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Late Interleukin-6 escalates T follicular helper cell responses and controls a chronic viral infection[#]

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Abstract

Multiple inhibitory molecules create a profoundly immunuosuppressive environment during chronic viral infections in humans and mice. Therefore, eliciting effective immunity in this context represents a challenge. Here we report that during a murine chronic viral infection, interleukin-6 (IL-6) was produced by irradiation resistant cells in a biphasic manner, with late IL-6 being absolutely essential for viral control. The underlying mechanism involved IL-6 signaling on virus-specific CD4 T cells that caused up-regulation of the transcription factor Bcl6 and enhanced T follicular helper (Tfh) cell responses at late, but not early, stages of chronic viral infection. This resulted in escalation of germinal center reactions and improved antibody responses. Our results uncover an antiviral strategy that helps to safely resolve a persistent infection *in vivo*.

Chronic viral infections, such as human immunodeficiency virus (HIV)-1, Hepatitis B and C viruses (HBV and HCV) in humans and lymphocytic choriomeningitis virus (LCMV) in rodents create an altered immune environment in the infected host. This is characterized by deletion and functional exhaustion of T cell responses (1, 2), delayed and often dysfunctional appearance of antibodies (3, 4) and dysregulation of innate immunity (5, 6). These enable the virus to persist and make the host extremely susceptible to a range of secondary infections, inflammatory disorders and cancers (7, 8). Despite this inhibitory environment the remaining immune responses can often elicit partial (or even complete) control over persistent infections, but the molecules promoting such responses remain poorly understood. Classical anti-viral mediators such as type I interferons are attenuated early and throughout the course of chronic viral infection (4, 5, 9), whereas CD4-derived IL-21 is critical for helping CD8 T cell responses and viral control during chronic LCMV and HIV-1 infections (10–14). This suggests that the host immune system uses only select antiviral strategies to contain a pathogen once it has productively spread in vital tissues. A greater understanding of such strategies may lead to more effective, and safer, therapeutic approaches to alleviate chronic infections.

To gain insight into the molecules governing immunity during chronic viral infections we infected mice with LCMV Clone 13 (Cl 13), a persistent variant of LCMV (15), and analyzed cytokine production throughout infection. We determined the serum levels of over 30 different cytokines and chemokines between day 1 and 30 post infection (p.i.) with higher resolution between days 20 to 30 p.i., a time period that precedes the decline in viremia during LCMV Cl 13 infection. As we have previously reported (5), type I IFN levels rapidly increased on day 1 p.i., with little or no detectable IFN-α in the serum between day 5

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p.i. and the end of the study at day 30 p.i. (Fig. S1A). A similar pattern of acute secretion was observed with most cytokines studied during Cl13 infection (Fig. S1B). In contrast, a profile of two wave inflammation was revealed by interleukin-6 (IL-6) and G-CSF, with a strong initial peak on days 1 and 3 p.i., followed by a second significant peak around day 25 p.i.(Fig. 1A and S1C). Acute infection with LCMV Armstrong 53b (ARM) resulted solely in the initial peak of both IFN-α and IL-6 (Fig. S2). Remarkably, IL-6 was essential for clearing LCMV Cl 13 from blood and all tissues studied. IL-6 knockout (ko) mice (16) had between 10^5 to 10^7 Plaque Forming Units (PFU) of virus up to 450 days p.i., in stark contrast to wild type (WT) mice that had eradicated the virus from most tissues, except kidneys where low levels remained (Fig. 1B & Fig. S3A). As previously reported, IL-6 ko mice showed normal viral clearance during acute ARM infection ((16) & Fig. S3B). These data revealed a biphasic inflammatory response that was specific for chronic LCMV infection and involved IL-6 production, which was vital for viral control.

