IL-21 Restricts Virus-driven Treg Cell Expansion in Chronic LCMV Infection

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Abstract

Foxp3+ regulatory T (Treg) cells are essential for the maintenance of immune homeostasis and tolerance. During viral infections, Treg cells can limit the immunopathology resulting from excessive inflammation, yet potentially inhibit effective antiviral T cell responses and promote virus persistence. We report here that the fast-replicating LCMV strain Docile triggers a massive expansion of the Treg population that directly correlates with the size of the virus inoculum and its tendency to establish a chronic, persistent infection. This Treg cell proliferation was greatly enhanced in IL-21R-/- mice and depletion of Treg cells partially rescued defective CD8+ T cell cytokine responses and improved viral clearance in some but not all organs. Notably, IL-21 inhibited Treg cell expansion in a cell intrinsic manner. Moreover, experimental augmentation of Treg cells driven by injection of IL-2/anti-IL-2 immune complexes drastically impaired the functionality of the antiviral T cell response and impeded virus clearance. As a consequence, mice became highly susceptible to chronic infection following exposure to low virus doses. These findings reveal virus-driven Treg cell proliferation as potential evasion strategy that facilitates T cell exhaustion and virus persistence. Furthermore, they suggest that besides its primary function as a direct survival signal for antiviral CD8+ T cells during chronic infections, IL-21 may also indirectly promote CD8+ T cell poly-functionality by restricting the suppressive activity of infection-induced Treg cells.

Introduction

The immune system has to efficiently eliminate pathogens but simultaneously needs to avoid the potential self-damage and immunopathology caused by excessive immune activation. Therefore, a tight regulation of immune responses is critical for host survival. The subset of CD4+CD25+ regulatory T (Treg) cells exerts key negative regulatory mechanisms of the immune system that prevent autoimmunity and T cell mediated inflammatory disease [1]. Treg cells are best defined by expression of the signature transcription factor forkhead box P3, Foxp3 [2,3,4,5,6,7]. Their fundamental role in the maintenance of immune homeostasis and tolerance is well established [8,9,10] and unambiguously demonstrated by the severe multi-organ autoimmune disease, allergy and inflammatory bowel disease that develops in Foxp3-deficient mice or patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome [3,11,12,13]. However, the relevance of Treg cell responses for shaping adaptive immunity against pathogens, in particular in the context of chronic infections, remains much less understood.

Treg cells potentially have both beneficial and adverse effects on disease outcomes during viral infections. By dampening effector immune responses, Treg cell responses mitigate immunopathology resulting from exaggerated inflammation and tissue destruction during acute [14,15,16,17], or protracted infections [18,19,20,21,22]. In addition, Treg cells have been shown to support antiviral immunity by modulating T cell migration to the site of infection [15,23]. Conversely, Treg cells were shown to suppress CD8+ T cell responses in some infections [21,24], which may prevent immunopathology, but hampers effective pathogen control and ultimately promotes persistent infection [18,21,25,26]. Thus, while Treg cells favorably influence pathogen clearance in many acute infections [14,15,16,23], they seem to negatively regulate CD8+ T cell responses during chronic infections [18,19,20,24,26]. Furthermore, elevated numbers of Treg cells have also been associated with persistent viral infections in humans [27,28,29]. However, to date little is known as to whether Treg cell...
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Author Summary

T cell exhaustion represents a state of T cell dysfunction associated with clinically relevant diseases, such as persistent viral infections or cancer. Although the molecular signature of exhausted T cells has been characterized in detail at the functional and transcriptional level, the immunological mechanisms that lead to T cell exhaustion during chronic infections remain poorly understood. Our present study reports two major findings that illustrate a pathway that contributes to T cell exhaustion during viral infection, and indicate its modulation by both, the pathogen and the host. First, we show that a persistence-inducing virus triggers the massive proliferation of Foxp3γ regulatory T (Treg) cells and demonstrate the potential of Treg cells to promote T cell exhaustion and chronic infection. Second, we identify IL-21 as a crucial host factor that antagonizes this virus-driven expansion of the Treg population in a cell intrinsic manner independent of IL-2. Thus, in addition to its known pre-dominant direct positive effects on antiviral T cells, IL-21 can also alleviate the suppressive activity of Treg cells. Together, these results suggest enhanced Treg cell responses as a mechanism of immune evasion that could be therapeutically targeted with IL-21.

Results

Dose-dependent expansion of the Treg cell population in response to chronic viral infection

We examined the role of Foxp3γ Treg cell responses in chronic viral infections in mice infected with the fast-replicating strain of LCMV-DOC. LCMV-DOC is characterized by its potential to establish a chronic, persistent infection depending on the size of the virus inoculum. Although low doses of LCMV-DOC (2 × 10^2–2 × 10^4 PFU) induce potent CD8γ T cell-mediated immunity and are cleared in immunocompetent hosts, infection with intermediate and high doses (2 × 10^4–2 × 10^6 PFU) results in virus persistence due to the exhaustion of the virus-specific CD8γ T cell response [30,31]. By choosing different virus inocula, we determined dynamics of the Treg cell population during an acute, resolving infection and a chronic infection. After an initial decline of Treg cell numbers between days 0–7 that was independent of the dose of infection and the LCMV strain used (i.e. DOC or WE) (Figure 1A and data not shown), we observed a striking dose-dependent expansion and recovery of the Treg cell population in LCMV-DOC infected mice (Figure 1B, C) that directly correlated with the ability of the virus to establish persistence (Figure 1D). Compared to animals infected with a low dose of LCMV-DOC (200 PFU), mice infected with intermediate (2 × 10^4 PFU) or high (2 × 10^6 PFU) virus doses exhibited markedly increased proportions of Treg cells in spleen and peripheral organs that amounted up to 20% of all CD4γ T cells at 15 days post infection (Figure 1B, C). At this time, infectious virus had been cleared from the blood and organs of all mice infected with 200 PFU and some of the animals infected with 2 × 10^5 PFU of LCMV-DOC (Figure 1D and data not shown); while mice infected with persistence-inducing doses (2 × 10^4–2 × 10^6 PFU) of LCMV-DOC still exhibited high viral titers in blood, spleen, liver and kidney, and subsequently failed to control the infection (Figure 1D and data not shown). As expected, we also detected a dose-dependent reduction in the frequency of gp33-specific CD8γ T cells, which was indicative of the progressing exhaustion of the T cell response, particularly in the spleen (Figure 1E), and the resulting inability to resolve the infection (Figure 1D).

