functional redundancies for IL-21 induction.

To bypass such functional redundancies, we examined the effect of genetic ablation of *Il6st* (which encodes gp130) in T cells during persistent LCMV Cl 13 infection in mice. In the absence of gp130 on T cells, mice were incapable of controlling infection, had a profound reduction in the numbers of virus-specific CD8$^+$ and CD4$^+$ T cells, and compromised antibody responses. In contrast to CD8$^+$ T cells, which appeared functionally unaltered by gp130 deficiency, *Il6st*$^{-/-}$ CD4$^+$ T cells also had greatly reduced Tfh proportions and IL-21 production. Although the CD8$^+$ T cell defects were in most part extrinsic, direct gp130 signaling on CD4$^+$ T cells was essential for their survival and function at late stages of chronic LCMV infection. We identified IL-27 as the gp130 signaling cytokine promoting CD4$^+$ T cell survival and that (similarly to IL-6) was capable of rapidly inducing IL-21 ex vivo but was redundant for its in vivo production. Our data indicate that gp130 signaling cytokines play a vital role during late stages of chronic viral infection including regulation of CD4$^+$ T cell survival and IL-21 production to orchestrate antiviral responses.

RESULTS

Gp130 Signaling on T Cells Was Essential for Control of Chronic Viral Infection

To investigate the role of T cell-specific gp130 signaling on control of a chronic viral infection, we infected *Cd4*-cre *Il6st*$^{fl/fl}$ (where *Il6st* is deleted in CD4$^+$ and CD8$^+$ T cells) or wild-type (WT) mice with LCMV Cl13. Loss of gp130 signaling did not adversely affect the proportion of regulatory T (Treg) cells, CD4$^+$ or CD8$^+$ T cells, or their capacity to produce TNF-α or IFN-γ, in the spleen prior to infection (see Figure S1 available online). Initial and peak viremia were identical; however, mice lacking T cell gp130 showed a complete failure to control viremia while WT mice had significantly reduced viral loads from day 45 postinfection (p.i.) onward (Figure 1A). By day 130 p.i., virus was readily detectable across multiple tissues in *Il6st*$^{-/-}$ mice while it was only detected in the brain of WT animals (Figure 1B). Gp130 deficiency did not affect the clearance of acute LCMV Armstrong infection (data not shown). In contrast to the decline in viremia that we previously observed in WT and *Il6*$^{-/-}$ mice between days 30 and 45 p.i., mice deficient in T cell gp130 showed little or no decline in viremia between these two time points (Figure 1A) (Harker et al., 2011). These results demonstrated that gp130 signaling on T cells played a critical role in controlling chronic (but not acute) LCMV infection in vivo. In addition, the fact that viremia was higher in mice lacking gp130 compared with *Il6*$^{-/-}$ mice suggested that gp130 signaling cytokines (other than IL-6) significantly contributed to viral containment during chronic LCMV infection.

T Cell gp130 Signaling Promotes CD8$^+$ and CD4$^+$ T Cell Accumulation at Late Stages of Chronic Infection

IL-6 deficiency does not affect the total numbers of virus specific CD8$^+$ or CD4$^+$ T cells throughout chronic LCMV infection (Harker et al., 2011). In the next series of experiments, we aimed to identify the immune defects that resulted in the more severe failure of *Cd4*-cre *Il6st*$^{fl/fl}$ mice compared to *Il6*$^{-/-}$ mice to control LCMV Cl13. For that, we first monitored the expansion of virus-specific CD8$^+$ and CD4$^+$ T cells after infection.

We observed similar numbers of H2-D^b LCMV GP$_{276–284}$ and H2-D^b LCMV GP$_{33–41}$-specific CD8$^+$ T cells in blood and spleens of WT and *Cd4*-cre *Il6st*$^{fl/fl}$ animals at day 9 p.i. (Figures 2A and 2B). By day 15 p.i., however, there were significantly fewer virus-specific CD8$^+$ T cells in the blood of *Cd4*-cre *Il6st*$^{fl/fl}$ animals, a trend that continued until day 60 p.i., the last time point analyzed (Figure 2A). These findings were confirmed in the spleen where *Cd4*-cre *Il6st*$^{fl/fl}$ mice had significantly fewer H2-D^b LCMV GP$_{33–41}$ and GP$_{276–284}$-specific CD8$^+$ T cells compared to infection matched controls at day 30 (but not day 9) p.i. (Figure 2B).

"Antigen experienced" PD-1$^+$ CD4$^+$ T cells in the blood also showed normal development in *Cd4*-cre *Il6st*$^{fl/fl}$ mice on days 9 and 15 p.i., but a significantly reduced number was seen from day 30 onward compared to WT mice (Figure 2C). The number of H2-A^b LCMV GP$_{67–77}$-specific CD4$^+$ T cells was also dramatically reduced in the absence of gp130 signaling, with a profound reduction of virus specific cells observable at day 30 p.i. (16% of WT numbers), but unaltered numbers at day 9 p.i. (Figure 2D). Combined these results show that gp130 cytokines, while not required for priming and initial expansion of virus specific T cell responses, are essential for their accumulation at late stages of chronic LCMV infection. Given that neither CD4$^+$ nor CD8$^+$ T cell numbers are affected by the absence of IL-6 alone (Harker et al., 2011), the aforementioned results highlight a critical role for

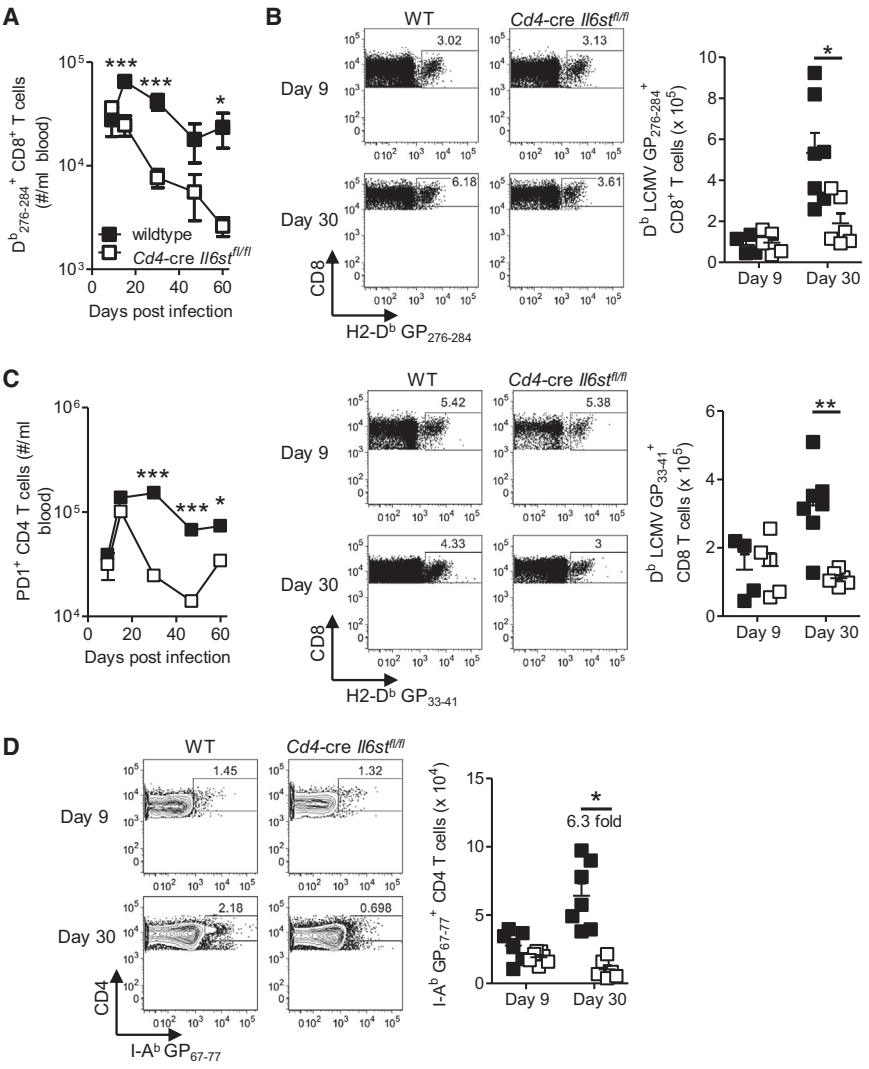


Figure 2. T Cell-Specific gp130 Signaling Is Required for Accumulation of Virus-Specific CD8 and CD4 T Cells during Chronic LCMV Infection

