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T cell-intrinsic IL6R signaling is required for optimal ICOS expression and viral control during chronic infection

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Abstract

The pleiotropic cytokine interleukin-6 (IL-6) plays an integral role not only in innate inflammatory responses but also in the activation and differentiation of lymphocyte subsets. Here, by using a conditional knockout model with selective IL-6 receptor deletion in T cells (IL6R-cKO), we demonstrated that T cell-specific IL6R signaling is essential for viral control during persistent lymphocytic choriomeningitis virus (LCMV) Clone 13 infection. Strikingly, we observed that in contrast to previous studies with ubiquitous IL-6 deletion or blockade, specific IL6R deletion in T cells did not affect T follicular helper (Tfh) accumulation unless IL6R-deficient T cells were competing with wildtype cells in mixed bone marrow chimeras. On the other hand, Tfh cells from IL6R-cKO infected mice exhibited reduced ICOS expression in both chimeric and non-chimeric settings, and this sole identifiable Tfh defect was associated with reduced germinal centres, compromised immunoglobulin switch and low avidity of LCMV-specific antibodies despite intact IL6R expression in B cells. We posit that IL6R *cis*-signaling is absolutely required for appropriate ICOS expression in Tfh cells and provides a competitive advantage for Tfh accumulation, enabling generation of optimal B cell and antibody responses, and ultimately viral control during *in vivo* chronic infection.

INTRODUCTION

The acute phase cytokine IL-6 has received much attention due to its meaningful role during infection, vaccination, autoimmune diseases, and cancer among others (1). IL-6 signaling is mediated by the heterodimeric IL-6 receptor, composed of the ligand-binding subunit IL6Ra and the signal-transducing subunit gp130. Gp130 is a membrane-bound common co-receptor for cytokines of the IL-6 family and others, including IL-6, IL-11, IL-27, leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1), as well as IL-35 (2). Intriguingly, in addition to membrane-bound IL6Ra *cis*-signaling, mRNA splicing or proteolytic cleavage can produce a soluble form of IL6Ra which, upon secretion, can bind to extracellular IL-6 and associate with gp130 on cells that may not intrinsically express the

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IL6Ra chain, thereby inducing a *trans*-signaling IL-6 pathway (3). Importantly, studies using global IL-6 or IL6Ra deletion fail to distinguish between these two forms of IL6R signaling (4).

IL-6's ability to direct the differentiation of CD4 T cell subsets has been increasingly appreciated. In particular, IL-6 has been highlighted to direct CD4 T cells away from $Foxp3^+$ regulatory T cell (Treg) and towards IL-17-expressing Th17 cells in the presence of TGF-β signaling (5–7). More recently, IL-6 has been implicated in the differentiation of T follicular helper (Tfh) cells, known for their specialized capacity to provide the necessary help for B cells in their response to T-dependent antigens (8, 9). In particular, *in vitro* IL-6 signaling is able to induce Tfh related genes, although insufficiently for full Tfh differentiation (10, 11). Furthermore, by using wildtype (WT):IL6R-KO mixed bone marrow (BM) chimeras, we demonstrated that *in vivo* cell-intrinsic IL-6 signaling is necessary to drive optimal Tfh accumulation and ICOS expression during chronic infection with lymphocytic choriomeningitis virus Clone 13 (LCMV Cl13) (12). Finally, *in vivo* IL-6 has also been shown to be necessary for the early induction of the transcription factor Bcl6, which is crucial for driving the Tfh differentiation program via STAT1 during acute LCMV infection (10, 11, 13, 14). Despite this early IL-6 activity (day 2–3 post-infection – p.i.), Tfh and germinal center responses are apparently unaltered in IL-6 deficient mice at the peak Tfh response during acute infection (day 7–8 p.i.) (12, 15), unless IL-21 signaling is removed at the same time. A T cell-specific deletion of the IL6R has only been explored within the context of an immunization model where T cell-specific IL6 signaling has been implicated in defective Tfh, Th1 and Th17 functions, CD4 T cell memory, and CD4 T cell resistance to Treg suppression (16).

In the context of infections, IL-6 was first reported to provide essential host defence against vaccinia virus and *Listeria monocytogenes* and to promote T-dependent antibody responses against vesicular stomatitis virus (VSV) (17). Ubiquitous deletion of IL-6 as well as late IL-6 or IL6R blockade with mAbs during LCMV Cl13 infection results in life-long viremia and high viral loads in multiple tissues (12). To directly interrogate whether IL-6's antiviral effect is a consequence of its cell-intrinsic signaling on T cells (or putative non-T cell IL-6 targets), we analyzed mice with an exclusive IL6R signaling deletion in T cells (herein referred to as IL6R-cKO) during LCMV Cl13 infection. We found that exclusive deletion of IL6R in T cells was sufficient to recapitulate the life-long viremia and higher viral loads in tissues that we previously reported in IL-6 KO versus WT mice after LCMV Cl13 infection (12). IL6R-cKO mice exhibited seemingly normal induction of CD8 T cell and Treg cells at late timepoints during chronic LCMV Cl13 infection. Surprisingly, and in contrast to what we had observed upon global IL-6 blockade or deletion (12), IL6R-deficient T cells showed unaltered Tfh accumulation late after LCMV Cl13 infection, except for when IL6R-cKO T cells were placed in competition with WT counterparts in chimeric settings. Importantly, IL6R-deficient T cells exhibited reduced ICOS expression on Tfh cells in both chimeric and non-chimeric settings. This singular Tfh cell defect was associated with defective B cell and virus-specific antibody responses, helping to explain the aforementioned lack of LCMV Cl13 control in IL6R-cKO mice.

MATERIALS AND METHODS

Mice and viral stocks.

Il6ra$^{fl/fl}$ mice (on a C57BL/6 background) were kindly provided by Dr. Angela Drew (University of Cincinnati (4)) and crossed to *Cd4*-cre mice (on a C57BL/6 background). CD45.1^{+} (B6.SJL-*Ptprc*^{a}*Prpc*^{b}/BoyJ) and inbred C57BL/6 mice were obtained from The Jackson Laboratory. All mice were bred and maintained in a closed breeding facility and mouse handling conformed to the requirements of the National Institutes of Health and the Institutional Animal Care and Use Guidelines of UCSD. Unless otherwise stated, 6–8 week old mice were infected intravenously (i.v.) with 2×10^6 pfu of LCMV Cl13. LCMV Cl13 was grown, identified and quantified as described in (18, 19). Viral quantification was carried out by six well plate plaque assay on VERO cells (ATCC). For mixed bone marrow (BM) chimeras, recipient CD45.1^{+} mice were exposed to 1000 Rads. The following day they were injected with 5×10^6 BM cells i.v. (a mix of 40% CD45.1^{+}WT and 60% of either CD45.2^{+}WT or CD45.2^{+}*Cd4*$^{cre/+}$*Il6ra*$^{fl/fl}$ donor cells). Mice were maintained on oral antibiotics for 2 weeks and reconstitution was continued for an additional 6 weeks prior to infection.

LCMV specific antibody ELISAs.

LCMV specific ELISAs and Ab avidity assays were done as we and others have previously described using antigen prepared by purifying LCMV on a renografin gradient (12, 20).

Flow cytometry.