Consumption and bioavailability of IL-2 by Treg cells has been suggested to restrict IL-2-dependent effector T cell differentiation and expansion [43,44,45,46]. Conversely, IL-2-driven expansion of CD8γ T cell expansion during an immune response can occur at the expense of Treg proliferation/survival [47,48]. Moreover, IL-21 has been suggested to interfere with Treg-mediated suppression by inhibition of IL-2 [49]. To monitor IL-2 and IL-21 production during acute and chronic infection, we took advantage of Il2-emGFP-/Il21-mCherry dual reporter transgenic mice [50] that were infected with low dose (i.e. 200 PFU) LCMV-WE or high dose (i.e. 2 × 10^6 PFU) LCMV-DOC. Both Il2 (GFP) and Il21 (mCherry) were predominantly expressed by CD4γ compared to CD8γ T cells and frequencies increased from days 7–15 post infection with low dose LCMV-WE (Figure 1F–H). Interestingly, chronic infection with high dose LCMV-DOC potently...
suppressed Il2-emGFP expression, but did not affect Il21-mCherry expression by CD4+ T cells (Figure 1F–H).

Together, these data demonstrate a direct correlation between the size of the virus inoculum, CD8+ T cell dysfunctionality, virus persistence and the expansion of Treg cells, thus underlining a potential contribution of Treg cells to the impaired T cell function and the induction of viral persistence.

IL-21 antagonizes the virus-induced Treg cell expansion during chronic infection

IL-21 receptor (IL-21R) signaling is essential for the maintenance and sustained functionality of antiviral T cell responses in chronic infections [39,40,41]. As a consequence, IL-21R−/− animals have an impaired control of LCMV-DOC, and exhibit much higher virus loads [40]. In LCMV-DOC infected WT mice, the main Treg cell expansion was observed between days 10 to 20 post infection, peaking at 15 days before returning to almost naive levels by five weeks post infection (Figure 2A, B). Strikingly, this virus-driven Treg cell expansion was much more pronounced and longer lasting in mice lacking IL-21R expression, which suggested that the pro-inflammatory cytokine IL-21 restricted the proliferation of Treg cells in viral infections (Figure 2A, B). Indeed, the Treg cells of naive and LCMV-DOC infected WT mice expressed the IL-21R as assessed by flow cytometry (Figure 2C). We thus investigated whether the increased Treg expansion observed in IL-21R−/− mice represented a direct inhibitory effect of IL-21 on Treg cells or rather was related to the increased viral replication in these mice. To address this issue, we generated mixed bone marrow (BM) chimeras by reconstituting lethally irradiated WT (CD45.1+) mice with a 1:1 ratio of WT (CD45.1+) and IL-21R−/− (CD45.2+) BM, and evaluated their Treg cell responses to infection with LCMV-DOC. Analysis of naive BM chimeras confirmed similar reconstitution efficacy of the CD8+ and CD4+ T cell populations including Treg cells from both WT and IL-21R−/− donor BMs at 8 weeks after BM transfer (Figure 2D, and data not shown). However, upon infection of the population of IL-21R−/− Treg cells expanded 3-fold over that of WT Treg cells to represent 30% versus 10% of all CD4+ T cells, respectively (Figure 2E–G). Since this augmented proliferation of IL-21R−/− Treg cells was detected side by side to WT Treg cells in the same animals and identical viral loads, it clearly established the direct inhibitory effect of IL-21 on Treg cells in vivo. Nevertheless, we considered that the higher Treg cell numbers in infected IL-21R−/− mice could not only result from the absence of inhibitory IL-21R signaling but might also indicate a compensatory proliferation to overcome a potential functional deficit of the IL-21R−/− Treg cells. To exclude the latter possibility, we isolated CD4+CD25+ GFP+ Treg cells from WT DEREG and IL-21R−/− DEREG mice by FACS-sorting 15 days post infection with 2×105 PFU LCMV-DOC, and compared their suppressive activity in a classical T cell inhibition assay. As shown in Figure 2H, both WT and IL-21R−/− Treg cells comparably inhibited the proliferation of anti-CD3/CD28-stimulated naive CD25−/−CD4+ T cells in vitro, suggesting a normal function of IL-21R−/− Treg cells.

Thus, the increased expansion of IL-21R−/− Treg cells in LCMV-DOC infected mice highlighted an important inhibitory role of IL-21 in restraining Treg cell expansion during chronic LCMV infection. Except for a slightly reduced CD25 expression, IL-21R−/− Treg cells were comparable to WT Treg cells with respect to the expression or characteristic Treg cell surface markers (Figure 2I). We did not detect any IL-10 producing or gp61-specific Treg cells in infected mice, suggesting that the suppressive activity of Treg cells in LCMV-DOC infection involved neither IL-10-mediated suppression nor virus-specific Treg cells (Figure 2J and data not shown).