WT (C57B/6 or *Il6st*$^{fl/fl}$) and *Cd4*-cre *Il6st*$^{fl/fl}$ mice were infected with 2 × 10^6 pfu of LCMV Cl13 i.v.

(A) PBMCs were analyzed to determine the number of GP$_{276–284}$ CD8$^+$ T cells.

(B) At days 9 and 30 p.i., splenocytes were analyzed by flow cytometry to determine the number of H2-D^b GP$_{276–284}$ and GP$_{33–41}$ CD8$^+$ T cells.

(C) As in (A), PBMC were analyzed to determine the number of PD-1$^+$ CD4$^+$ T cells.

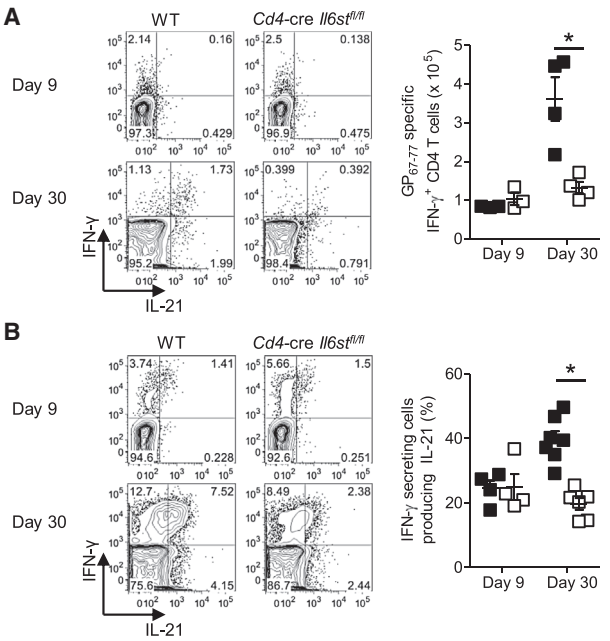
(D) As in (B), I-A^b GP$_{67–77}$$^+$ CD4$^+$ T cells numbers were determined in the spleen, the fold increase from *CD4*-cre *Il6*$^{fl/fl}$ to WT cells at day 30 p.i. is indicated. Data is representative of three experimental repeats n ≥ 4 mice per group with mean ± SEM depicted. This figure is supported by Figure S2.

IL-6 independent gp130 signaling in the maintenance of virus-specific CD8$^+$ and CD4$^+$ T cell numbers at later stages of chronic infection.

Gp130 Deletion in T Cells Alters CD4$^+$T Cell Functions at Late Stages of Chronic Infection

We next investigated the consequences of ablated gp130 signaling on the function of CD8$^+$ and CD4$^+$ T cells. Chronic infection not only leads to deletion of virus-specific CD8$^+$ T cells but also a hierarchical (IL-2 > TNF-α > IFN-γ) loss of function of those cells that remain (Wherry et al., 2003). The loss of gp130 signaling on T cells did not, however, alter the level of functional exhaustion in virus specific CD8$^+$ T cells (Figure S2).

During chronic LCMV infection there is a progressive increase in the proportion of virus specific CD4$^+$ Tfh cells (Fahey et al., 2011; Harker et al., 2011). In this context, IL-6 signaling is required for maximal upregulation of Bcl6 and Tfh cell numbers but is not required for these cells to produce IL-21 (Harker et al., 2011), a cytokine vital to the maintenance of virus-specific CD8$^+$ T cells (Elsaesser et al., 2009; Fröhlich et al., 2009; Yi et al., 2009). As expected, loss of gp130 signaling resulted in significant loss of Tfh cell differentiation and Bcl6 expression in both virus-specific and total CD4$^+$ T cells at day 30 p.i., although the expression of Bcl6 within the remaining CXCR5$^+$ virus-specific CD4$^+$ T cells was not significantly affected (Figures S3A–S3C). In contrast to the reduced number of Tfh cells, the proportion of FoxP3$^+$ T regulatory cells (Tregs) was increased at day 30, but not day 9, p.i. in the absence of gp130 signaling (Figure S3D). However, the total number of Treg cells was unaltered in gp130-deficient animals at both time points studied. Stimulation of CD4$^+$ T cells from both WT and *Cd4*-cre *Il6st*$^{fl/fl}$ mice at day 9 p.i. with LCMV GP$_{67–77}$ peptide resulted in moderate IFN-γ production and low IL-21 secretion as previously reported (Figure 3A; Elsaesser et al., 2009). At day 30 p.i., we observed fewer peptide-responsive IFN-γ$^+$ CD4$^+$ T cells, consistent with the reduced number D^b GP$_{67–77}$ $^+$ CD4$^+$ T cells in *Cd4*-cre *Il6st*$^{fl/fl}$ mice. *Cd4*-cre *Il6st*$^{fl/fl}$ IFN-γ$^+$ CD4$^+$ T cells also produced significantly less IL-21 than WT CD4$^+$ T cells after GP$_{67–77}$ ex vivo peptide stimulation. Similar observations were seen in response to polyclonal PMA and Ionomycin stimulation (Figure 3B). Consistently, *Il21* transcripts were significantly reduced in purified D^b GP$_{67–77}$$^+$ CD4$^+$ T cells from *Cd4*-cre *Il6st*$^{fl/fl}$ mice compared to WT controls (Figure 3C). These results indicated that gp130 signaling is not only essential for CD4$^+$ T cell maintenance and differentiation, but it is also necessary for them to produce IL-21 at late stages of chronic LCMV infection. The defective IL-21 production in the absence of T cell gp130 signaling contrasted with unchanged amounts observed in *Il6*$^{-/-}$ mice (Harker et al., 2011). This implied the presence of gp130 cytokines (other than IL-6) that regulated this fundamental CD4$^+$ T cell function during an established chronic viral infection.



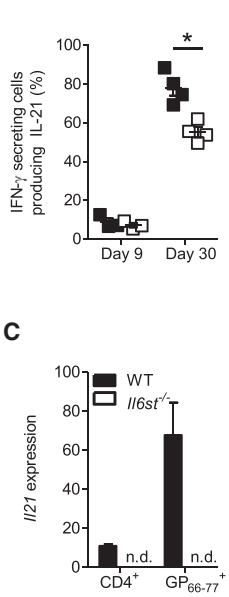


Figure 3. T Cell Specific gp130 Signaling Is Required for IL-21 Production during Chronic LCMV Infection

WT (C57B/6 or *Il6st*$^{fl/fl}$) and *Cd4*-cre *Il6st*$^{fl/fl}$ mice were infected with 2 × 10^6 pfu of LCMV Cl13 i.v. (A and B) At days 9 and 30 p.i., splenocytes were stimulated with (A) LCMV GP$_{67–77}$ peptide or (B) PMA and ionomycin and the number of IFN-γ secreting cells (GP$_{67–77}$-specific only), and the proportion of IFN-γ$^+$ cells that were also secreting IL-21, were determined by flow cytometry. (C) At day 30 p.i., CD4$^+$ and I-A^b LCMV GP$_{67–77}$$^+$ cells were isolated by flow cytomtery, and *Il21* expression, relative to *Gapdh*, was determined by qPCR. n.d. = not detectable; data are representative of three experimental repeats, n ≥ 4 mice per group with mean ± SEM depicted. This figure is supported by Figure S3.