Flow cytometry was done as previously described (21). The following fluorochrome labelled antibodies purchased from Biolegend, eBioscience/ThermoFisher, or BD Biosciences were used to stain blood or spleen cells: anti-CD8-Pacific blue, -CD4-APC-Cy7, -CD19-PE, -B220-PE-CF594, -CD38-Alexafluor700, -CD38-PE-Cy7, -GL7-FITC, -GL7-efluor660, -CD138-PE, -IgM-APC-Cy7, -IgD-PB, -PD1-PE-Cy7, -PD1-BV605, -ICOS-PE, -ICOS-PE-Cy7, -CD11a-FITC, -CD49d-PerCP-Cy5.5, -KLRG1-FITC, -CD127-PerCP-Cy5.5, -CD8-efluor450, -CD45.1-PE-CF594, -CD45.2-BV650, -IgG1-FITC, -IgG2a^{b}-biotin followed by Strepavidin-BV650, -IFN-γ-APC, -TNF-α-FITC, -IL-2-PE, -CXCR5-BV421. Two-step CXCR5 (BD), Foxp3 (eBioscience), and Bcl6 (BD, K112–91) staining were done as previously described (22). Biotinylated D^{b} GP$_{33–41}$ and D^{b} NP$_{396–404}$ monomers along with APC-I-A^{b} GP$_{67–77}$ tetramers were kindly provided by the NIH tetramer core facility (Atlanta, GA). Monomers were folded using SA-PE or SA-APC (Molecular Probes, Life Technologies). Class I tetramers were stained 1:100 for 75 mins on ice, and Class II tetramers were stained 1:100 for 3 hours at 37°C, followed by normal antibody staining. Cells were acquired using the Digital LSR II flow cytometer (BD Biosciences, San Jose, CA). Staining for fluorescence assisted cell sorting was conducted in an identical fashion and cells were isolated on a FACSAria (BD Biosciences, San Jose, CA) to >95% purify. Flow cytometric data were analyzed with FlowJo software (TreeStar, CA).

Confocal microscopy.

Spleen segments were immediately fixed with 4% paraformaldehyde and 5% sucrose, followed by 10% and then 30% sucrose overnight. The segments were then flash-frozen in OCT compound (Tissue Tek). 7um sections were cut, re-fixed with 4% paraformaldehyde, then stained with antibodies (IgD, clone 11–26C; GL7, clone GL7; CD4, clone RM4–5; B220, clone RA3–6B2) and with DAPI (Invitrogen). Confocal images were captured with an Olympus FV1000. Whole-spleen 10x images were constructed using the Olympus FlowView software. Individual germinal center (GC) images were captured at 40x. ImageJ was used to quantify $GL7^+$ and $B220^+$ areas, as well as to demarcate GC boundaries.

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 in the presence of Brefeldin A (BFA) as we have previously described (21). For intracellular IL-21 staining, cells were permeabilized with saponin and incubated with 1:25 dilution of mouse IL-21R-human Fc (R&D Systems) for 30 minutes at 4°C, washed twice and stained with 1:200 anti-human Fc-PE (BD Pharmingen).

Real-time RT-PCR.

Total RNA was extracted from splenocytes using RNeasy kits (Qiagen), and reverse transcribed into cDNA using superscript III RT (Invitrogen). cDNA quantification was performed using SYBR Green PCR kits (Applied Biosystems) and a Real-Time PCR Detection System (ABI). Primers for the genes assessed are described in (12) as well as *Maf*-Forward, AAATACGAGAAGCTGGTGAGCAA; *Maf*-Reverse, CGGGAGAGGAAGGGTTGTC.

Statistical analysis.

Non-parametric Mann Whitney tests, ANOVA tests, or paired Student t-tests were performed using the Prism 5.0 software (GraphPad, CA) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ in all data shown.

RESULTS

IL6R deficient on T cells resulted in prolonged viral persistence

In order to explore the impact of T cell-specific deletion of the IL-6 receptor alpha subunit (IL6R) during chronic viral infection, we crossed $Cd4^{cre/+}$ mice with $Il6ra^{fl/fl}$ mice and infected them with LCMV Cl13 (4). In all experiments, the $Cd4^{+/+}$ littermates (herein referred to as WT) were used as IL6R-sufficient controls for the IL6R-deficient $Cd4^{cre/+}$ mice (IL6R-cKO). Mice were analyzed at day 0 or day 30 p.i. to evaluate surface expression of IL6R on PBMCs. At both time points IL6R expression was ablated on all CD4 and CD8 T cells in IL6R-cKO mice (Fig. 1A). In contrast IL-6 receptor was expressed at WT level and frequency on B cells, dendritic cells (DCs), and myeloid cells in IL6R-cKO mice (Fig. 1A). Importantly while WT littermates were able to control LCMV Cl13 viremia by day 120 p.i., deletion of IL6R from T cells resulted in the inability to control viremia (Fig. 1B) with

viral titers in IL6R-cKO mice beginning to deviate from WT mice as early as day 45 p.i., and remaining high through day 120 p.i., the last timepoint analyzed. Likewise there was marked persistence of virus in all organs of IL6R-cKO mice at day 120 p.i. when most WT mice had cleared virus from spleen, liver, lung and brain (Fig. 1C). Together these data indicate that IL6R signaling in T cells is essential for controlling chronic infection with a persistently replicating virus.

CD8 T cell responses were unaffected prior to viremia divergence in the context of T-cell-IL6R deficiency

As CD8 T cells are critical effectors in the immune response to control LCMV Cl13 viremia (23), we first sought to determine whether deficiencies in this compartment were contributing to the inability of IL6R-cKO mice to control serum virus levels. There was no significant difference in the number of circulating D^b $NP_{396–404}^+$ or $GP_{33–41}^+$ CD8 T cells between WT and IL6R-cKO mice at days 5 and up to 30 p.i., although WT mice had significantly more D^b $GP_{33–41}^+$ CD8 T cells than IL6R-cKO mice on days 45 and 60 p.i., once viremia had diverged (Fig. 2A). Likewise, PD-1 expression by $GP_{33–41}$ specific CD8 T cells was similar between days 8 and 30 p.i., but significantly elevated in IL6R-cKO versus WT mice on days 45 and 60 p.i. (Fig. 2A). We next focused on day 30 post-infection with LCMV Cl13 in order to study a late time-point prior to the divergence of viral titers between the IL6R-cKO mice and their WT littermates (Fig. 1B). Day 30 p.i. was also analogous to the time points we have analyzed previously in both IL-6 deficient or T cell specific gp130 deficient animals (12, 22).

Both the number of total CD8 T cells and frequencies of the virus-specific CD8 T cell compartments (identified by H-$2D^b$ $GP_{33–41}$ or $NP_{396–404}$ tetramers) were equivalent in WT and IL6R-deficient CD8 T cells (Fig. 2B). In addition upon re-stimulation of whole splenocytes *in vitro* with $GP_{33–41}$ peptide, CD8 T cells lacking IL6R were equally as capable as WT cells in their production of IFN-γ and IL-2 (Fig. 2C). PD-1 expression by splenic virus-specific CD8 T cell was also similar between WT and IL6R-cKO mice (Fig. 2D). Overall these data suggest that virus-specific CD8 T cell responses were initially unaltered by the absence of IL-6R signaling, and only appeared to become more dysfunctional in IL6R-cKO versus WT mice at later time points (day 45 and 60) p.i., likely due to lack of viral control in the IL-6R deficient setting.

CD4 T cell accumulation and Treg differentiation were unaffected by T-cell-IL6R deficiency

We next evaluated CD4 T cell responses in IL6R-cKO mice infected with LCMV Cl13 at day 30 p.i. There were similar numbers of total and activated ($CD44^+PD\text{-}1^+$) CD4 T cells in IL6R-cKO mice when compared to WT mice during LCMV Cl13 infection (Fig. 3A&B). Likewise there was no difference in the number or proportion of splenic I-A^b $GP_{67–77}^+$ CD4 T cells in IL6R-cKO compared to WT infected mice (Fig. 3C). Furthermore, the numbers of CD4 T cells making IFN-γ and IL-21 when re-stimulated with the $GP_{67–77}$ peptide *in vitro* (either co-expressing cells or single producers) was unaltered in the absence of T cell-specific IL6R signaling (Fig. 3D).