Infection of fully reconstituted bone marrow (BM) chimeras of IL6-ko BM into lethally irradiated WT hosts (IL6-ko>WT) resulted in similar serum IL-6 levels to those seen in the WT>WT mice (Fig. 1C & Fig. S4). In contrast, WT>IL-6ko mice showed only minor IL-6 production at day 1 p.i., and no detectable IL-6 for the remainder of infection. Viremia in these mice mirrored serum IL-6, with WT>WT and IL-6ko>WT mice showing significantly reduced viral loads by day 60 p.i. and thereafter compared to either WT>IL-6ko or IL-6ko>IL-6ko mice (Fig. 1D). The spleen appeared to be an important source for IL-6 (Fig. S5A) and this was consistent with up-regulated *Il6* transcript in splenic leukocytes at day 1 p.i. (Fig. S5B) and $CD45^-$ cells at day 1 and 25 p.i. (Fig. 1E). Notably, $CD45^-$ FDC-$M1^+$ $CD21/35^+$ cells, which showed size, granularity, and gene expression associated with follicular dendritic cells (FDCs) (Fig. S6) (17, 18), exhibited the highest levels of *Il6* RNA at day 25 (but not day 1) p.i. (Fig. 1E), suggesting that irradiation resistant FDCs were an important source of late IL-6 during chronic LCMV infection.

IL-6 is a pleiotropic cytokine with described roles in cell survival, differentiation, proliferation and inflammation (19). This includes induction of IL-21 in $CD4^+$ T cells that could aid CD8 T cell responses (11–13, 20, 21). We, however, observed no difference between IL-21 RNA or protein levels in WT *versus* IL-6 ko virus-specific CD4 T cells (Fig. S7A&B). Moreover, CD8 T cell responses in WT and IL-6ko mice at day 30 p.i. (i.e. after the second wave of IL-6 but before viremia became different) were indistinguishable in the number of H2-D^b $NP_{396–404}$ or H2-D^b $GP_{33–41}$ specific $CD8^+$ T cells, their surface expression of the T cell exhaustion marker PD-1 (22) and the degree of functional exhaustion (Fig. S7C–F & S8). There was also no significant reduction in the numbers of I-A^b $GP_{67–77}$ specific $CD4^+$ T cells in IL-6 ko Cl 13 infected mice compared to WT mice at day 30 p.i. (Fig. S9A). At this time virus specific IFN-γ, IL-2 and TNF-α production from CD4 T cells was also similar, implying IL-6 had no role in conventional $CD4^+$ T helper type 1 (Th1) cell development (Fig. S9B). IL-6 can also inhibit TGF-β dependent development of regulatory T cells (Tregs) while driving the differentiation of IL-17 secreting T helper (Th17) cells (23). We previously reported sustained TGFβ activity during chronic LCMV infection (24), but neither the $FoxP3^+$ $CD4^+$ T cell responses nor the RNA levels of the Th17 master regulator *Rorc* were affected by IL-6 deficiency during Cl 13 infection (Fig. S9C&D).

T follicular helper (Tfh) cells are defined by a combination of cell surface markers including antigen specificity, CXCR5, PD-1, CD200, ICOS and the absence of SLAM in $CD4^+$ T cells (25). The Tfh transcription factor, *Bcl6*, has recently been identified as being required and sufficient for Tfh differentiation (26–28), but the signals that lead to *Bcl6* upregulation during viral infection *in vivo* remain unclear. During Cl 13 infection in WT mice we and others (29) observed a significant increase in virus-specific Tfh cells (defined as I-A^b