Consistent with defective CD4$^+$ T cell accumulation and function we observed dramatic effects on humoral immunity in *Cd4*-cre *Il6st*$^{fl/fl}$ compared to WT mice. This included lower numbers of germinal center B cells, reduced concentrations of LCMV-specific immunoglobulin (Ig), IgG, IgG1, and IgG2a antibodies and weakened avidity of LCMV-specific IgG (Figure S4). These results further demonstrate the need for gp130 signaling in CD4 T cells for optimal antibody responses.

Cell-Intrinsic gp130 Is Essential for Virus Specific CD4$^+$ T Cells Accumulation and IL-21 Production

The loss of T cell-specific gp130 signaling, although not affecting T cell development or the initial expansion of either virus-specific CD4$^+$ or CD8$^+$ T cell responses during LCMV infection, had significant impact on the number of virus-specific T cells at later stages of infection (day 30 p.i.) and prevented viral control. To determine the role of intrinsic gp130 signaling on either CD8$^+$ or CD4$^+$ T cells, we generated mixed WT:*Cd4*-cre *Il6st*$^{fl/fl}$ bone marrow (BM) chimeric mice. Injection of an equal ratio of WT and *Cd4*-cre *Il6st*$^{fl/fl}$ BM cells favored development of WT T cells, with an approximately 70:30 (WT:*Il6st*$^{-/-}$) ratio of CD8$^+$ and CD4$^+$ T cells at 8 weeks after reconstitution (Figure S5A). The bias to WT cells seen was similar to that described in mixed chimeras reconstituted with equal proportions of WT and *Il6ra*$^{-/-}$ deficient BM cells (Harker et al., 2011) suggesting that IL-6 played a role in the development of T cells under competitive conditions.

At day 30 after LCMV Cl13 infection, the numbers of H2-D^b LCMV GP$_{33–41}$ CD8$^+$ T cells were reduced in the gp130-deficient compartment versus WT in some, but not all, mixed chimeras, reaching a p value equal to 0.0649 (Figure 4A). In contrast, as in *Cd4*-cre *Il6st*$^{fl/fl}$ mice, the number of D^b GP$_{67–77}$$^+$ CD4$^+$ T cells were significantly and consistently reduced in the *Il6st*$^{-/-}$ compared to WT cells, and the total number of gp130-deficient virus-specific cells was only 3.5% of WT cells in this setting (Figure 4B). Tfh cell differentiation in the remaining virus-specific CD4$^+$ T cells, and total CD4$^+$ T cells, was also dramatically reduced (Figure S5B). Consistently, there was low or undetectable expression of *Bcl6* in virus-specific CD4$^+$ T cells in the absence of gp130, whereas *Prdm1* (Blimp-1) expression was lower but detectable (Figure S5C).

Deletion of T cell-specific *Il6st* during intestinal nematode infection, in otherwise highly susceptible IL-10-deficient animals, resulted in enhanced Th2 CD4$^+$ T cell and reduced Th1 and Th17 CD4$^+$ T cell, mediated immunity, and accelerated parasite expulsion and protection (Fasnacht et al., 2009). In this study, we saw slight reductions in the expression of both *Tbx21* (T-bet) (Th1) and *Gata3* (Th2) in virus-specific CD4$^+$ T cells in the absence of *Il6st*, but no change in the balance of the immune response (Figures S5E–S5F), whereas we saw little to no *Rorc* expression indicating few or no Th17 cells were present (data not shown). This difference is most likely as a result of the different infectious systems used, because this is the first report of the overall role of T cell-specific gp130 signaling during a viral infection.

The proportion of IFN-γ-producing cells that were also producing IL-21 was significantly reduced in the absence of gp130, indicating that cell intrinsic gp130 signaling is required for IL-21 production after ex vivo peptide stimulation (Figure 4C). Accordingly, amounts of *Il21* transcript measured directly ex vivo were undetectable in *Il6st*$^{-/-}$ I-A^b GP$_{67–77}$ CD4$^+$ T cells from mixed chimeras, whereas they were readily detectable in their WT counterparts (Figure 4D). These results indicate that although CD8$^+$ T cells might be modestly influenced by intrinsic gp130 signaling, CD4$^+$ T cells are greatly dependent on direct gp130 signaling to both accumulate and acquire key helper properties during late chronic LCMV infection.

Direct gp130 Signaling Promotes CD4$^+$ T Cell Survival during Chronic Viral Infection

To discern whether the reduced numbers of virus-specific CD4$^+$ T cells resulted from increased cell death, reduced proliferation, or both, we analyzed apoptosis and cell cycle entry in WT *versus* *Cd4*-cre *Il6st*$^{fl/fl}$ mice 30 days after LCMV Cl13 infection. Annexin