IL-6 has been suggested to regulate the balance between Treg and pro-inflammatory Th17 cell responses [51,52] similar to IL-21 [53]. While we have previously shown that IL-17–producing CD4+ T (Th17) cells were barely detectable in LCMV-DOC infected mice [40], it remains possible that IL-21 inhibits Treg cell expansion by regulation of IL-6. However, comparing IL-6+ WT and WT mice we found no differences in Treg cell expansion (Figure 2K), antiviral CD8+ and CD4+ T cell responses (Figure 2L, M), and virus titers (Figure 2N) up to day 30 post infection with 2×105 PFU LCMV-DOC. Together with the Treg cell-intrinsic negative IL-21R−/− signaling observed in WT:IL-21R−/− mixed BM chimeras, these data suggest that IL-21 restricted the virus-driven Treg cell expansion in LCMV-DOC infection independently of IL-6 signaling.

Depletion of Treg cells partially restores CD8+ T cell functionality and increases disease severity during chronic viral infection

To directly evaluate the impact of Treg cells on viral persistence, we next sought to analyze antiviral T cell responses and viral clearance in the absence of Treg cells. We therefore studied LCMV-DOC infection using the DEREG mouse model, in which Treg cells can be ablated by diphtheria toxin (DT) treatment due to transgenic expression of a high affinity DT receptor under control of the Foxp3 promoter [54]. DEREG and nontransgenic WT control mice were treated with DT and infected with 2×105 PFU LCMV-DOC on day 0, and the DT treatment was continued throughout the experiment (Figure 3A). Since a single DT injection depleted Treg cells in naive mice for 3 days, we injected DT every 3 days to achieve complete Treg cell ablation. While this treatment effectively depleted all Foxp3+ Treg cells in naive DEREG mice ([54], and data not shown), we consistently observed in LCMV-DOC infected DEREG mice the emergence of a residual GFP+Foxp3+ Treg cell population not depleted by DT, presumably due to lacking expression of the GFP-DTR fusion protein (Figure 3B). Compared to DT-treated WT controls, Treg cell-depleted DEREG mice exhibited a greatly enhanced morbidity in response to high dose LCMV-DOC infection, as indicated by an increased weight loss (Figure 3C). As a
result, a significant number of DT-treated, LCMV-DOC–infected DEREG mice had to be prematurely removed from the experiment and euthanized, whereas no equivalent morbidity was observed in DT-treated WT mice (Figure 3D). Thus, Treg cell depletion — even if not absolute — substantially aggravated the disease severity in LCMV-DOC–infected DEREG mice. Depletion of Treg cells did not affect the numbers of virus-specific CD8\(^+\) T cells, as indicated by the percentage of gp33-specific CD8\(^+\) T cells in spleens and livers (Figure 3E). However, Treg cell depletion to some extent restored the functionality of the antiviral CTL and significantly increased the frequencies of gp33-specific and overall splenic CD8\(^+\) T cells producing IFN-\(\gamma\) upon restimulation in vitro (Figure 3F). In comparison, Treg cell-depletion did not enhance frequencies of IFN-\(\gamma\)-producing virus-specific CD4\(^+\) T cells (Figure 3G). In spite of partly restoring CD8\(^+\) T cell cytokine responses, Treg cell depletion did not influence virus control, and LCMV-DOC replicated to comparably high levels in the blood and organs of DT-treated WT and DEREG mice at 15 days post infection (Figure 3H). Thus, Treg cells appeared to inhibit the functionality rather than the expansion of antiviral T cells. Consistent with the observed onset of immunopathology, the effect of Treg cell depletion on the antiviral T cell response was more pronounced at 10 days post infection (Figure 3I-M). Depletion of Treg cells resulted in higher percentages of gp33-specific CD8\(^+\) T cells (Figure 3J) and increased percentages of cytokine-producing antiviral CD8\(^+\) and CD4\(^+\) T cells (Figure 3K, L), yet did not affect viral titers (Figure 3M).

We next assessed the impact of increased Treg cell numbers in IL-21R\(^{-/-}\) mice and treated both IL-21R\(^{-/-}\) and WT mice with DT between days 8 to 15 post infection with 2000 PFU LCMV-DOC (Fig. 4A). Although Treg cells were considerably (but not entirely) depleted, frequencies of gp33-specific CD8\(^+\) T cells remained unchanged (Figure 4B, C) similar to the results obtained by depletion through the entire course of infection (Figure 3B, E). However, Treg cell depletion partially restored efficiencies of IFN-\(\gamma\)-producing gp33-specific cells in IL-21R\(^{-/-}\) mice to levels found in WT mice (Figure 4D). Furthermore, Treg cell depletion lowered virus titers significantly in liver and lung of IL-21R\(^{-/-}\) mice, although viral loads in spleen and kidney remained unaffected (Figure 4F).

Whether the differences in antiviral CD8\(^+\) T cells were too small to result in better virus control or whether the failure to detect differences in viral titers (Figure 3H, M) has to be attributed to the suppressive activity of the residual Treg cells that resisted DT depletion (Figures 3B and 4B) remains to be clarified. Regardless, our results establish a link between the functional impairment of the CD8\(^+\) T cell response and the elevated Treg cell levels observed during chronic infection in absence of IL-21.