$GP_{67–77}$tetramer$^+$CXCR5$^+$CD200$^+$ICOS$^+$SLAM$^-$PD1$^+$), with the majority of virus-specific CD4 T cells showing a Tfh phenotype by day 30 p.i. (Fig. 2A and Fig. S10A). The loss of IL-6 led to a significant reduction in percentage and number of LCMV-specific Tfh cells at day 30 (but not day 9) p.i. (Fig. 2A & Fig. S10B). Specifically, ICOS and CD200 expression on Tfh cells were significantly reduced in the absence of IL-6 at day 30 p.i. (Fig. S10C). While *Bcl6* transcript and protein levels in LCMV specific CD4$^+$ T cells normally increased from day 9 to day 30 p.i., this increase was absent in IL-6ko mice (Fig. 2B&C and S10D&E); a result also seen when CXCR5$^+$BCL6$^+$ CD4 T cells were analyzed (Fig. S11). Notably, as described for *Rorc* (Fig S9D), the expression of *Tbx21* and *Gata3* master transcriptional regulators for T helper (Th)1 and Th2 subsets and the BCL6 antagonist, *Prdm1*, were mostly similar in WT and IL-6 ko LCMV specific CD4$^+$ T cells (Fig. S12). A limitation in their inducing signals (e.g. IL-2 (30)) combined with repression by residual Bcl6 expression (Fig. 2C) (27) may explain the lack of up-regulation in the aforementioned transcriptional regulators. Again, we did not find differences in *Il21* even when virus-specific Tfh and non-Tfh cells were separately analyzed in WT versus IL-6 ko mice *in-vivo* (Fig. S13).

Tfh are central in the development of fully matured germinal center (GC) B cells and the production of high affinity antibodies (25). Consistent with Tfh kinetics, GC B cell responses increased over time in WT mice during Cl 13 infection (Fig. S14A&B). IL-6ko mice had significantly reduced GC B cells at day 30, but not day 9, after Cl 13 infection (Fig. 2D & Fig. S14C). LCMV specific Ig was reduced in IL-6ko Cl 13 infected mice with a significant decrease in the LCMV-specific IgG1 subtype but minimal difference in LCMV specific IgG2a antibodies (Abs) (Fig. 2E and S14D). Antibody avidity was also reduced in IL-6 ko mice (Fig. 2F). IL-6 produced by irradiation-resistant cells was sufficient for Tfh differentiation and reconstitution of the GC B cell response and anti-LCMV Ab levels (Fig S15). As previously shown (31) *Bcl6* expression and GC responses were not affected by IL6 deficiency during acute LCMV infection (Fig. S16). In conclusion, despite the pleiotropic functions ascribed to IL-6, we identified *Bcl6* up-regulation in CD4 T cells and induction of Tfh-B cell responses as the central effects of IL-6 during chronic viral infection.

We next investigated whether the second wave of IL-6 production was responsible for escalating Tfh/B cell responses during chronic LCMV infection. Administration of IL-6 or IL-6R monoclonal (m) Abs into WT Cl 13 infected mice at days >20 p.i. resulted in a significant drop in the number and proportion of Tfh cells and *Bcl6* expression in LCMV-specific CD4 T cells compared to isotype control administration (Fig. 3A&B and Fig. S17A–C). GC B cells and LCMV-specific Abs were also reduced (Fig. 3C & S17D). No changes were observed in virus-specific CD8 T cell number, their PD1 expression or CD4 Treg numbers during late treatment with IL-6 or IL-6R mAbs when analyzed at day 30 p.i. (Fig. S17 E–G). Additionally we could not observe any change in late Tfh responses, GC reactions, or CD8 T cells responses when IL-6R mAb was administered early on day −1 to day 5 p.i. (Fig. S18A–F). Importantly, late treatment of WT Cl 13 infected mice with anti-IL-6R or anti-IL-6 mAbs resulted in prolonged viremia revealing that IL-6 signaling during this period was essential for optimal viral control (Fig. 3D&E). These results indicated that late (rather than early) IL-6 was vital for maximizing Tfh and GC responses restraining viral replication in the face of the profound immunosuppressive environment that characterizes established chronic infections (1).