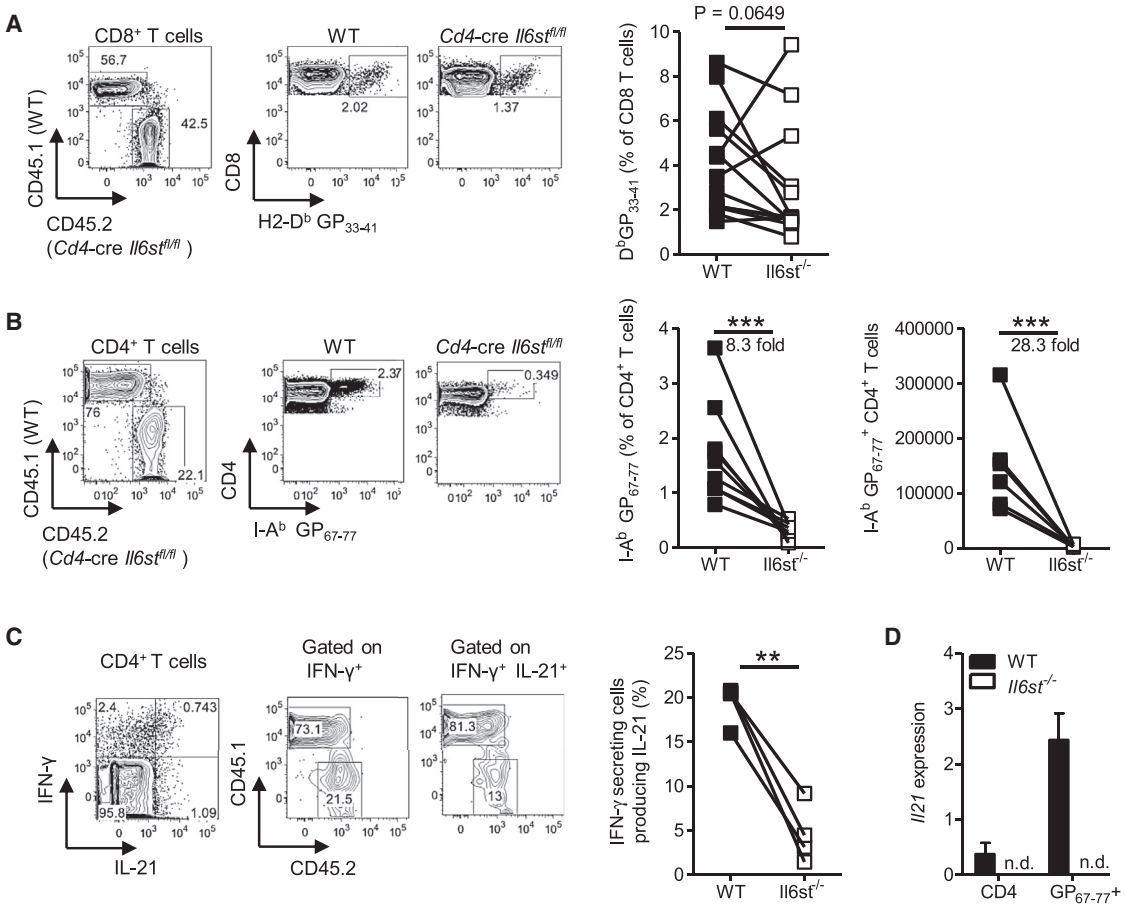


Figure 4. Cell-Intrinsic gp130 Signaling Is Required for CD4$^+$ T Cell Accumulation and IL-21 Production

Congenic WT (CD45.1) mice were lethally irradiated and reconstituted with a 50:50 mix of CD45.1 and *Cd4*-cre *Il6st*$^{fl/fl}$ (CD45.2) bone marrow cells. Eight weeks later, chimeras were infected with 2 × 10^6 pfu of LCMV Cl13. At day 30 p.i., the percentages of splenic CD8$^+$ T cells that were H2-D^b GP$_{33–41}$ $^+$ (A) and the percentage and number of splenic CD4$^+$ T cells that were I-A^b GP$_{67–77}$$^+$ (B) in CD45.1$^+$ or CD45.2$^+$ compartments were determined by flow cytometry. The fold increase between WT and *Il6st*$^{-/-}$ is depicted.

(C) Splenocytes were stimulated ex vivo with GP$_{67–77}$ peptide and the proportion of IFN-γ-producing CD4$^+$ T cells that were also producing IL-21 was determined by flow cytometry and (D) *Il21* expression determined in I-A^b GP$_{67–77}$$^+$ CD4$^+$ T cells by qPCR. n.d. = not detectable. Data are representative of four independent repeats from pooled samples of n ≥ 4 mice, with mean ± SEM depicted. This figure is supported by Figure S4.

V staining revealed that a higher proportion of virus-specific CD4$^+$ T cells were undergoing apoptosis in *Cd4*-cre *Il6st*$^{fl/fl}$ compared to WT mice; and this difference was further accentuated in the competitive environment generated in WT:*Cd4*-cre *Il6st*$^{fl/fl}$ mixed chimeras (Figures 5A and 5B). These results were validated by the presence of increased cleaved caspase-3 in *Il6st*$^{-/-}$ PD1$^+$ CD4$^+$ T cells compared to WT cells, another indicator of increased cell-intrinsic apoptosis (Figure 5C).

In contrast, analysis of bromodeoxyuridine (BrdU) incorporation revealed that the proliferation of virus-specific CD4 T cells was enhanced (likely as a result of a compensatory effect) in *Cd4*-cre *Il6st*$^{fl/fl}$ mice, but similar in WT and *Il6st*$^{-/-}$ cells in the mixed chimeric setting (Figures 5D and 5E). There was a very low frequency of BrdU$^+$ virus-specific CD4$^+$ T cells at day 30 p.i. in the mixed chimera; however, in the same system WT PD1$^+$ CD4$^+$ T cells and *Il6st*$^{-/-}$ PD1$^+$ CD4$^+$ T cells showed detectable, and similar proportions of, Ki67$^+$ cells supporting the absence of an intrinsic proliferation defect when gp130 signaling is not present (Figure 5F). Overall, these data indicate that cell-intrinsic gp130 signaling was fundamental in promoting CD4$^+$ T cell survival at late stages of chronic LCMV infection.

IL-27 Drives Accumulation of Virus-Specific CD4$^+$ T Cells and Promotes IL-21 Production Ex Vivo

To determine which of the cytokines that use gp130 was involved in promoting CD4$^+$ T cell survival and IL-21 production, we examined their individual capacity to phosphorylate the downstream molecules STAT-1 and STAT-3. In CD4$^+$ T cells taken from naive animals IL-6, IL-27, OSM, and LIF were all capable of STAT-3 phosphorylation, while IL-6 and IL-27 also caused phosphorylation of STAT-1 (Figure 6A). As we have shown previously, ex vivo IL-6 stimulation of transgenic LCMV GP$_{61–80}$-specific CD4$^+$ T (Smarta) cells taken from WT mice 18 days after LCMV Cl13 infection also caused rapid phosphorylation of STAT-3 (Figure 6A) (Harker et al., 2011). However, IL-6 stimulation of Smarta cells did not promote STAT-1 phosphorylation. In comparison, IL-27 stimulation of the same Smarta cells caused slightly weaker phosphorylation of STAT-3, but

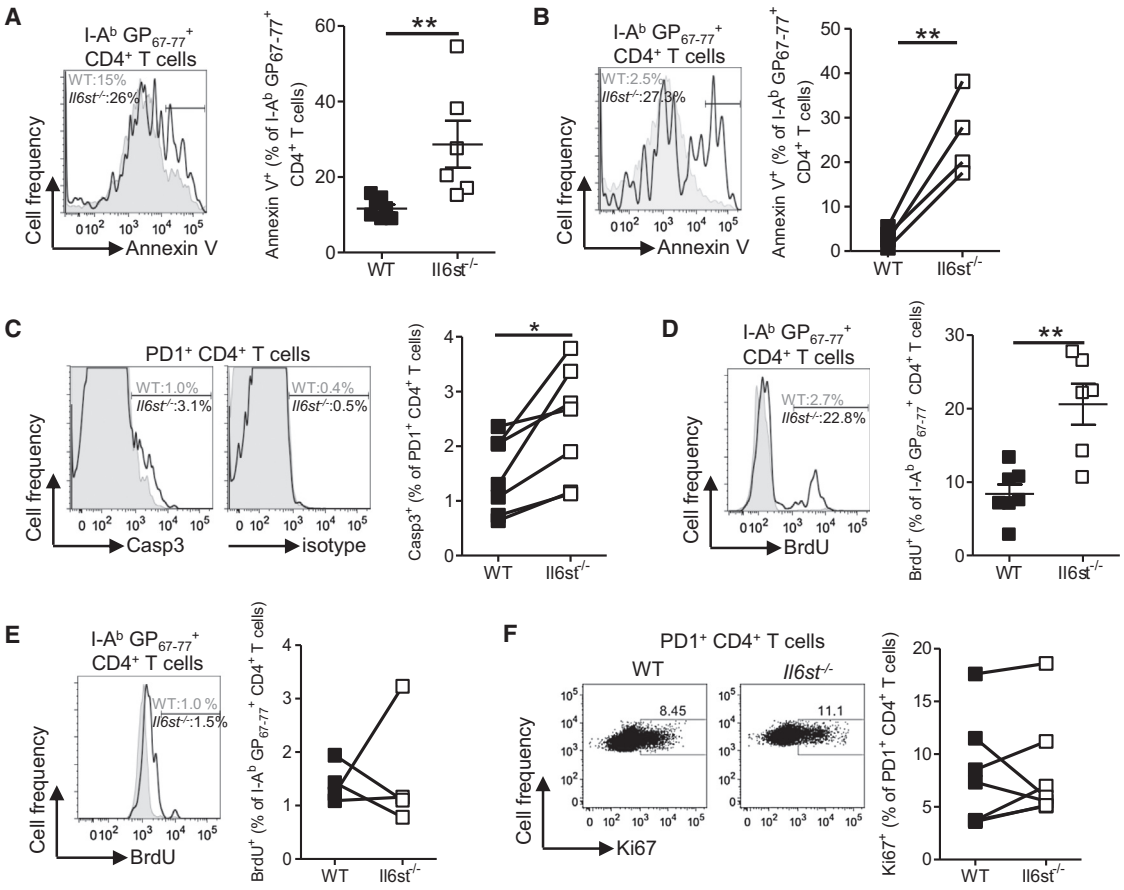


Figure 5. Virus-Specific CD4$^+$ T Cells Exhibit an Intrinsic Survival Defect in the Absence of gp130 Signaling

WT, *Cd4*-cre *Il6st*$^{fl/fl}$.