IL-6 signaling is known to participate in the suppression of regulatory T cell (Treg) development (5–7) but conversely can promote suppressive capacity of thymic Tregs *in vivo* (24), we therefore assayed a variety of Treg markers. The absence of IL6R signaling did not affect the differentiation of Foxp3$^+$ Tregs during LCMV Cl13 infection (Fig. 3E). Within the Foxp3$^+$ compartment, there were normal proportions of Helios, Neuropilin-1 and activated KLRG-1-expressing subsets, as well as unchanged expression of the suppressive molecule CTLA-4 on the KLRG-1 subset (Fig. 3F&G).

Overall these data indicate that the accumulation of virus-specific CD4 T cells and Treg differentiation were unaltered in the absence of T cell-IL6R signaling during chronic LCMV infection.

ICOS expression was down-regulated in the absence of T-cell-IL6R signaling

Tfh are critical in driving T-dependent antibody responses that can restrain chronic viral infections (25). Tfh accumulation is compromised in IL-6 KO mice as well as in WT:IL6R-KO mixed chimeras infected with LCMV Cl13 (12). We therefore expected reduced accumulation of Tfh cells during LCMV Cl13 infection in the absence of T-cell specific IL6R signaling. Strikingly, similar numbers of Tfh cells (gated either by CXCR5$^+$SLAM$^-$ or CXCR5$^+$Bcl6$^+$) were observed in WT and IL6R-cKO mice either within activated or within LCMV-specific CD4 T cell compartments (Fig. 4A&B). In addition, analysis of PSGL-1 and Ly6C (which were proposed to respectively identify terminally differentiated Th1 and Tfh cells during acute LCMV infection (26, 27)) in infected IL6R-cKO mice showed unaltered proportions of Ly6C$^+$PSGL-1$^+$ terminally differentiated Th1 cells, the Ly6C$^-$PSGL-1$^+$ compartment that contains memory precursors, and Ly6C$^-$PSGL-1$^-$ Tfh cells within activated CD44$^+$PD-1$^+$ CD4 T cells (Fig. 4C).

When we sorted either the total activated CD4 T cell compartment (CD4$^+$PD1$^+$) or the virus-specific CD4 T cells (CD4$^+$ I-A^{b}GP$_{67–77}$ tetramer$^+$), we could not identify differences in the gene expression of a variety of Tfh-related transcription factors including *Bcl6, Batf*, *Irf4*, and *Maf*, or Tfh effector molecules *Cxcr5, Il21* and *Il4* (Fig. 5A). Similarly, loss of IL6R did not affect protein expression of the Tfh master transcription factor Bcl6 in the polyclonal or virus-specific Tfh compartment (Fig. 5B). However in the absence of T cell-specific IL6R signaling, there was a significant defect in surface expression of ICOS, a molecule that is important for both Tfh development and function (27–34), in both activated and LCMV-specific CD4 T cells (Fig. 5C). This defect in ICOS expression was, however, not seen in the non-Tfh compartment (Fig. 5D). These results suggest that while numerous Tfh-specific functionally relevant molecules remain unchanged in the absence of IL6R signaling, optimal Tfh expression of ICOS appears to rely on IL6R signaling.

Cell-intrinsic IL6R cis-signaling promotes Tfh accumulation and ICOS expression in competitive chimeric setting

Unlike WT:IL6R-KO BM chimeras (12), the aforementioned experiments in IL6R-cKO mice indicated that IL6R signaling in T cells was dispensable for Tfh accumulation and Bcl6 expression. To discern whether this seemly contradictory observation was due to absence of a competing WT T cell compartment in the non-chimeric IL6R-cKO mice analyzed above,

we next evaluated T cell responses in WT(CD45.1$^+$):IL6R-cKO(CD45.2$^+$) versus WT(CD45.1$^+$):WT(CD45.2$^+$) mixed chimeras at day 30 after LCMV Cl13 infection. As with WT:IL6R-KO BM chimeras (12), we detected similar numbers of LCMV-specific D^b GP$_{33–41}$$^+$ CD8 T cells in both WT and IL6R-cKO compartments, and these virus-specific CD8 T cells expressed similar levels of PD-1 (Fig 6A). We also observed similar numbers of LCMV-specific I-A^{b}GP$_{67–77}$$^+$ CD4 T cells (Fig. 6B) but reduced Tfh proportion within PD-1$^+$ activated CD4 T cells in the IL6R-cKO compartment compared to WT cells (Fig. 6C). As in the non-chimeric animals, ICOS expression was decreased on IL6R-cKO Tfh cells in the mixed chimeric setting, further implicating intrinsic IL6R signaling upstream of the induction of ICOS in Tfh cells (Fig. 6D). IL6R-deficient CD4 T cell frequencies in the Foxp3$^+$ Treg compartment were unaltered compared to WT cells in the chimeric mice (data not shown). These data highlight that under competitive mixed chimeric settings, cell-intrinsic *cis*-IL6R signaling promotes Tfh accumulation and ICOS expression, without affecting CD8 T cell responses, at late stages of chronic LCMV infection.

Decreased germinal center and antibody responses in the absence of T cell-specific IL6R deletion

ICOS in CD4 T cells plays predominant roles in Tfh differentiation and function, allowing optimal CD4 T cell help for B cell responses in both acute and chronic inflammation (27–35). Given the compromised ICOS expression in Tfh cells from IL6R-cKO LCMV Cl13 infected mice, B cell and antibody responses in the absence of T cell-IL6R signaling were investigated. Despite normal IL6R expression on B cells in IL6R-cKO mice at day 30 after LCMV Cl13 infection (Fig. 1A), we observed a reduction in the frequencies of IgD$^+$IgM$^+$ switched B cells in IL6R-cKO versus WT mice (Fig. 7A, 4 out of 7 experiments; absolute number comparisons did not reach significance). Concomitantly there was a significant reduction in the proportions of GL7$^+$CD38$^–$ germinal center B cells from IL6R-cKO versus WT mice (Fig. 7B, 5 out of 7 experiments; absolute number comparisons reached significance in 4 of 7 experiments).

Fitting with the observation that IL6R-cKO mice had similar frequencies of Tfh to WT mice, the number of CD4 T cells per splenic GC was similar in both WT and KO mice (Fig. 7C). However, there was a significant reduction in the area of the GC (GL7$^+$) within the B cell zone (B220$^+$) as observed by confocal microscopy (Fig. 7D), correlating with the reduced frequencies of GC B cells as seen by flow cytometry.

We next assayed virus-specific antibody titers in the serum at day 30 and day 60 p.i. At both time-points analyzed, endpoint titers of IgG2b were significantly decreased in IL6R-cKO compared to WT LCMV Cl13-infected mice (Fig. 7E). Virus-specific IgG1 titers were slightly decreased at day 30 p.i., but not different at day 60 p.i. while IgG2a/c titers and total Ig were decreased at day 60 p.i. in IL6R-cKO versus WT mice. Moreover, in the absence of T cell-specific IL6R signaling, the avidity of LCMV-specific IgG was decreased both at day 30 and day 60 p.i. (Fig. 7F). Finally, in agreement with a previous report (36), antiviral-neutralizing antibody levels were negligible in the serum from day30-LCMV-Cl13-infected mice and were similarly undetectable in the IL6R-cKO group at day 30 p.i., the last time-point before divergence of viremia (Fig. 2B and data not shown). Taken together, these data

indicate that IL6R signaling in T cells is necessary for optimal germinal center responses and the generation of high avidity anti-viral antibodies. Given that late control of chronic LCMV infection is dependent on effective antibody responses (37), it is likely that the aforementioned B cell and antibody defects caused, at least in part, the inability of IL6R-cKO mice to control late LCMV Cl13 viremia (Fig. 1B&C).