Finally, we sought to elucidate whether CD4 and/or B cells were the direct IL-6 targets during Cl 13 infection. *Ex vivo* IL-6 stimulation of total or LCMV-specific CD4$^+$ T cells, but not B cells, led to rapid phosphorylation of the main IL-6 transcription factor, STAT-3, regardless of infection status (Fig. 4A & S19A). *Ex vivo* IL-6 stimulation of total or LCMV-specific CD4$^+$ T cells isolated at day 0, 8 or 18 after Cl 13 infection resulted in similar

increase in the IL-6 prototypical target genes *Il6ra* and *socs3* (Fig. 4B& S19B). *Bcl6* and *Il-21*, however, were more rapidly and/or strongly induced in $CD4^+$ T cells isolated at day 18 p.i. (Fig. 4B and S19B). These data indicated that despite comparable signaling, the outcome of IL-6 stimulation in virus-specific $CD4^+$ T cells was dynamic, and resulted in rapid *Bcl6* induction only at late stages of chronic LCMV infection.

To determine the cell-intrinsic effect of IL-6 signaling *in vivo* we generated mixed chimeras of WT and IL-6 receptor (IL-6R) ko mice (32). WT and IL-6R ko cells showed successful T cell and B cell reconstitution before infection (Fig. S20) and total as well as LCMV specific $CD8^+$ and $CD4^+$ T cells were similarly represented in WT *versus* IL6R ko compartments at day 30 after Cl 13 infection (Fig S21 A&B). The proportions of LCMV-specific Tfh cells (analyzed with two different sets of Tfh markers) were, however, significantly biased towards WT respect to IL-6R ko cells (Fig. 4C). WT LCMV-specific CD4 T cells also exhibited upregulated Bcl6 RNA and protein expression compared to their IL-6R ko counterparts at day 30 p.i. (Fig. 4D&E). On the other hand, the proportion of total and GC B cells were comparable in WT *versus* IL-6R ko compartments (Fig. S21C). These data demonstrated that IL-6R promoted virus-specific Tfh responses in a cell-intrinsic fashion but did not directly control GC differentiation, suggesting that the decreased GC responses observed in IL-6 ko mice were secondary to Tfh impairment. Accordingly, adoptive transfer of Tfh enriched cells from day 30-Cl13 infected WT mice into infection-matched-IL6 ko recipients resulted in improved GC and Ab responses and enhanced viral control; contrasting with either untreated mice or mice that received non-Tfh cells from the same WT donors (Fig. 4F and S22). Conversely, SAP ko mice that showed impaired Tfh responses during Cl 13 infection exhibited reduced GCs and failed to clear viremia, despite normal IL-6 levels and enhanced CD8 T cell responses (Fig. S23) (33, 34). Altogether, these results support the idea that Tfh are central to IL-6 mediated viral control.

The mechanism of *Bcl6* upregulation and Tfh cell generation remain unclear. CFA immunization requires IL-6 for Tfh differentiation, but alum immunization or acute virus infection does not (21, 31, 35). A recent report described functional redundancy between IL-6 and IL-21 to induce Tfh cells during acute LCMV infection (36) and this may explain the residual Bcl6 expression and Tfh features in IL-6 ko chronically infected mice. However, IL-6 was absolutely essential to reach the optimal Bcl6 and Tfh up-regulation during late chronic infection. While differential location or cell source (i.e. FDCs) of IL-6 may play a role in determining the effect of late, versus early, IL-6 signaling, our data suggests that CD4 T cells are also intrinsically more prone to upregulate Bcl6 in response to IL-6 at later stages of infection This may be determined by a combination of precise TCR affinity (37), sustained TCR stimulation (29, 38), low IL2Rα signaling (30, 39) and possibly other signals (or lack thereof) that integrate with the IL-6 pathway at different times during infection. On the other hand, as IL-21 was unchanged in IL6 ko mice but *ex-vivo* IL-6 stimulation is capable of driving IL-21 in non-infectious (21, 35) as well as chronically infectious conditions; it is conceivable that redundancy may occur *in vivo* to secure IL-21 induction during persistent viral infection.