(A and D) or WT:*Cd4*-cre *Il6st*$^{fl/fl}$ mixed chimeric (B, C, E, and F) mice were infected with 2 × 10^6 pfu of LCMV Cl13 i.v. and splenocytes analyzed by flow cytometry at day 30 p.i.

(A and B) The percentage of Annexin V binding in I-A^b GP$_{67–77}$$^+$ CD4$^+$ T cells was determined.

(C) The percentage of PD1$^+$ CD4$^+$ T cells expressing cleaved Caspase-3$^+$ was determined.

(D and E) The proportion of I-A^b GP$_{67–77}$$^+$ CD4$^+$ T cells that had incorporated BrdU was quantified.

(F) The proportion of Ki67$^+$ was analyzed within PD1$^+$ CD4$^+$ T cells. Data are representative of two or three independent experiments of n ≤ 4 mice per group, with mean ± SEM depicted. This figure is supported by Figure S5.

pronounced phosphorylation of STAT-1. Stimulation with LIF, IL-11, and OSM phosphorylated neither STAT-1 nor STAT-3.

Because IL-27 was the only tested member of the IL-6 family of cytokines, other than IL-6, capable of directly signaling on virus-specific CD4$^+$ T cells during LCMV Cl13 infection, we evaluated its cell-intrinsic effects on CD4$^+$ T cells in vivo. In WT:*Il27ra*$^{-/-}$ mixed bone marrow chimeras, unlike *Cd4*-cre *Il6st*$^{fl/f}$ and *Il6ra*$^{-/-}$ mixed bone marrow chimeras, the *Il27ra*$^{-/-}$ CD4$^+$ T cell compartment was similar in proportion to the WT CD4$^+$ T cell compartment after reconstitution (Figure S6A). There was, however, a slight bias toward WT CD8 T cells and *Il27ra*$^{-/-}$ B cells during reconstitution, which could indicate a role for IL-27 during lymphocyte development (Figure S6A). After LCMV Cl13 infection, direct IL-27 signaling on CD4$^+$ T cells was vital for the accumulation of virus specific CD4$^+$ T cells, indicated by the reduced proportion of I-A^b GP$_{67–77}$$^+$ CD4$^+$ T cells in the *Il27ra*$^{-/-}$ compared to the WT compartment, as was seen in *Cd4*-cre *Il6st*$^{fl/fl}$ mice (Figure 6B). However, IL-21 production in the remaining *Il27ra*$^{-/-}$ virus-specific CD4$^+$ T cells was similar to that seen in WT CD4$^+$ T cells (Figure 6C) as was the proportion of virus-specific CD4 T cells that expressed Bcl6 and differentiated into Tfh cells (Figures S6B and S6C).

Stimulation of polyclonal virus-specific PD-1$^+$ CD4$^+$ T cells taken 18 days after LCMV Cl13 infection with recombinant IL-27 resulted in rapid upregulation of *Il21*, with the amount of transcript peaking at 6 hr after stimulation, whereas as we have previously shown, IL-6 also caused the upregulation of *Il21* transcript, but this peaked at 12 hr after stimulation (Figure 6D). Increased *Il21* transcript was also seen 6 hr after IL-27 stimulation in Smarta CD4$^+$ T cells (Figure 6E). Ex vivo IL-27 stimulation also lead to upregulation of *Bcl6* expression in PD-1$^+$ and Smarta CD4$^+$ T cells (Figures S6D and S6E). Together, these data show that IL-27 plays an essential role in the accumulation of virus-specific CD4$^+$ T cells at late stages during chronic viral infections. Furthermore, both IL-27 and IL-6 were capable of rapidly upregulating IL-21 in virus-specific CD4$^+$ T cells but

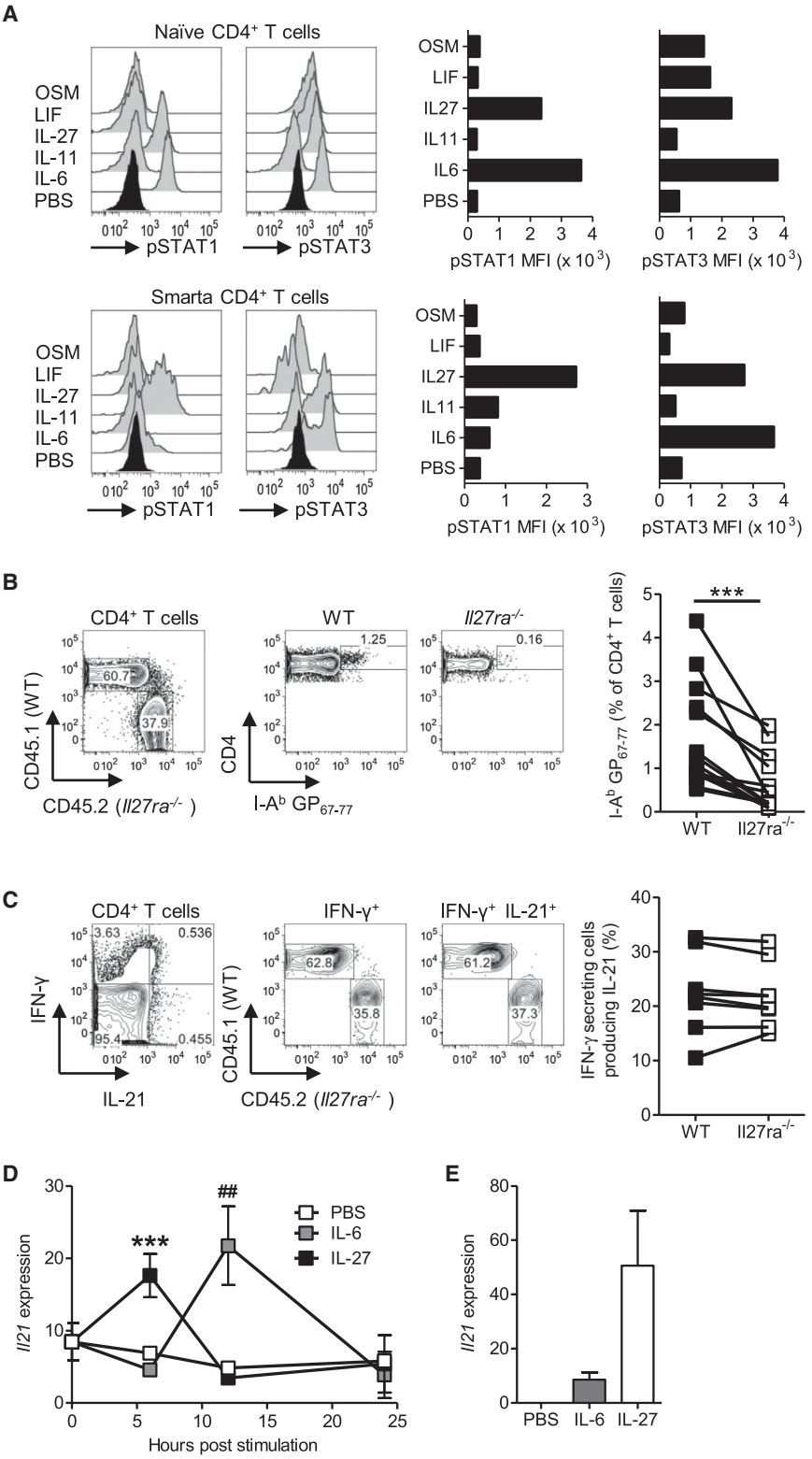


Figure 6. IL-27 Signaling on Virus-Specific CD4$^+$ T Cells Is Vital for Their Accumulation during Chronic Viral Infection