**Figure 2.** IL-21 antagonizes virus-induced Treg cell expansion during chronic infection. (A) Frequencies of splenic Foxp3\(^{+}\)CD25\(^{+}\)CD4\(^+\) T cells from WT and IL-21R\(^{-/-}\) mice at indicated days after infection with indicated doses of LCMV-DOC. (B) Total numbers of Foxp3\(^{+}\) cells (2000 PFU LCMV-DOC, 15 dpi). (C) IL-21R expression on GFP\(^{+}\)CD4\(^{+}\)Treg cells of naive and LCMV-DOC–infected DEREG mice (2×10\(^{10}\) PFU, 15 dpi). The mean fluorescent intensity (MFI) of single mice (left panel) and averages ± SEM of groups (n = 4) (right panel) are shown. (D-G) Analysis of WT (CD45.1) IL-21R\(^{-/-}\) and Foxp3\(^{+}\)CD25\(^{+}\)CD4\(^+\) T cell populations derived from WT and IL-21R\(^{-/-}\) BM 8 weeks after BM transfer prior LCMV infection. Reconstitution efficiency of WT and IL-21R\(^{-/-}\) Treg cells of naïve and LCMV-DOC–infected DEREG mice (2×10\(^{10}\) PFU, 15 dpi). The mean fluorescent intensity (MFI) of single mice (left panel) and averages ± SEM of groups (n = 4) (right panel) are shown. (D-G) Dot plots show CD25 and Foxp3 expression of CD4\(^+\) T cells, (F) graphs show frequencies and (G) total numbers of splenic Foxp3\(^{+}\)CD25\(^{+}\) Treg cells 35 dpi with 2000 PFU LCMV-DOC. (H) Suppressive activity of FACS-sorted CD4\(^{+}\)CD25\(^{+}\) Treg cells from infected DEREG or DEREG-xFGF-CD4\(^{+}\)CD25\(^{+}\) mice was assessed as described in Materials and Methods. (I) Cell surface expression of putative Treg markers and (J) IL-10 production by Foxp3\(^{+}\)CD4\(^+\) T cells isolated from WT or IL-21R\(^{-/-}\) mice infected with 2×10\(^{10}\) PFU LCMV-DOC 15 dpi. (J) Dot plots depict IL-10 production of splenic Foxp3\(^{+}\) T cells of WT or IL-21R\(^{-/-}\) mice after restimulation with gp61. Shown are representative individuals of groups (n = 4). (K-N) IL-6\(^{-/-}\) and WT mice (n = 5/group) were infected with 2×10\(^{10}\) PFU LCMV-DOC and analyzed 30 dpi. Shown are frequencies of Foxp3\(^{+}\) cells (K), gp33-specific CD8\(^{+}\) T cells (L), IFN-\(\gamma\)-producing CD8\(^+\) and CD4\(^+\) T cells (M), and virus titers in organs indicated (N). (A–N) Data represent one of two independent experiments.

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IL-2 driven Treg cell expansion inhibits antiviral T cell responses and promotes persistent viral infection

We next examined the potential of Treg cells to limit the antiviral immune response and to promote virus persistence in a gain of function approach. For this purpose, we injected immune complexes (ic) comprised of recombinant IL-2 and the anti-IL-2 antibody JES6-1 [42], to selectively expand the subset of Foxp3\(^{+}\)CD4\(^+\) Treg cells in vivo (Figure 5A). In naïve mice, three injections of IL-2ic drastically expanded the Treg population to represent 40–50% of all CD4\(^+\) T cells in blood, spleen and liver within 5 days after the first injection (Figure S1 and Figure 5B). Similarly, IL-2ic treatment triggered a pronounced expansion of Treg cells in mice infected with 2×10\(^{10}\) PFU LCMV-DOC, established stably elevated levels of Treg cells in blood for at least 30 days (Figure 5B), and thus appeared comparable to that observed during high dose LCMV-DOC infection. The Treg cell population of infected, IL-2ic-treated mice was fully comparable to that of untreated, infected mice, with respect to cell surface expression of Fr4, GITR, CD103 and CD25 (Figure 5C) as well as TCR-\(\beta\)-V\(\beta\) profiles (data not shown). The IL-2ic-stimulated expansion of the Foxp3\(^{+}\) Treg cell population profoundly interfered with generation and maintenance of gp33-specific CD8\(^+\) T cells, CD62L downregulation, and their capacity to produce IFN-\(\gamma\) and TNF-\(\alpha\) as measured in spleen and liver at days 15, 30, and 65 post infection (Figure 5D–G, and Figure S2), which is reminiscent of the state of exhaustion that usually coincides with viral persistence in high dose LCMV-DOC infection. Accordingly, this long lasting impairment of antiviral T cells in presence of the enhanced Treg cell expansion prevented IL-2ic–treated animals from controlling a low dose LCMV-DOC infection. While infectious virus was readily cleared from the blood and most organs in control mice within 15 days, IL-2ic-induced Treg cell expansion resulted in a failure to clear virus in spleens, livers, kidneys and lungs for more than 2 months (Figure 5H–J). Notably, IL-2ic expanded Treg cells also impaired antiviral CD8\(^+\) T cell effector responses and viral clearance of low dose LCMV-WE infection, which is otherwise rapidly cleared irrespectively of the viral inoculate (Figure S3).