DISCUSSION

Late IL-6 signaling is required for both optimal Tfh responses and for control of chronic LCMV infection (12, 22) but it remained unclear whether the antiviral IL-6 effect was a consequence of IL-6 signaling in T cell or non-T cell populations. Here we compellingly establish that IL-6 signaling specifically in T cells is absolutely required for viral control during chronic LCMV infection. Importantly, while global IL-6 signaling deficiency promotes broader defects in T and B cell responses, lack of *cis*-IL6R signaling in T cells resulted in a narrower defect on Tfh cells (i.e. compromised ICOS expression), which was still sufficient to prevent optimal antibody responses and explained the lack of late viral control.

CD8 T cells are vital in the containment of LCMV Cl13 (23). However, the CD8 T cell responses from IL6R-cKO mice appeared unaltered respect to WT controls, both in terms of number and functionality, from day 5 and up to day 30 p.i. (the last time point analysed prior to divergence of viremia). Although CD8 T cells from IL6R-cKO versus WT mice showed reduced numbers and higher PD1 expression at day 45 and 60 p.i., these results were confounded and likely a consequence of higher viral titers in the T-cell-specific-IL-6R deficient setting. Overall, our results fit with previous CD8 T cell data from studies in IL-6 deficient mice during either Influenza A virus or LCMV infections (12, 38). On the other hand, it has been recently shown that IL-6 is detrimental for the generation of memory bystander CD8 T cell responses during chronic LCMV infection (39). Together, the aforementioned results argue against an antiviral role for IL-6 signaling on CD8 T cells during chronic viral infection.

More surprisingly, specific deletion of IL6R signaling on CD4 T cells had minor effects on the differentiation and accumulation of virus-specific CD4 T cells. Indeed, lack of IL6R signaling in T cells did not seem to affect key transcription factors *Bcl6*, *Batf*, *Maf* or *Irf4* known to be needed in the differentiation and function of Tfh cells (8). Nor did T-cell-specific IL6R deficiency affect a number of key functional molecules such as CXCR5, IL-21 or IL-4. Despite this, T cell-specific IL6R signaling was required for optimal B cell responses after infection with LCMV Cl13, regulating both isotype switch and the generation of high avidity antibodies. Virus-specific antibodies are known to be important in restricting LCMV Cl13 persistence (37). Sustained Tfh dependent somatic hypermutation of antibodies at late stages of LCMV infection is required to prevent the emergence of antibody resistant viruses and restrain viral load (40). Given the undetectable defects in CD8 T cell-mediated immunity, it is therefore likely that loss of antibody quality and quantity ultimately lead to viral persistence in the absence of T cell-specific IL6R signaling. We cannot, however, rule out the possibility that defects in IL6R-deficient T cells, other than the ones

uncovered in our study, might contribute to the lack of viral control in chronically infected IL-6R-cKO mice.

We have previously shown that late IL-6 is essential for the accumulation of Tfh cells at late stages of chronic viral infection (12) however the only observable difference in Tfh cell phenotype in the T-cell-specific absence of IL-6 *cis*-signaling was reduced ICOS expression. ICOS expression by CD4 T cells is vital for the mounting of T-dependent antibody responses (41, 42) largely due to the importance of ICOS signaling in the differentiation, accumulation and function of Tfh cells (27–35). Moreover, ICOSL signaling on B cells is important for participation in the GC response as well as optimal selection and affinity maturation (43). Importantly, the ICOS-ICOSL interaction appears to be tightly regulated in order to achieve appropriate Tfh and B cell responses and suboptimal ICOS or ICOSL expression can result in Tfh and GC defects that could contribute to pathology (44). Indeed during acute LCMV infection, T cell-specific deletion of the common gp130 signaling molecule also leads to a defect in ICOS expression on Tfh cells and a dysregulated memory B cell compartment (45). Consequently, inadequate ICOS expression on CD4 T cells can result in downstream defects in B cell responses essential for viral control.

Global blockade or deletion of IL-6 and T cell-specific deletion of gp130 result in dramatic defects in CD4 Tfh cell responses during chronic LCMV infection (12, 22). In contrast, we detected only ICOS expression alterations when non-chimeric IL6R-cKO mice were analyzed while compromised Tfh cell accumulation was only evidenced in a competitive mixed chimera settings. These results imply that IL6R signaling is dispensable for Tfh cell accumulation in non-competitive settings during chronic infection, likely due to the existence of redundant factors that can promote Tfh differentiation in a chronic inflammatory environment. Indeed, it is likely that at late time points during chronic LCMV infection CD4 T cells receive numerous other signals beside IL-6 that can provide adequate STAT1 and STAT3 signaling that in turn would allow for appropriate Tfh development in the absence of IL6R signaling (46, 47). For example IL-27 also signals via gp130 to activate STAT1 and STAT3, and IL-27R deficient animals fail to control LCMV Cl13 and have dampened virus-specific antibody responses (48). Nonetheless, results from competitive settings in WT:IL6R-cKO mixed chimeras indicate that cell-intrinsic IL6R signaling actively contributes to Tfh cell accumulation during chronic LCMV infection, providing CD4 T cells that receive IL6R signaling with a greater competitive advantage.

Importantly, through the IL6R-cKO mice and the mixed BM chimera system, we could also interrogate the contribution of IL-6 *cis*- versus *trans*-signaling in Tfh cell differentiation since in these system WT cells could generate soluble IL6R that could bind to surface gp130 on the IL6R-deficient T cells. In other systems, such as in a transfer model of colitis, IL-6 *trans*-signaling has been implicated in controlling Treg activity (49). More recently IL6R signaling has also been shown to occur via *trans*-presentation, where DCs present their own IL6-IL6R complexes to cognate T cells in order to trigger T cell gp130 signaling (50). In our study, we decisively demonstrated a non-redundant role of IL-6 *cis*-signaling by surface-bound IL6R that could not be complemented neither by *trans*-signaling via soluble IL6R or *trans*-presentation by DCs. Indeed, in WT:IL6R-cKO mixed chimeras, the IL6R-deficient CD4 T cells did not efficiently populate the Tfh compartment nor did they express normal

levels of ICOS despite the possible release of soluble IL6R secreted by neighbouring WT cells. Moreover, *trans*-IL-6 signaling provided by WT cells in IL6R-cKO mice was insufficient for late LCMV Cl13 control, indicating that cis-IL-6 signaling in T cells is essential for controlling a persistently replicating virus.

It has also been proposed that IL-6 signaling on CD4 T cells is required to counteract Treg-mediated suppression (16) and consequently in the absence of the IL6R signal Tregs would be more suppressive. Increased Treg activity during chronic LCMV infection has been implicated in limiting antiviral CD8 T cell responses (51, 52). However, in our hands, there were no differences in either Treg or effector CD4 T cell numbers or cytokine production. While the possibility remains that IL6R-dependent Treg-mediated mechanism is important at another stage of infection, we did not observe any CD8 T cell defects in the absence of T cell-specific IL6R signaling prior to loss of viral control, in agreement with our previous reports with global blockade or deletion of IL-6 (12, 22).

Overall, we have demonstrated that T cell-specific IL-6 signaling is required to instigate effective B cell mediated immunity, likely through optimal ICOS expression on Tfh cells, and that the selective alteration of IL6R signaling pathway in T cells is sufficient for preventing control of a persistently replicating virus. Our work established the necessary knowledge for future studies on the mechanisms underlying the induction of optimal ICOS expression downstream *cis*-IL6R signaling in Tfh cells and how this regulatory axis could be exploited to manipulate Tfh cell responses in infectious and non-infectious settings.

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Abbreviations used in this article:

LCMV	lymphocytic choriomeningitis virus
Cl13	Clone 13
cKO	conditional knockout
KO	knockout
WT	wildtype
p.i.	post-infection
BM	bone marrow
GC	germinal center

Tfh	T follicular helper cells
Treg	regulatory CD4 T cells

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KEY POINTS:

- IL-6R signaling on T cells is essential for controlling chronic viral infection.
- IL-6R signaling on T cells is required for optimal antibody development.
- IL-6 signaling on T cells regulates ICOS expression on Tfh.