(A) LCMV-specific CD45.1$^+$ transgenic CD4$^+$ T cells (Smarta) were transferred i.v. 1 day prior to LCMV Cl13 infection, and splenocytes were isolated at 18 days p.i. The amount of pSTAT1 and pSTAT3 were then determined in naive and Smarta CD4$^+$ T cells 30 min after ex vivo stimulation with 50 ng/ml of the indicated recombinant cytokines by flow cytometry.

(B and C) WT:*Il27ra*$^{-/-}$ mixed bone marrow chimeras were generated and infected with LCMV Cl13. 30 days p.i., the proportion of splenic CD4$^+$ T cells that were I-A^b Gp$_{67–77}$$^+$ (B) and CD4$^+$ T cells that were IFN-γ$^+$ IL-21$^+$ after GP$_{67–77}$ peptide stimulation (C) were determined by flow cytometry.

(D and E) At day 18 after LCMV Cl13 infection, PD1$^+$ CD4$^+$ T cells (D) or Smarta cells (E) prepared as in (A) were isolated by flow cytometry. Expression of *Il21* relative to *Gapdh* was determined 6, 12, and 24 hr (D) or 6 hr (E) after ex vivo stimulation with IL-6, IL-27, or PBS. (A) is representative of two independent experiments with pools of five mice per group. (B), (C), and (D) and (E) are representative of five, three, and two independent experiments, respectively, with mean ± SEM depicted. This figure is supported by Figure S6.

We next quantified the number of virus-specific CD4$^+$ T cells in the spleens of WT versus *Il27ra*$^{-/-}$ nonchimeric mice at days 9 and 30 after infection (Figure 7A). We observed higher percentage and numbers of virus-specific CD4$^+$ T cells in *Il27ra*$^{-/-}$ compared to WT mice at day 9 p.i. Because this early increase in the CD4$^+$ T cell responses was not observed in *Cd4*-cre *Il6st*$^{fl/fl}$ mice (Figure 2D), it may result from CD4$^+$ T cell-extrinsic effects due to lack of IL-27R signaling in other cell populations. *Il27ra*$^{-/-}$ mice showed a significant decline in the proportion and numbers of LCMV-specific CD4$^+$ T cells from day 9 to day 30 p.i., whereas in WT mice the number of LCMV-specific CD4$^+$ T cells was maintained or slightly increased. This defect in anti-viral CD4$^+$ T cells maintenance in *Il27ra*$^{-/-}$ mice was comparable with the one observed in *Cd4*-cre *Il6st*$^{fl/fl}$ mice and was also detected in blood samples, where the number of PD1$^+$CD4$^+$ T cells was significantly lower at day 45 p.i (Figures 7B and 7C, respectively). These results were consistent with our conclusions from *Cd4*-cre *Il6st*$^{fl/fl}$ mice, WT:*Cd4*-cre *Il6st*$^{fl/fl}$ and WT:*Il27ra*$^{-/-}$ mixed chimeras that cell-intrinsic IL-27R and gp130 signaling promotes the maintenance of virus-specific CD4$^+$ T cell numbers at late stages of chronic viral infection.

were redundant for its production in vivo, where IL-21 production could only be reduced by removal of IL-6 and IL-27 common coreceptor, gp130.

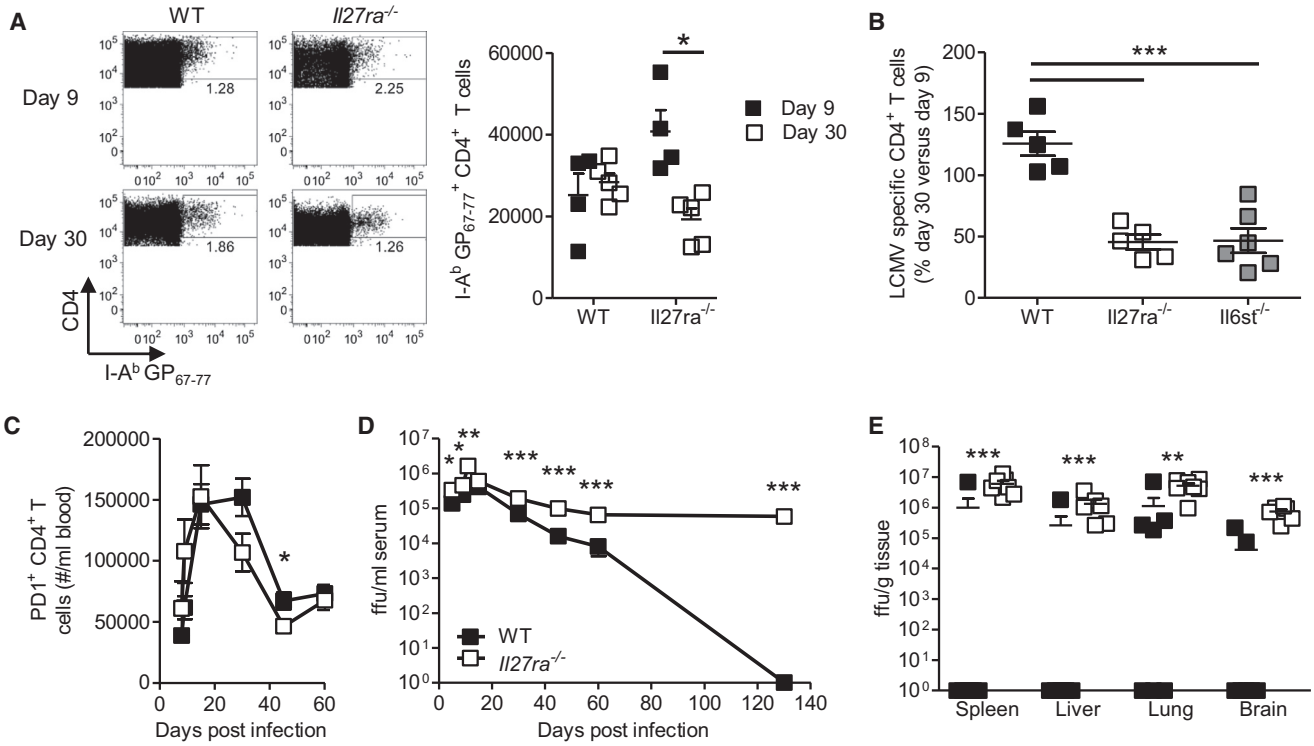


Figure 7. IL-27 Is Critical in Controlling Viral Load during Chronic LCMV Infection

WT or *Il27ra*$^{-/-}$ animals were infected with LCMV Cl13.