Taken together, the experimental expansion of Treg cells recapitulated both the long lasting functional impairment of the antiviral T cell response and the viral persistence that characterize high-dose LCMV-DOC infection in the setting of a low dose inoculum, and thus emphasized the remarkable potency of Treg cells to facilitate persistent viral infections. While Sprent and colleagues have clearly shown that IL-2:IL-2mAb (JES6-1) primarily target high affinity IL-2R\(^{\alpha}\) Treg cells and have minimal effects on low affinity IL-2R\(^{\alpha}\) naïve and memory CD8\(^+\) T cells [42], we cannot completely rule out the possibility that the IL-2:IL-2mAb also target high affinity antiviral effector CD8\(^+\) T cells resulting in terminal differentiation and exhaustion. However, this scenario appears
Figure 3. Depletion of Treg cells partially restores CD8^+ T cell functionality and increases disease severity. DEREG and C57BL/6 WT mice were infected with 2 x 10^5 PFU LCMV-DOC and treated with diphtheria toxin (DT) for depletion of Foxp3^+ cells as illustrated in (A, L). (B) Dot plot of GFP and Foxp3^+ expressing cells gated on CD4^+ T cells from naive and infected mice treated or untreated with DT. (C) Morbidity indicated by weight change and survival.
weight loss and (D) percentage of surviving mice for groups of 8 mice. (E) Expansion of virus-specific CD8\(^+\) T cells and cytokine production of splenic (F) CD8\(^+\) and (G) CD4\(^+\) T cells as detected with gp33-specific tetramers or intracellular cytokine staining after restimulation with specific peptide or PMA/ionomycin. (H) Virus titers in blood (left panel) and organs (right panel) of individual mice at 15 dpi. Dotted lines indicate the detection limit (DTL). (J) Percentages of gp33-specific CD8\(^+\) T cells in spleen, liver, and lungs 10 dpi. Frequencies of splenic IFN-\(\gamma\)-producing CD8\(^+\) T cells (K) and CD4\(^+\) T cells (L) after restimulation with specific peptide (left graph) or PMA/ionomycin (right graph). (M) Virus titers in blood and organs of individual mice 10 dpi. Dotted lines indicate the detection limit (DTL). Data are representative of two independent experiments including n = 8 (A–H) or n = 4 (I–H) mice per group. Symbols depict individual mice and lines indicate averages of groups.

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unlikely considering the time of treatment with the IL-2:IL-2mAb complexes (i.e. days 0–2), their short half-life (i.e. 4 h) [42], and the normal expansion of antiviral CD8\(^+\) T cells until day 7.

The above data demonstrate the differential regulation of the Treg cell population during viral infection by IL-2 and IL-21. To better understand counter-regulation of Treg cells by IL-2 and IL-21 in the absence of confounding virus dynamics, we delivered the IL-21 gene by hydrodynamic injection to IL-2ic treated mice in the absence of viral infection (Figure 6). Indeed, IL-21 significantly inhibited IL-2ic driven expansion of Treg cells. Similar results were obtained by co-injection of an engineered IL-21-Fc fusion protein together with IL-2ic. Together, these data indicate that IL-21 interferes with IL-2 driven expansion of Treg cells to optimize antiviral effector T cell responses.

**Discussion**

T cell exhaustion represents a state of T cell dysfunction associated with clinically relevant diseases, such as persistent viral expansion of Treg cells.
Figure 5. Forced Treg cell expansion inhibits antiviral T cell responses and interferes with effective virus clearance. (A) Mice were infected with 2000 PFU LCMV-DOC and treated with IL-2/anti-IL-2mAb immune complexes (IL-2ic) or untreated (ctrl) as illustrated. (B) Frequencies of Treg cells measured longitudinally in blood (left) and at 15 dpi in spleen and liver (right). (C) Cell surface expression of putative Treg markers on Foxp3^+ CD4^+ T cells of individuals out of a group (n = 4) of mice. (D) Percentages of gp33-specific CD8^+ T cells in blood at days indicated. (E–G) Frequency of IFN-γ- and TNF-α-producing CD8^+ and CD4^+ T cells after in vitro restimulation with gp33 and gp61 peptides at day 15 (E–F) and day 65 (G) post infection. Dotted lines indicate the detection limit (DTL). Data are representative of two (A–B) or three (C–I) independent experiments. Symbols represent individual mice and lines averages of the groups. doi:10.1371/journal.ppat.1003362.g005
and lines indicate averages (*p < 0.05).

Figure 6. IL-21 delivery impairs IL-2ic driven expansion of Treg cells. Expression vectors encoding the IL-21 gene fused with the hlgG1 (IL-21pDNA) or a control hlgG1 gene (ctrl pDNA) alone were delivered to C57BL/6 mice by hydrodynamic injection 24 h prior injection of IL-2ic. Alternatively, IL-2ic treated C57BL/6 WT mice were injected with 2 µg of IL-21-hlgG1 fusion protein consecutively every 12 h from days 0–5. Foxp3+ Treg cells were measured (A) in the blood at days indicated and (B) in spleen and liver at day 6 by flow cytometry. (C) Marginal zone B cells in the spleen at day 6. Values show percentages of Foxp3+ T cells of CD4+ T cells (A, B) and CD21+CD23low of CD19+ cells (C). Dots represent individual mice and lines indicate averages (*p<0.05).

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infections or cancer. Even though the molecular signature of exhausted T cells has been characterized in detail at the functional and transcriptional level [32,34], we are only beginning to understand the immunological mechanisms that support or counteract the development of T cell exhaustion during chronic infections [35]. In this study we report two major findings that establish a pathway of T cell exhaustion mediated by Treg cells during viral infection, and indicate its modulation by both, the pathogen and the host. First, we show that a persistence-inducing virus triggers the massive proliferation of Treg cells between days 9–20 post infection and demonstrate the potential of Treg cells to promote T cell exhaustion and chronic infection. Second, we identify IL-21 as a crucial host factor that antagonizes this virus-driven expansion of the Treg cell population. Together, these results suggest enhanced Treg cell responses as a mechanism of immune evasion that could be therapeutically targeted with IL-21.