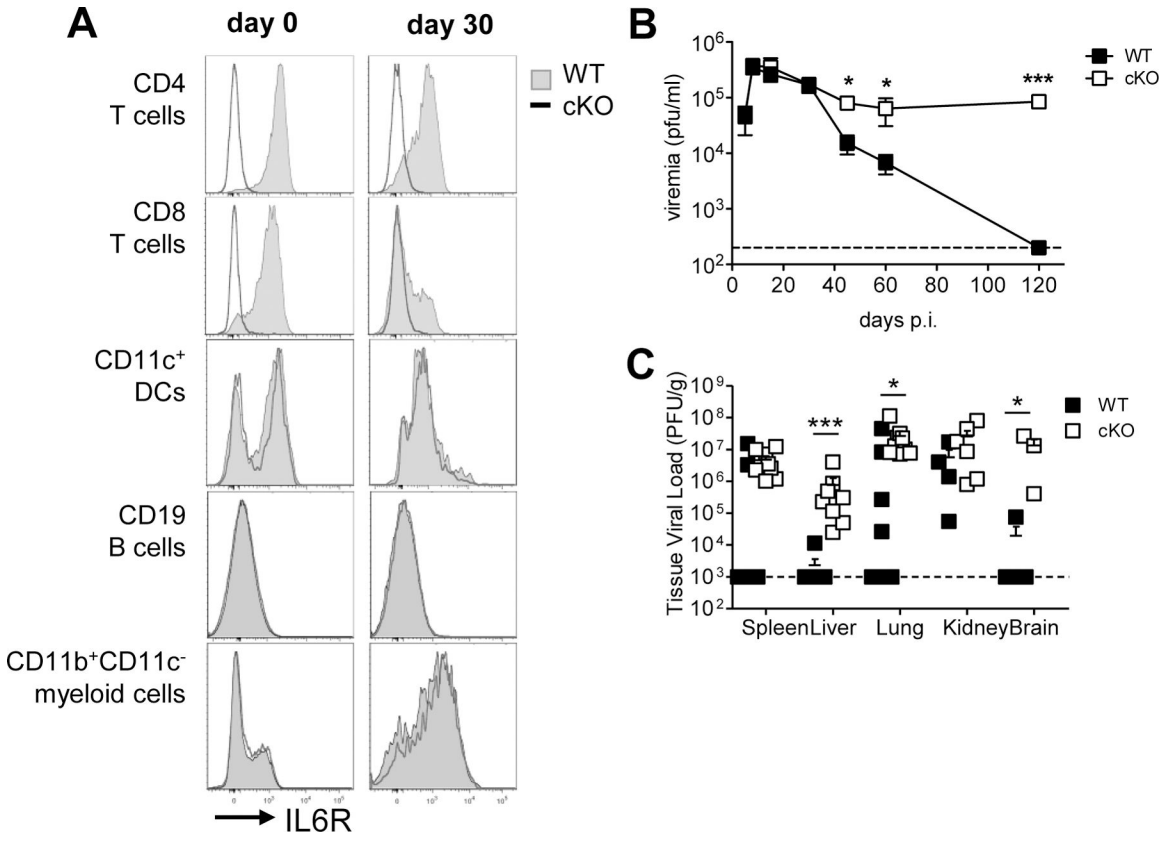

Figure 1. IL6R signaling on T cells is required for control of chronic LCMV viremia

WT or IL6R-cKO (cKO) mice were infected i.v. with 2×10^6 pfu LCMV Cl13. (A) At day 0 or day 30 post-infection, spleens were harvested and surface expression of IL6R was quantified by flow cytometry on CD4 T cells ($CD3^+CD4^+$), CD8 T cells ($CD3^+CD8a^+$), DCs ($CD11c^+CD19^-CD3^-$), B cells ($CD19^+$), and myeloid cells ($CD11b^+CD11c^-CD19^-CD3^-$). (B) Serum was collected at various timepoints up until day 120 post-infection. (C) Tissues were harvested from mice at day 120 post-infection, the dotted line represents the limit of detection. Serum (viremia) and tissue viral titers were quantified by VERO cell-based plaque assay. A is representative of 3 independent experiments. B and C. Data represent n = 6–8 mice per group per tissue from 2 independent repeats.