(A and B) Splenic I-A^b GP$_{67–77}$$^+$ CD4$^+$ T cells at days 9 and 30 p.i. were enumerated by flow cytometry (A). Example flow plots are shown and the proportion of D^b GP$_{67–77}$$^+$ CD4$^+$ T cells at day 30 p.i. relative to day 9 p.i. was compared to that observed in *Cd4*-cre *Il6st*$^{fl/fl}$ animals (B).

(C) PD-1$^+$ CD4$^+$ T cells were enumerated in the blood by flow cytometry.

(D and E) Viral load was determined in the blood (D) at various time points and in tissues (E) at day 130 after infection. Data is representative of n = 2 experimental repeats of n = 5 mice per group, with mean ± SEM depicted.

Finally, we assessed the importance of IL-27R signaling in overall viral control. *Il27ra*$^{-/-}$ mice exhibited significantly higher viremia than their WT counterparts as early as between days 5 and 9 p.i. (Figure 7D). Given that deletion of *Il6st* exclusively in T cells did not affect early control of LCMV Cl13 (Figure 1A), it is likely that this initial difference in viral loads is a consequence of deficient IL-27 signaling in non-T cells. Infection of *Il27ra*$^{-/-}$ mice also resulted in a failure of the long term control of persistent LCMV with virus detectable in the blood and a range of tissues up to 130 days after infection, when the majority of WT animals had cleared virus (Figure 7D and 7E). Altogether, these data indicate that IL-27 signaling plays a critical role in the maintenance of CD4$^+$ T cell numbers and viral control during chronic viral infection in vivo.

DISCUSSION

The presence and function of antigen-specific CD4$^+$ T cells is vital for optimal immune responses in many infectious contexts, but especially during chronic viral infections. Here we showed that direct gp130 signaling was vital for the survival of virus-specific CD4$^+$ T cells and for their key functional properties such as IL-21 production during chronic viral infection in vivo. Further, we demonstrated that different members of the IL-6 family have both unique and redundant roles in this process; IL-27 was essential for CD4$^+$ T cell accumulation, while we have previously shown that IL-6 plays a dominant role in the differentiation of Tfh cells. On the other hand, although both IL-6 and IL-27 were capable of promoting IL-21 production ex vivo they were redundant in vivo, suggesting that multiple gp130 signaling cytokines secure optimal IL-21 production.

Higher concentrations of IL-21 are associated with lower viral loads in HIV and HCV infections (Chevalier et al., 2011; Iannello et al., 2010; Yue et al., 2010) and with an increased success rate in vaccination of chronically infected individuals (Pallikkuth et al., 2011). STAT-3 is heavily associated with the transcription of *Il21* (Kaplan et al., 2011). Engagement of gp130 with cytokine-specific receptors results in rapid phosphorylation of STAT-3 (Ernst and Jenkins, 2004) and our study revealed a vital role for gp130 signaling in IL-21 production in vivo at late stages of chronic LCMV infection. STAT-3 binds not only at the *Il21* promoter but also at *Batf* and *Maf* loci (Durant et al., 2010), two other transcription factors known to promote IL-21 (Hiramatsu et al., 2010; Schraml et al., 2009), suggesting multiple molecular pathways through which gp130 signaling might trigger and maintain IL-21 production. Once produced, IL-21 itself could act in an autocrine fashion by further phosphorylating STAT-3. Both IL-6 and IL-27, were capable of inducing STAT-3 phosphorylation and *Il21* transcription in virus-specific CD4$^+$ T cells taken from chronically infected mice but none of the other gp130 cytokines

tested (i.e., IL-11, OSM, and LIF) were capable of phosphorylating STAT-3 in LCMV-specific $CD4^+$ T cells. Given the essential nature of gp130 but nonessential roles for IL-6R or IL-27R, our data strongly suggests that IL-6 and IL-27 act redundantly to promote IL-21 production in $CD4^+$ T cells. Although our study focused on chronic viral infection, it is conceivable that gp130 signaling may trigger IL-21 production in the context of autoimmune diseases such as diabetes, multiple sclerosis, and Sjogren's syndrome where IL-21 is associated with increased pathology (Jones et al., 2009; McGuire et al., 2011; Spolski et al., 2008).

IL-27-gp130 signaling promoted the survival of virus-specific $CD4^+$ T cells at late stages of chronic LCMV infection. However, it was dispensable for $CD4^+$ T cell accumulation early after infection, indicating that other signaling pathways must be involved in inducing $CD4^+$ T cell proliferation and survival at these time points. Likely candidates are the common γc cytokines such as IL-2, IL-7, and IL-15, which are potent mediators of both naive and activated T cell survival and proliferation (Rochman et al., 2009). Indeed provision of either IL-2 or IL-7 is sufficient to rescue mice from LCMV Cl13 infection (Blattman et al., 2003; Pellegrini et al., 2011). Many of their effects are mediated by activation of STAT-5, which also blocks the differentiation of Tfh cells (Johnston et al., 2012; Nurieva et al., 2012). Tfh cells accumulate in LCMV Cl13 infected mice during late stages of infection, at a time when IL-2 production is compromised by T cell exhaustion (Brooks et al., 2005), which may indicate reduced γc-STAT5 signaling, leaving $CD4^+$ T cells reliant on gp130 signaling for survival. In $CD8^+$ T cells, although STAT-3 is not required for initial expansion, it is essential for the formation of long-lived memory cells, providing evidence that T cells can progressively alter their STAT requirements for accumulation and survival (Cui et al., 2011). Both IL-6 and IL-21 can phosphorylate STAT-3, however, whereas only IL-27 signaling results in enhanced $CD4^+$ T cell survival in vivo. Meanwhile, only IL-27 phosphorylated STAT-1 in virus-specific $CD4^+$ T cells, suggesting that STAT-1 could promote $CD4^+$ T cell survival late during chronic infection. Phosphorylation of STAT-1 by IL-27 in this context is most likely due to its use of WSX-1, which contains a box 1 janus kinase (JAK) domain capable of activating the STAT family independently of gp130 (Sprecher et al., 1998). In many contexts, STAT-1 is thought to activate an antiproliferative, proapoptotic pathway in T cells (Bromberg et al., 1996; Gil et al., 2006; Tanabe et al., 2005); thus, exploration of STAT-1's functions at late stages of infection is worthy of further investigation. IL-27 is also known to phosphorylate STAT-4 and STAT-5, although less potently than it does STAT-1, both of which have potent effects on $CD4^+$ T cell differentiation and could have pro-survival effects via these pathways (Kaplan, 2005; Lucas et al., 2003).

The loss of IL-27R signaling across the whole mouse resulted in reduced viral control and a slight increase in the number of virus-specific $CD4^+$ T cells at early stages of viral infection. Because this was not observed when *Il6st* was conditionally ablated in T cells, nor in mixed chimeras generated from either *Il27ra*$^{-/-}$ or *Cd4*-cre *Il6st*$^{fl/fl}$ mice, it suggests that IL-27 signaling also has a vital non-T cell role in antiviral immunity early during LCMV Cl13 infection. Fitting with this, effective IL-27 signaling has also been shown in natural killer cells, NK T cells, B cells, and macrophages (Imamichi et al., 2008; Villarino et al., 2005; Yoshimoto et al., 2004); however, the effects of IL-27 signaling on cells other than $CD4^+$ T cells during chronic viral infection is yet to be explored. It is possible that the presence of higher viral loads early during LCMV Cl13 infection plays a role in the increased virus-specific $CD4^+$ T cell numbers observed in LCMV Cl13 infected *Il27ra*$^{-/-}$ mice at day 9 p.i. Alternatively, this could be a consequence of IL-27's regulation of cell populations that directly influence $CD4^+$ T cell expansion (e.g., antigen-presenting cells). Our data therefore not only show the importance of IL-27 in the maintenance of virus-specific $CD4^+$ T cell responses and viral control during chronic viral infection in vivo but also highlight the complex and significant role that IL-27 plays in different aspects of immune responses and the need to use models that allow discrimination of cell-intrinsic versus -extrinsic effects (such as mixed bone marrow chimeras) in the exploration of these processes.