Treg cells are essential for immune homeostasis and T cell tolerance [8,9], yet their contribution to anti-infectious immune responses is poorly defined. In the setting of persistent viral infections that was investigated here, Treg cells appear to down-regulate antiviral T cell responses, and thus prevent the potentially lethal immunopathology caused by prolonged immune activation in presence of highly replicating virus (Figure 3). Supporting this notion, clinical studies have associated high numbers of Treg cells to chronic infection with HIV [29], HCV [28,55], or HBV [56], whereas low Treg cell numbers have been reported for elite HIV controllers [57], which collectively suggests that Treg cells modulate the equilibrium between host immune response and persisting virus [27][34]. Our study has now recapitulated these observations in a murine model of persistent viral infection and applied experimental manipulation of the Treg cell response to define the direct link between virus-induced Treg cell expansion and T cell exhaustion. Our initial observation that titrated doses of LCMV-DOC induce graded degrees of Treg cell proliferation and T cell exhaustion allowed us to assess the impact of such virus-induced Treg cell expansion under “non-persisting conditions” using the identical LCMV strain. Although the expansion of Treg cells with IL-2ic achieved very high Treg cell numbers, it is important to note that this level of Treg cell expansion was fully comparable to that induced with LCMV-DOC at high doses or in IL-21R−/− mice. The Treg cell responses triggered by LCMV-DOC in presence or absence of IL-2ic treatment did not phenotypically differ with respect to their cell surface marker expression and exhibited TCRβ profiles similar to that described for LCMV clone 13 infection [58]. Thus, the IL-2ic-elicited Treg cell proliferation appeared to truly mimic the physiological Treg cell expansion during chronic LCMV-DOC infection.

These experiments exposed two facets of the antiviral Treg cell response in LCMV-DOC infected mice that are central for our understanding of the role and potential of Treg cells within antiviral and anti-tumoral responses. First, the increased morbidity in Treg-depleted DEREG mice infected with LCMV-DOC clearly revealed the critical role of Treg cells for preventing lethal immunopathology caused by potent immune responses to persisting antigen. Second, both the depletion as well as the gain of function approach demonstrated that Treg cells primarily modulate the functionality (e.g. cytokine response, antiviral activity) of antiviral T cells rather than influencing their priming consistent with an earlier report showing compromised cytolyis but no defect in priming, proliferation and motility of regulated CTLs [59]. This finding is especially promising, since it implies that the primed but exhausted antiviral T cells present in chronically infected subjects could be therapeutically rescued by removal of Treg cell-mediated suppression.

Dynamics of regulatory and effector T cell populations in homeostasis and during an immune response are very sensitive to the availability of IL-2. Competition for IL-2 between effector and regulatory T cells has been suggested to control tolerance and immunity or the outcome of infectious disease. Associated with the rapid expansion of virus-specific CD8+ T cells in the early phase of infection between days 0 to 8, we observed a remarkable drop in Treg cells below naïve levels irrespective of the LCMV inocula and strain used. This has also been observed in other infections and suggested to be due to consumption of IL-2 by expanding CD8+ T cells and required for efficient clearance of the invader [47,48]. While this appears feasible, it should be noted that expansion of CD8+ T cells in the acute phase of LCMV infection is independent of IL-2 [60,61] and that hyper-proliferation of Treg
cells between days 10–20 in chronic LCMV infection was associated with potent suppression of IL-2 production by CD8\(^+\) T cells (Figure 1F, G) arguing that IL-2 availability does not sufficiently explain cross-regulation of effecter CD8\(^+\) T cells and Treg cell proliferation in acute and chronic LCMV infection. In \textit{vivo} experiments suggested that IL-21 could inhibit Treg cells by suppression of IL-2 production in CD4\(^+\) effecter T cells [49]. However, our results in the WT/IL-21R\(^-/-\) mixed BM chimeric mice demonstrate that IL-21 inhibits Treg cell expansion directly in a cell intrinsic manner (Figure 2E-G). The finding that expansion of Treg cells induced by IL-21c treatment was impaired by simultaneous (hydrodynamic) overexpression of the IL-21 gene (Figure 6) further supports this conclusion. Thus, IL-2 and IL-21 exert opposing activities on Treg cells, while they cooperate in driving effecter and memory T cell responses, which adds another level of complexity to theoretical and experimental models addressing the dynamics of Treg cells and effecter T cells [45].

Amongst the known Treg cell effector molecules, IL-10 has been shown to support the functional impairment of T cell responses during chronic infection with LCMV clone 13 [36,37]. However, we were unable to detect any IL-10-producing Treg cells in LCMV-DOC infected mice (Figure 2F). Furthermore, IL-10 blocking antibodies or genetic IL-10-deficiency did not prevent T cell exhaustion and viral clearance in response to LCMV-DOC, in contrast to LCMV clone 13 infection [62]. The exhausted T cells in chronically LCMV infected mice have been shown to upregulate expression of several co-inhibitory receptors, e.g. PD-1 and Tim3, which contribute to T cell exhaustion in the LCMV model [63,64] and in human HIV patients [65,66]. It will thus be important to test whether these pathways are involved in the Treg cell-induced T cell exhaustion described in our study. Though a detailed characterization is beyond the scope of the current analysis, the experiments described in this manuscript will provide the framework for further mechanistic studies.