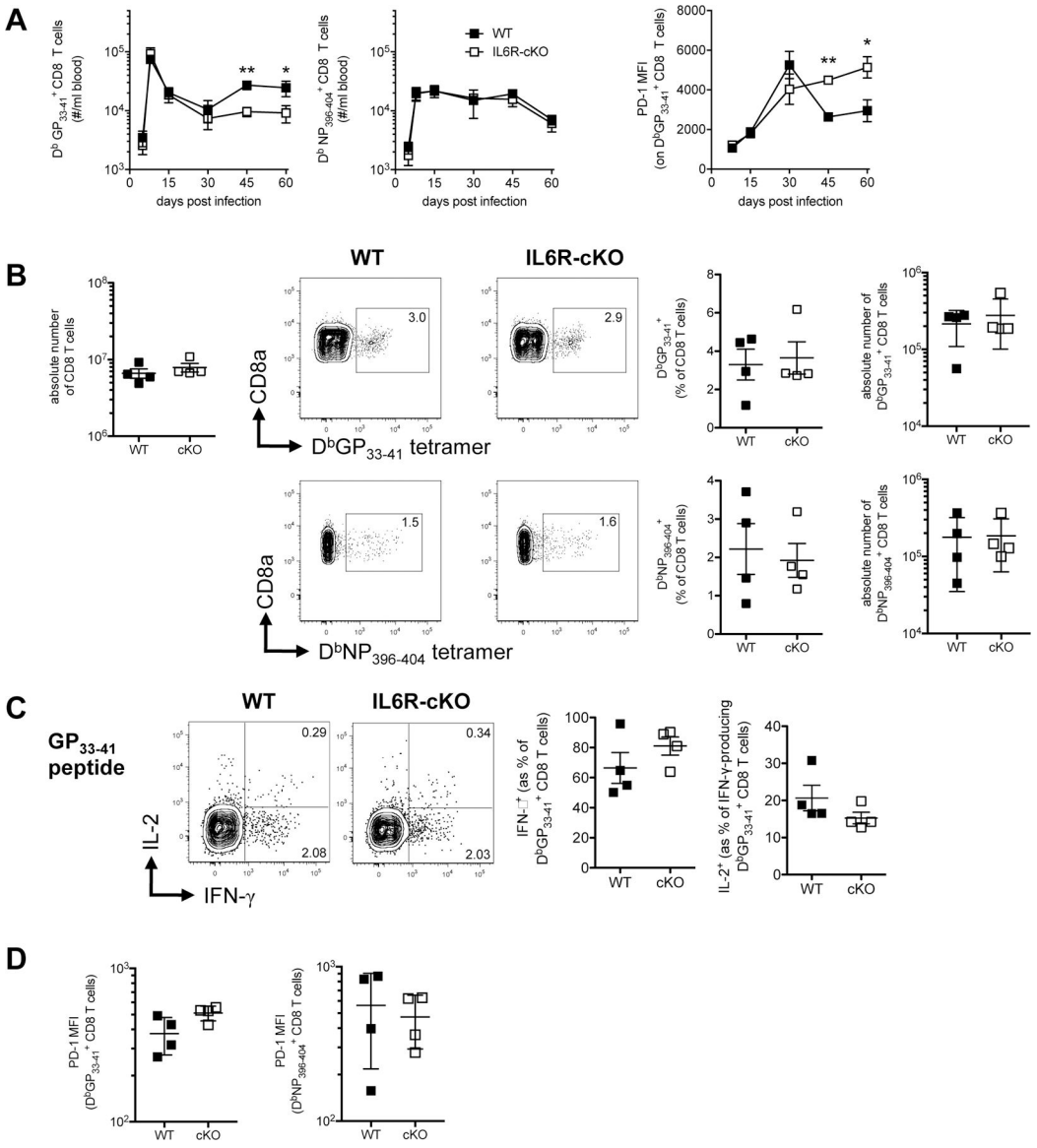

Figure 2. CD8 T cell responses are unaffected by the deletion of IL6R

WT or IL6R-cKO (cKO) mice were infected i.v. with 2×10^6 pfu LCMV Cl13. (A) The frequency of circulating $GP_{33–41}$MHCI-tetramer$^+$ and $NP_{396–404}$MHCI-tetramer$^+$ CD8 T cells, and the expression of PD-1 on the $GP_{33–41}$-specific CD8 T cells was determined at various time points post-infection. At day 30 post-infection, spleens were harvested and stained for phenotypic analysis by flow cytometry. (B) Frequency and absolute numbers of virus-specific CD8 T cells, identified using MHCI-tetramers loaded with $GP_{33–41}$ or $NP_{396–404}$ peptides. (C) Intracellular cytokines production was detected after splenocytes were restimulated with $GP_{33–41}$ peptide in the presence of BFA. (D) Median fluorescence intensity (MFI) of PD-1 expression on virus specific CD8 T cells. Data represent n = 4–5 mice per group and is representative of at least 3 independent experiments.

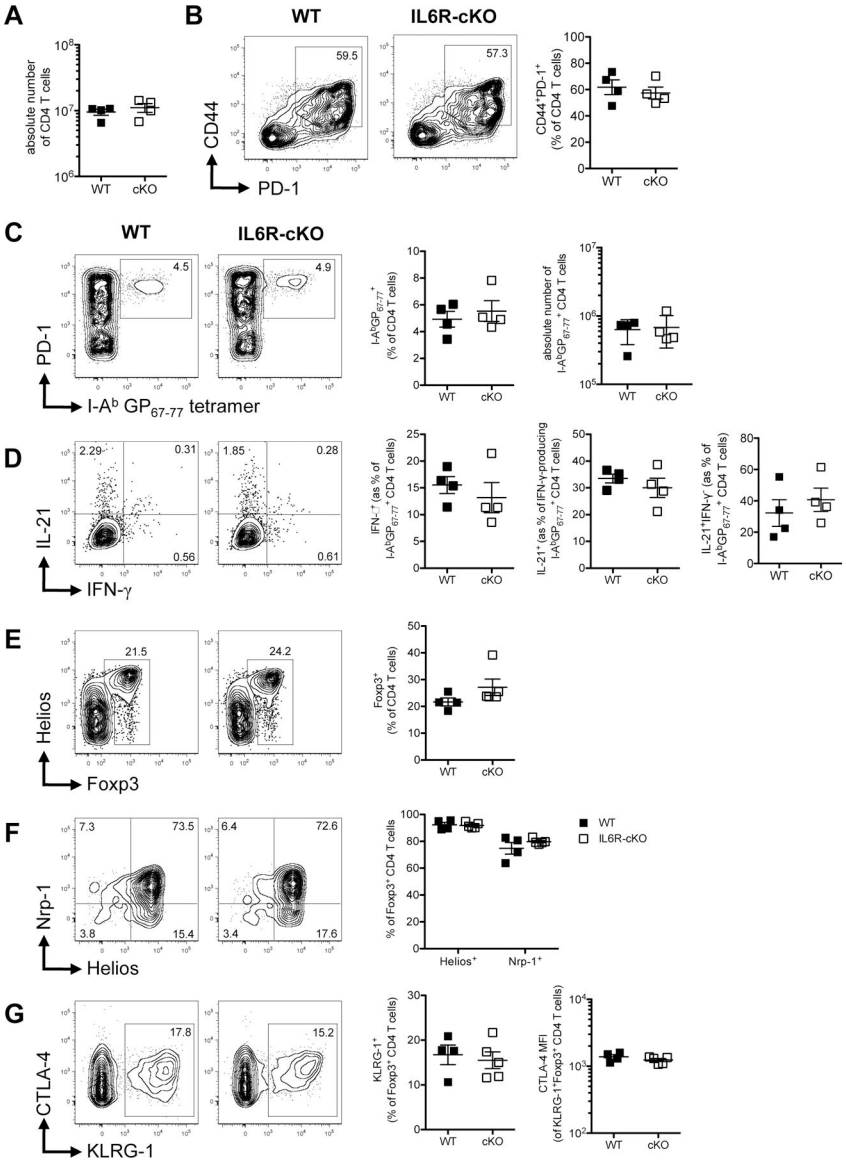

Figure 3. Effector and regulatory CD4 T cell responses are unaffected by the deletion of IL6R

WT or IL6R-cKO (cKO) mice were infected i.v. with 2×10^6 pfu LCMV Cl13. At day 30 post-infection, spleens were harvested and stained for phenotypic analysis by flow cytometry. (A) Absolute number of total CD4 T cells, (B) frequency of activated CD4 T cells ($CD44^+PD\text{-}1^+$), and (C) frequency of virus-specific CD4 T cells, identified using MHCII-tetramers loaded with $GP_{67–77}$ peptide. (D) Intracellular cytokines production was detected in CD4 T cells after whole splenocytes were restimulated with $GP_{67–77}$ peptide in the presence of BFA. (E) Frequency of $Foxp3^+$ CD4 T cells, (F) proportion of $Foxp3^+$ cells that also express Helios, or Nrp1, and (G) the proportion of $Foxp3^+$ cells that express KLRG-1, as well as MFI of CTLA-4 of the KLRG-1^+ compartment. Data represent n = 4–5 mice per group and is representative of at least 3 independent experiments.