IL-27 signaling has previously been implicated in controlling Tfh cells numbers after vaccination (Batten et al., 2010), whereas we have previously observed that IL-6 plays an essential role in Tfh cells differentiation during chronic viral infection (Harker et al., 2011). In this context, IL-27 appears to be essential for the survival of virus-specific $CD4^+$ T cells, the majority of which are Tfh cells, but does not appear to control Tfh cell differentiation or Bcl6 expression. Complete loss of gp130-mediated signaling, however, results in loss of both virus-specific $CD4^+$ T cells and Tfh cell differentiation. This leads us to hypothesize that during chronic viral infection, IL-6 is vital for Tfh cell differentiation, whereas IL-27 is required for their ongoing survival.

The number of $CD8^+$ and $CD4^+$ T cells is critical in determining the outcome of chronic viral infections in mice and humans (Rehermann and Nascimbeni, 2005; Virgin and Walker, 2010; Virgin et al., 2009). $CD4^+$ T cells are often not required for the initial generation of virus-specific $CD8^+$ T cell responses, but they are vital for the maintenance and quality of these cells and are also fundamental for B cell responses (Matloubian et al., 1994b; Shedlock and Shen, 2003). Notably, the use of vaccines that target both the cytotoxic and humoral arms of the immune system, such as the RV144 prime-boost HIV vaccine trial (Benmira et al., 2010), have demonstrated the efficacy of this approach compared to prior vaccines that aimed to target a single aspect of immunity. Our data places gp130 signaling at the center of $CD4^+$ T cell responses within the highly immunosuppressive environment of an established chronic viral infection and highlights the potential of this pathway as a target for simultaneously empowering both humoral and cellular immunity in this context.

EXPERIMENTAL PROCEDURES

Mice and Viral Stocks

WT C57BL/6 mice, C57BL/6 CD45.1$^+$ mice, and *Il27ra*$^{-/-}$ mice were purchased from The Jackson Laboratory. *Cd4*-cre *Il6st*$^{fl/fl}$ mice were kindly provided by Dr. Werner Mueller (University of Manchester, UK). Mice were bred and maintained in a closed breeding facility, and mouse handling conformed to the requirements of the National Institutes of Health (NIH) and the Institutional Animal Care and Use Guidelines of UCSD. Unless otherwise stated, 6- to 8-week-old mice were infected intravenously (i.v.) with 2×10^6 pfu of LCMV ARM or Cl13. Viruses were grown, identified, and quantified as described in (Ahmed et al., 1984; Borrow et al., 1995). For viral quantification by focus forming, assay vero cells (ATCC) were seeded in 96 well plates and

incubated with serial dilutions of serum or tissue homogenate for 20 hr. Cells were then fixed with 1% paraformaldehyde for 30 min, blocked with PBS containing 10% FBS for 1 hr, and incubated with supernatant from 113 hybridoma cells (A mouse B cell hybridoma producing monoclonal IgG1 against LCMV NP kindly provided by M.B. Oldstone, TSRI) for 1 hr. Finally, the cells were incubated with a Cy3 conjugated goat anti-mouse IgG (Jackson Immunoresearch) for 40 min and foci counted by fluorescent microscope.

Generation of BM Chimeras

BM chimeras were generated via transfer of a 50:50 ratio of BM cells from *Cd4*-cre *Il6st*$^{fl/fl}$ or *Il27ra*$^{-/-}$ mice and C57BL/6 CD45.1^{+} mice into lethally irradiated C57BL/6 CD45.1^{+} as described previously (Harker et al., 2011).

LCMV-Specific Antibody ELISAs

LCMV-specific ELISAs and avidity assays were done as we and others have previously described (Hammond et al., 1997; Harker et al., 2011).

Flow Cytometry

Flow cytometry was done as previously described (Tinoco et al., 2009). The following fluorochrome labeled antibodies purchased from ebioscience or BD biosciences were used to stain blood or spleen cells: anti-CD8-pacific blue, -CD4-pacific blue, -CD19-PE, -CD38-Alexa Fluor 700, -GL7-FITC, -PD1-PE-Cy7, -ICOS-PE, -SLAM-FITC or PE-Cy7, -CD45.2-APC-Cy7, -CD45.1-PerCy5.5, -CD200-PerCP-eFlour 610, -IFN-γ-APC, -TNF-α-FITC, -IL-2-PE, -CD107a-FITC, -CD107b-FITC, Annexin-V-PE, and -B220-FITC. CXCR5, FoxP3, and Bcl6 (BD, K112-91) staining were done as previously described (Harker et al., 2011). Cells were acquired by using the Digital LSR II flow cytometer (Becton Dickinson, San Jose, CA). To quantify incorporation of BrdU by tetramer^{+} T cells, we injected mice with 2 mg of BrdU (Sigma-Aldrich) 16 hr before analysis and splenocytes stained with BrdU Flow kit (BD Biosciences) following the manufacturer instructions. Staining for fluorescence assisted cell sorting was conducted in an identical fashion and cells were isolated on a FACSAria (Becton Dickinson, San Jose, CA) to >95% purify. Flow cytometric data were analyzed with FlowJo software (TreeStar, CA).

Ex Vivo T Cell Stimulation

For MHC class-I-restricted $GP_{33–41}$ peptide (2 μg/ml) or MHC class-II restricted $GP_{67–77}$ (5 μg/ml) stimulation and staining were carried out as we have previously described (Tinoco et al., 2009). For polyclonal stimulation, we used PMA (10 ng/ml) and ionomicyn (0.5 μg/ml) in place of peptide. For intracellular IL-21 staining, cells were permeabilized with saponin and incubated with 1:25 dilution of IL-21RhFc (R&D Systems) for 30 min at 4°C, washed twice and stained with 1:200 anti-human Fc-PE (BD PharMingen). For phosphorylation of STATs, 1,000 CD45.1^{+} Smarta CD4^{+} T cells were transferred i.v. 1 day prior to infection with 2 × 10^{6} pfu of LCMV Cl13. At 18 days p.i., splenocytes were isolated, rested, for 2 hr at 37°C, and stimulated with 50 ng/ml of recombinant human IL-6 (National Cancer Institute) or mouse IL-11, IL-27, LIF (eBioscience), or oncostatin M (Cell Signaling). Cells were fixed after 30 min, and pSTAT staining was done according to manufacturer's instructions (BD).

Real-Time RT-PCR

Total RNA was extracted from splenocytes by using RNeasy kits (QIAGEN), and reverse transcribed into cDNA by using superscript III RT (Invitrogen). cDNA quantification was performed by using SYBR Green PCR kits (Applied Biosystems) and a real-time PCR Detection System (ABI). Primers for the genes assessed are described in (Harker et al., 2011). *Il21* detection of IL-27 and IL-6 stimulated CD4^{+} T cells was carried out on 50,000 PD1^{+} CD4^{+} T cells and 10,000 Smarta cells per time point, resulting in *Il21* detection being 18-fold more sensitive in PD1^{+} CD4^{+} T cells compared to Smarta based on *Gapdh* expression.

Statistical Analysis

Unpaired Student's t tests or ANOVA tests were performed by using the InStat 3.0 software (GraphPad, CA). For mixed chimeras, paired t tests were used. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in all data shown.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.08.010.

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