IL-6 and IL-21 have similar activities and interact in the cross-regulation of inducible Treg and Th17 cell development \textit{in vitro} and \textit{in vivo} depending on the experimental model [51,52,53,67,68]. Interestingly, while IL-6 has recently also been shown to be essential for viral control by enhancing follicular T helper cell responses at late stages of chronic infection [69], virus titers and Treg numbers were comparable in LCMV-DOC infected IL-6\(^-/-\) and WT mice [70]. In contrast to LCMV clone 13 infection, the inhibition of virus-induced Treg cell expansion is a phenomenon that occurs in both early and late stages of LCMV infection, thus, it remains to be investigated whether the undeletable GFP\(^+\)/Foxp3\(^+\) T cells can compensate for the deleted Treg cells or represent a subpopulation of T cells that is responsible for maintenance of regulatory activity in LCMV-infected DERE\(^+\) mice.

Regardless, our findings suggest a dual importance of IL-21 for preventing T cell exhaustion during chronic viral infections, and demonstrate that IL-21 in addition to its known direct effects on antiviral T cells [39,40,41] also partially alleviates the suppressive activity of Treg cells. Notably, in a model of acute lung infection, it was recently demonstrated that IL-21R\(^-/-\) mice are protected from fatal lung immunopathology induced by pneumonia virus [70]. It is tempting to speculate that IL-21 might aggravate immunopathology by suppression of Treg cells in this infection model.

In summary, our data support the concept of virus-induced Treg cell expansion as an active immune evasion strategy, and thus highlight a novel pathway by which viruses exploit regulatory mechanisms of the immune system to establish persistent infection. In view of the relevance to human disease these results have direct therapeutic implications and suggest strategies that boost IL-21 signaling in T cells as novel treatment options for chronic viral infections and cancer.

**Materials and Methods**

**Viruses and mice**

The LCMV strains WE and DOC were originally provided by Rolf Zinkernagel (University of Zurich, Switzerland) and were propagated on L929 or MDCK cells, respectively. C57BL/6 WT and IL-6\(^-/-\) mice [71] were from Charles River Inc. SMARTA-2 mice (expressing a transgenic TCR specific for LCMV-GP 61–80; [72]) and IL-21R\(^-/-\) mice [73] were bred locally. DERE\(^+\) mice [54] were kindly provided by Tim Sparwasser (TWINCORE, Hannover, Germany) and crossed with the IL-21R\(^-/-\) strain at our facility. IL21-mCherry/IL2-emGFP dual-reporter transgenic mice [50] were kindly provided by Warren Leonard, National Institutes of Health, Bethesda, MD, USA. Mice were housed in individually ventilated cages under specific pathogen free conditions at BioSupport AG (Zurich, Switzerland). For the generation of BM chimeras, recipient mice were lethally irradiated (9.5 Gy, using a cesium source) one day before reconstitution with 1\(\times\)10\(^7\) CD4/CD8-depleted (Milenyi Biotec) BM cells. Mice were infected i.v. with the indicated virus doses.

**Ethics statement:** All animal experiments were approved by the local animal ethics committee (Kantonales Veterinäramt Zurich, licenses 217/2008 and 115/2012), and performed according to local guidelines (TschV, Zurich) and the Swiss animal protection law (TschG).

**Cells and reagents**

All cell lines were originally obtained from the American Tissue Culture Collection (ATCC). Chemicals were purchased from Sigma-Aldrich except where otherwise stated. PE and APC-conjugated peptide-MHC class I tetramers (H-2D\(^\beta\)/gp33-41) were generated as described [74] or kindly provided by the NIH tetramer core facility. The LCMV-GP peptides gp33-41 (KAVYNFATM) and gp65-80 (GLNGPDIYKGVYQFKSVFED) were bought from Mimotopes. The following antibodies (all eBioscience unless otherwise stated; clone names given in parentheses) were used for flow cytometry: FITC-labeled anti-CD4 (L3T4), anti-CD262L (MEL-14); PE-labeled anti-CD4 (GK1.5; conjugated in our laboratory), anti-CD8 (53-6.7; BioLegend), anti-CD25 (PC61), anti-GITR (DTA-1; BioLegend), anti-CD103 (2E7), anti-IFN-\(\gamma\) (12A5; BioLegend), anti-IL-21R-biotin (4-A9 – Streptavidin-RPE; BioLegend) and anti-TNF-\(\alpha\) (MP6-XT22; PerCP-labeled anti-CD4 (RM4-5; BioLegend), anti-CD45.1 (A20; BioLe-
gred), anti-CD8 (53-6.7; BD); APC-labeled anti-CD4 (GK1.5), anti-
CD127 (SB/199; Biolegend), anti-CD3 (53-6.7; BioLegend), anti-
CD45.2 (104), anti-IL-21R (4A9; BioLegend), anti-Foxp3 (JFK-16S),
antي-IFN-γ (XMG1.2; BioLegend), anti-IL-2 (JES6-5H4), anti-IL-10
(JES5-16E3).

Manipulation of Treg cells
In depletion experiments, DEREG and WT control mice were
treated with DT (Merck) diluted in PBS. After an initial dose of
200 ng DT, mice were treated with 100 ng DT every third day
without otherwise indicated. To boost Treg cells, mice received 5
daily i.p. injections of IL-2ic generated from carrier-free recombi-

ant mouse IL-2 and anti-IL-2 mAb (JES6-1A12; both from eBioscience) as described [42].