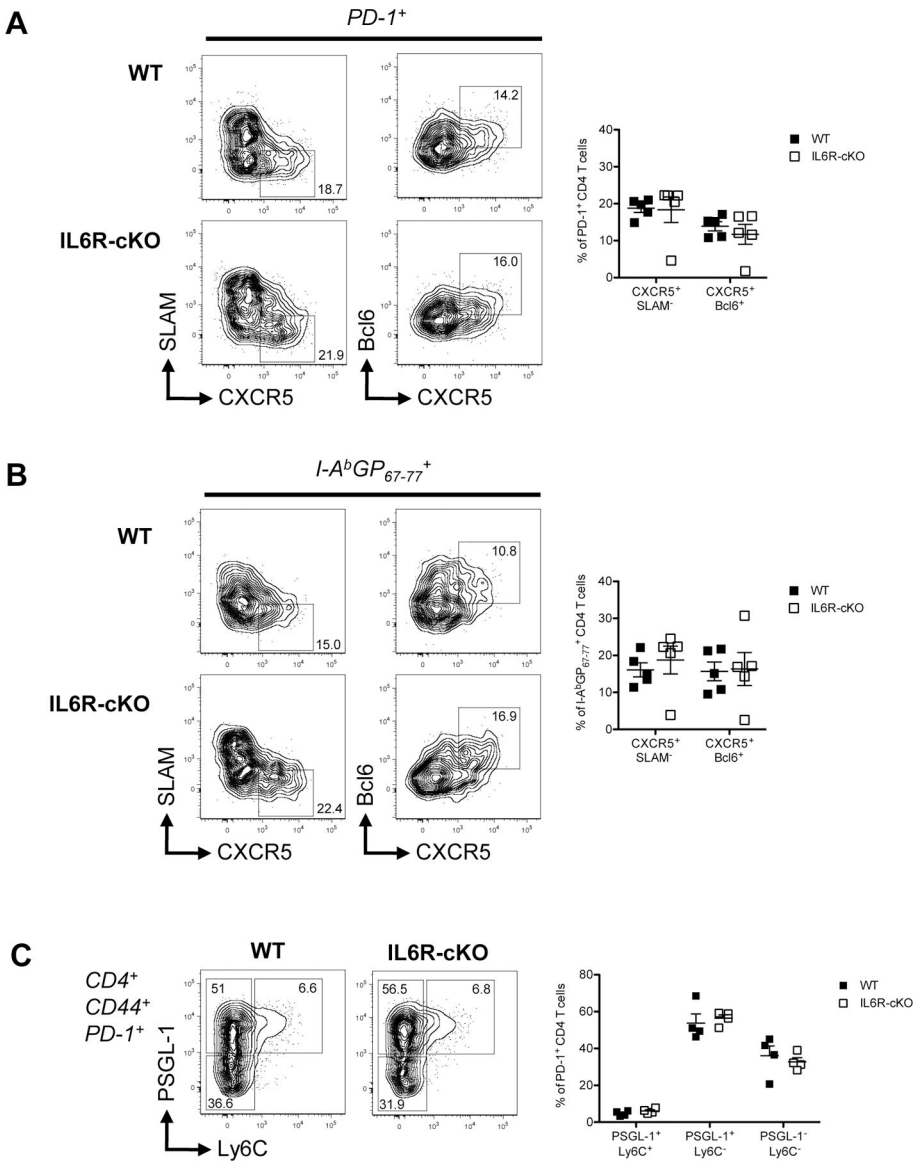

Figure 4. The size of the Tfh compartment is unaffected in the absence of IL6R signaling

WT or IL6R-cKO mice were infected i.v. with 2×10^6 pfu LCMV Cl13. At day 30 p.i., spleens were harvested and assayed by flow cytometry. Frequency of PD-1^+ (A) and $GP_{67–77}$MHCII-tetramer$^+$ (B) Tfh cells identified by CXCR5^+SLAM$^–$ or by CXCR5^+Bcl6^+ flow cytometry staining. (C) Frequency of CD4 T effector, memory precursor, and Tfh cells as phenotyped by expression of effector markers Ly6C and PSGL-1. Data represent n = 4–5 mice per group and is representative of at least 3 independent experiments.

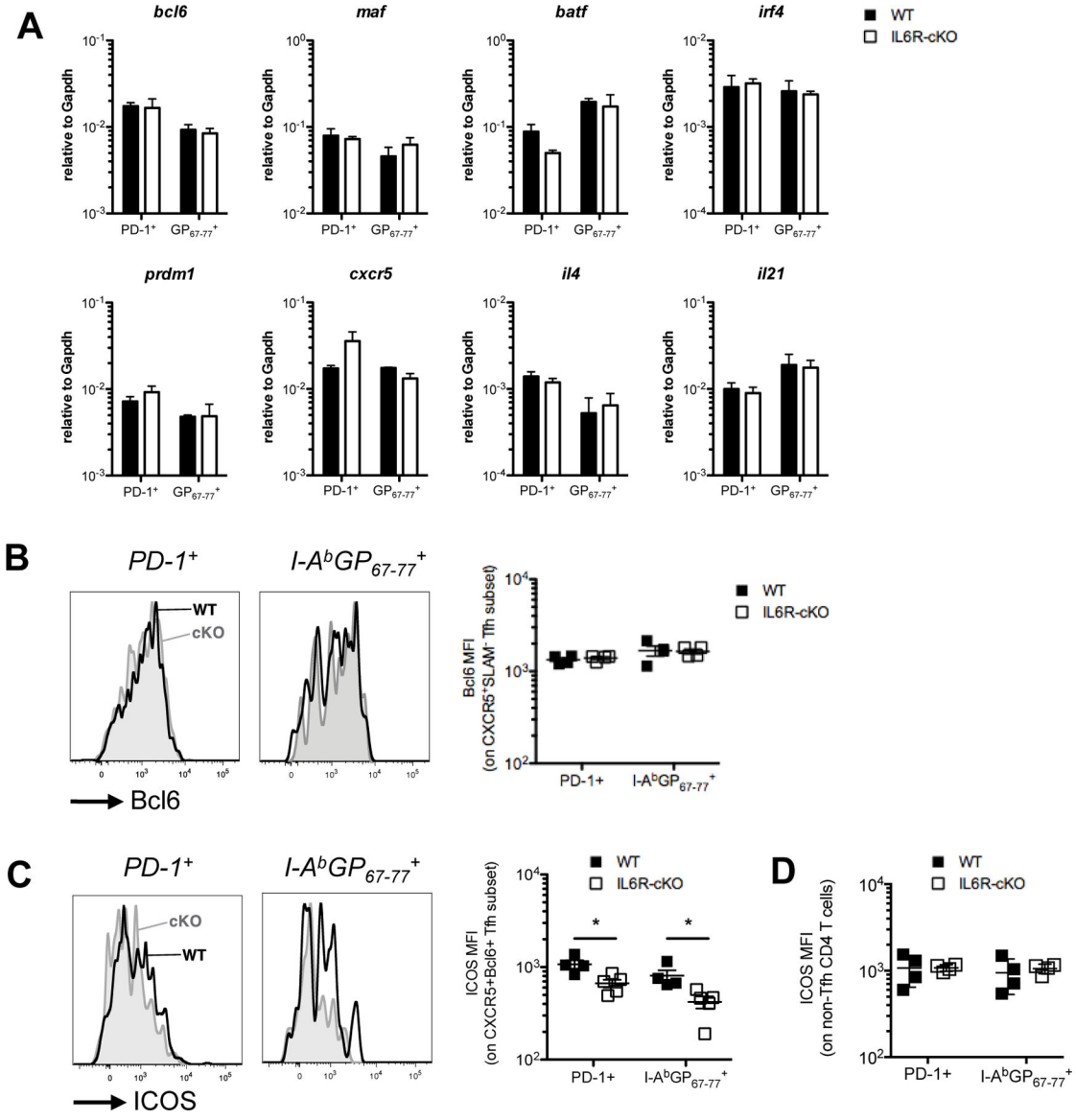

Figure 5. Suboptimal ICOS expression on the Tfh compartment in the absence of IL6R signaling during chronic LCMV infection

WT or IL6R-cKO mice were infected i.v. with 2×10^6 pfu LCMV Cl13. At day 30 p.i., spleens were harvested and assayed by flow cytometry. (A) At day 30 p.i., PD-1^+ or $GP_{67–77}$MHCII-tetramer$^+$ CD4 T cells were FACS-sorted and expression of Tfh-related transcription factors and functional genes were assayed by qPCR. (B) MFI of Bcl6 on activated PD-1^+ or virus-specific Tfh cells. (C) MFI of ICOS on activated PD-1^+ or virus-specific Tfh cells. (D) MFI of ICOS on non-Tfh cells (cells that were not CXCR5$^+$Bcl6$^+$) within the activated PD-1^+ or virus-specific CD4 T cell compartments. Data represent n = 4–5 mice per group and is representative of at least 3 independent experiments.

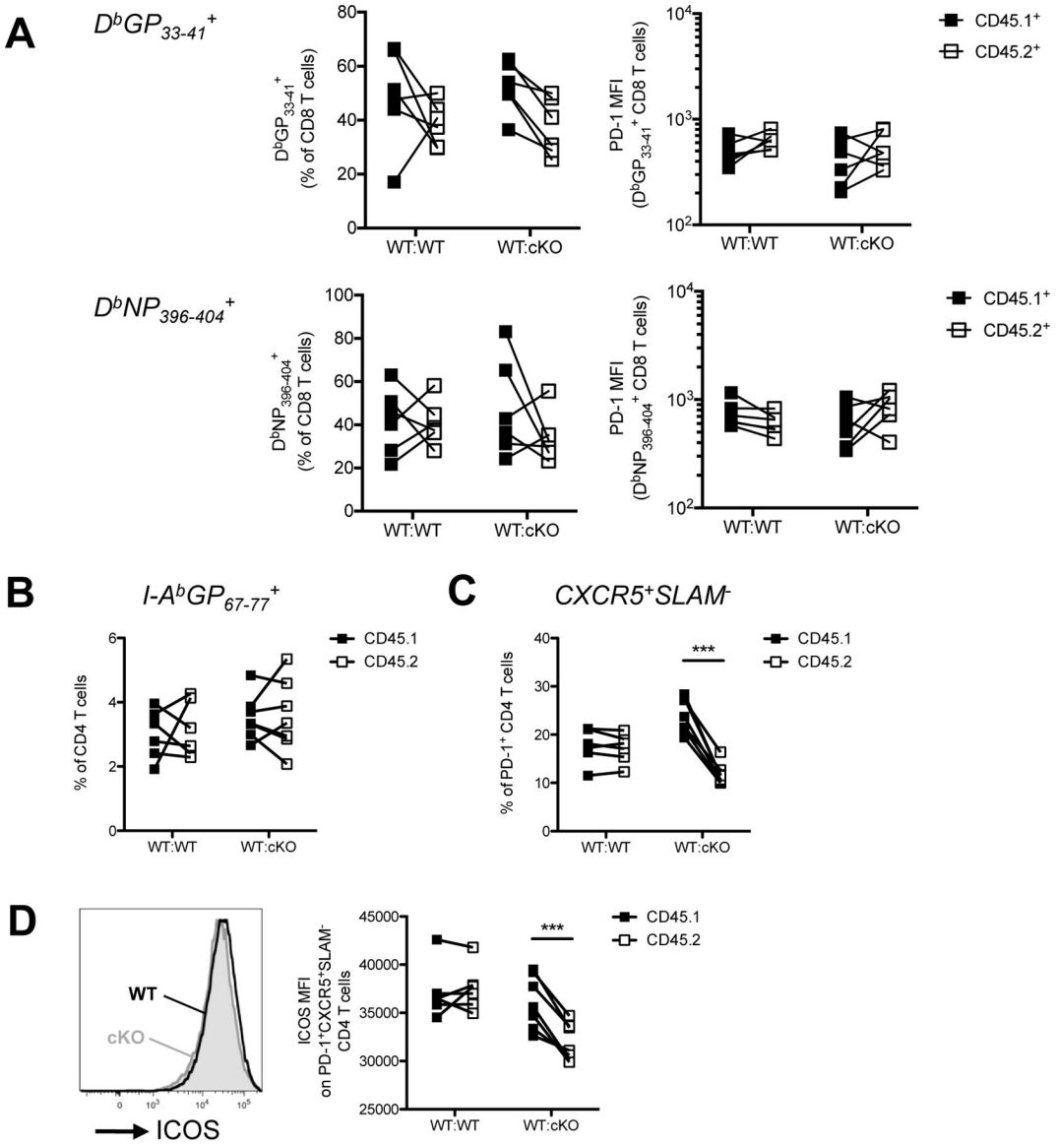

Figure 6. IL6R-deficient CD4 T cells have intrinsically defective responses during chronic viral infection.

Mixed BM chimeras were generated using a 40:60 ratio of BM cells from WT (CD45.1$^+$) and either WT or IL6R-cKO (CD45.2$^+$) donors. 8 weeks after reconstitution, chimeras were infected with 2×10^6 pfu of LCMV Cl13 and the spleen analyzed at day 30 p.i. (A) $GP_{33–41}$MHCI-tetramer$^+$ and $NP_{396–404}$MHCI-tetramer$^+$ CD8 T cells, and their expression of PD-1 and (B) $GP_{67–77}$MHCII-tetramer$^+$ virus-specific CD4 T cells were analyzed within their respective congenic compartments. (C) Proportion of activated PD-1$^+$ CD4 T cells that were Tfh cells (CXCR5$^+$SLAM$^-$). (D) MFI of ICOS on Tfh cells (PD-1$^+$CXCR5$^+$SLAM$^-$). Data represent 6 mice per group and is representative of 2 independent repeats.

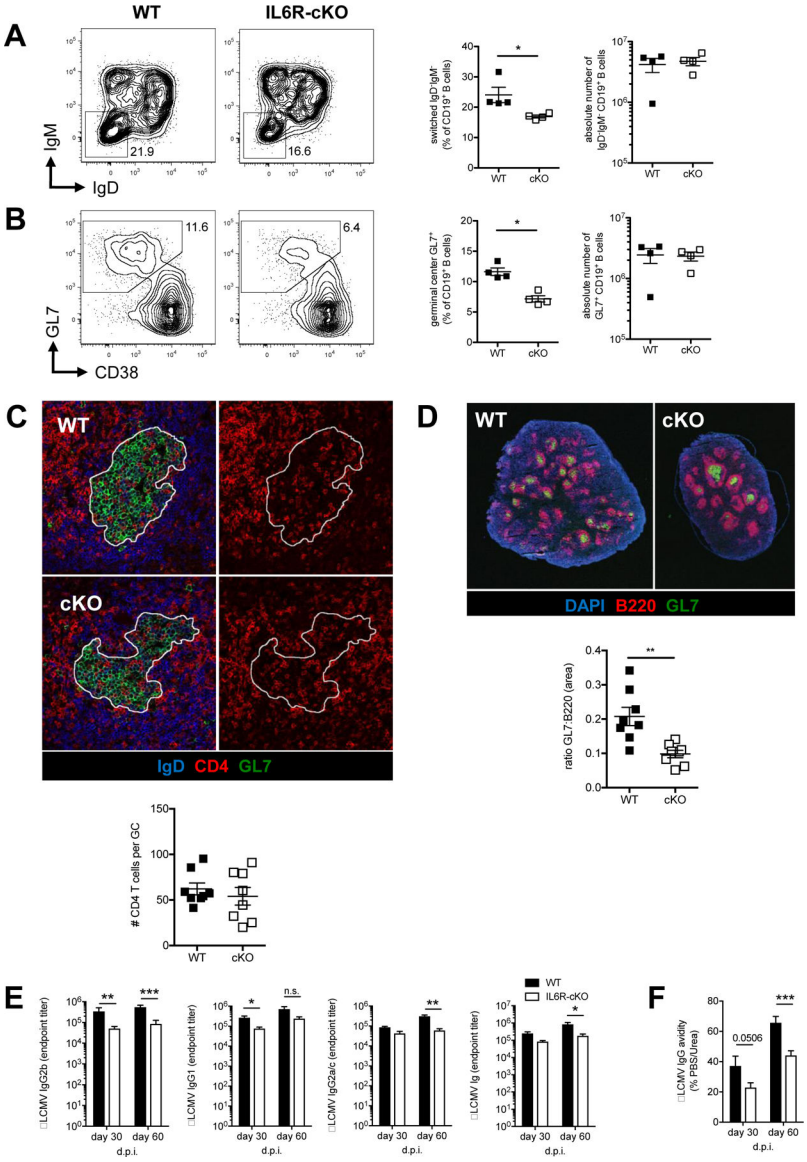

Figure 7. Altered B cell and antibody responses when T cells lack IL6R

WT or IL6R-cKO (cKO) mice were infected i.v. with 2×10^6 pfu LCMV Cl13. At day 30 p.i., spleens were harvested. Frequency and absolute numbers of (A) $CD19^+IgD^-IgM^-$ isotype switched B cells (gated on $CD19^+$) and (B) $CD19^+GL7^+CD38^-$ germinal center B cells were quantified by flow cytometry. (C-D) Spleens were imaged by confocal microscopy and (C) the number of $CD4^+$ cells within the GCs ($GL7^+$ area) was enumerated and (D) the proportion of GC ($GL7^+$) area within the area of B cell zones ($B220^+$) was quantified; example confocal images are shown. (E-F) At days 30 and 60 p.i., serum was collected and assayed for LCMV-specific Ab (E) as well as Ab avidity (F) by ELISA. A & B represent n = 4 mice per group and are representative of 3 independent repeats. C & D represent n = 8–9 mice per group from 2 independent experiments. E-F represent n = 9 mice from 2 independent repeats.