Flow cytometry
Tetramer and antibody staining was performed on blood cells and
single cell suspensions prepared from organs. Spleens and kidneys were
passed through a 70 μm cell strainer to obtain single cell suspensions.
Livers were first dissected into small pieces, and then passed through a
cell strainer before lymphocytes were purified by Lympholyte M
gradient centrifugation (Cedarlane Laboratories Ltd.). Blood samples
were pretreated with red blood lysis buffer (155 mM NH4Cl, 10 mM
KHCO3, 0.1 mM EDTA, pH 7) for 10 min at RT. Cells were
incubated with anti-CD16/CD32 mAb (2.4G2) to block Fc
receptors. Cells were stained with tetramers or antibodies for surface
staining, cells were incubated at RT with peptide MHC I
complexes. For intracellular staining, cells were incubated at 4°C
overnight. Intracellular staining was then performed in permeabilization
buffer at 4°C for 20 minutes. After 2 washes with permeabilization
buffer, cells were resuspended in FB. All samples were
acquired on a FACSCalibur with CellQuest software (both BD
Biosciences) and analyzed using the FlowJo software (Tree Star Inc.).

Determination of virus titers
Blood samples were obtained from LCMV-infected mice at
indicated times, diluted 5-fold in MEM (5% FCS) containing 50 U/L
of Liquemin (Drossapharm) and frozen. Organs were collected in 1 ml
MEM (5% FCS) and smashed with a Tissue Lyser (Qiagen). Samples
were stored at −80°C until further analysis by plaque forming assay
[75].

Treg cell suppression assay
Responder CD4+ T cells were purified from naïve spleens by
positive MACS separation (Miltenyi Biotec) and labeled with 25 μM
CFSE (Molecular Probes, C-1157) at a density of 106 cells/ml in PBS
containing 0.5% BSA for 7 min at RT. The labeling reaction was
stopped with pure FCS and cells were washed twice with IMDM
containing 10% FCS. As suppressor cells, CD25+ Treg cells were
FACS-sorted from MACS-purified CD4+ T cells isolated from
LCMV-DOC infected DEREG and IL-21R−/− DEREG mice.
CFSE-labeled responder CD4+ T cells (1×10^5/well) and sorted Treg
cells were then incubated at defined responder/suppressor ratios (1:1, 2:1, 4:1) in RPMI (10% FCS, 50 μM β- ME and 100 U/ml IL-2) for 6
days in the presence of 5×10^6 anti-CD3/CD28-coated (both
eBioscience) latex beads.

Hydrodynamic gene delivery
Mouse IL-21 coding sequence was amplified by PCR and linked
with hlgG1 Fc domain and cloned into pLIVE in vivo expression
vector (Mirus Bio). Endotoxin-free plasmid DNA (100 μg) was
injected i.v. in PBS in a volume equal to 10% body weight
(0.1 ml/g) within 5 s. As a control, a hlgG1 expression vector was
injected. To supplement IL-21, mice received i.p. injections of 2 μg
recombinant IL-21-hlgG1 fusion protein (kindly provided by
Daniel Christ, Garvan Institute for Medical Research, Sydney,
Australia) or PBS two times daily.

Statistical analysis
Data are shown as average ±SEM. Statistical analysis was
performed with the unpaired two-tailed t-test (except for Fig. 1B–
E) using the Prism 4.0 software (GraphPad Software). Differences
were considered significant for p<0.05 and were denoted as *,
** p<0.01; *** p<0.001.

Supporting Information
Figure S1 Expansion of Treg cells with IL-2ic. Mice were
treated with IL-2ic as depicted in (A). (B) Treg cell populations in
blood, spleens, and livers of treated as compared to untreated (ctrl)
mice. Shown are dot plots of representative individuals of groups
(n=4). Percentages and total cell counts of Foxp3+ cells are
indicated in quadrants.

Figure S2 Expanded Treg cell population impairs the
antiviral T cell response and promotes virus persistence.
(A–C) Kinetics of CD8+ and CD4+ T cells during infection
with 2000 PFU LCMV-DOC in presence or absence of IL-2ic-
mediated Treg cell expansion. Shown are percentages and
activation status of (A) CD8+ T cells, (B) gp33-specific CD8+ T
cells and (C) Foxp3-negative CD4+ T cells in blood. Lym,
lymphocytes. Lines indicate averages of groups. Data are
representative of two independent experiments (n = 4/group).

Figure S3 Treg cells promote virus persistence in a
model of acute viral infection. (A) Percentages of gp33-
specific CD8+ T cells in blood, spleens and livers of mice infected
with 200 PFU LCMV-WE in the presence or absence of IL-2ic-
mediated Treg cell expansion. (B, C) Percentages of IFN-γ- and
TNF-α-producing virus-specific CD8+ and CD4+ T cells at (B) 15
and (C) 29 dpi as assessed by intracellular cytokine staining after
restimulation with gp33 or gp61 peptide, respectively. Dot plots
show representative mice and bar graphs indicate means ±SEM
of groups (n = 3–4) of mice. (D) Virus titers in blood and organs of
individual mice as determined by plaque forming assay 15 dpi.
Dotted lines indicate the detection limit (DTL). Data are
representative of two independent experiments.

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Author Contributions
Conceived and designed the experiments: IS AO SF MK. Performed the
experiments: IS CS AF HF. Analyzed the data: IS CS AF HF AO SF MK.
Contributed reagents/materials/analysis tools: TS WJL DC. Wrote the
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