FDCs produce IL-6 that supports GC reactions during immunization (40, 41) and are likely the biologically relevant IL-6 source during late chronic viral infection. Whether late IL-6 production and escalation of Tfh cells occur in HIV-1, HCV and/or other infections during which delayed emergence of GC responses and/or neutralizing Abs have been observed (4, 8, 42) is worthy of further investigation. Indeed, elevated IL-6 has been found in serum from HIV, HCV and HBV infected patients but its immune functions in these contexts remain elusive (43–45). Boosting IL-6 signaling in CD4 T cells and/or the downstream Tfh responses could aid therapies to combat persistent viruses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The data reported in this paper are tabulated in the Supporting Online Material. The authors would like to thank Dr. A. Drew (University of Cincinnati), Dr. P. Schwartzberg (NIH), Dr. M. David (UCSD) and Dr. S. Crotty (LIAI) for providing IL-6R ko, SAP ko, stat3 ko mice and CD45.1^{+} Smarta mice, respectively. We are thankful to Dr. S. Crotty for insightful discussion, to L.-Y. Liou for technical help with initial experiment and to A. Dolgoter for technical assistance throughout. This work was supported by grants from the National Institutes of Health (AI072752 and AI081923 to EZ, and AI09484). JAH and EIZ have a provisional patent (no. 61/475,511) relating in part to methods of treating chronic viral infections by administering compounds that boost IL-6 signaling and/or Tfh responses.

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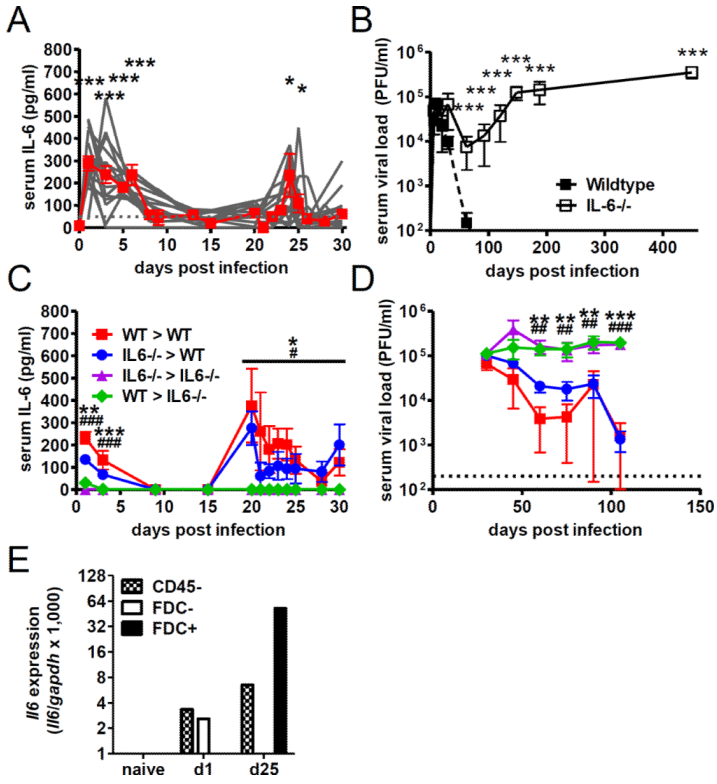

Fig 1.

Biphasic IL-6 is produced by radiation resistant cells and is essential for virus control during chronic LCMV infection. **(A)** C57BL/6 WT mice were infected with LCMV Cl 13 and serum IL-6 concentrations were determined by enzyme-linked immunosorbant assay (ELISA) throughout infection. **(B)** WT or IL-6 ko mice were infected with LCMV Cl 13 and virermia determined by plaque assay. **(C&D)** WT or IL-6 ko mice were lethality irradiated and reconstituted with BM from either WT or IL-6 ko mice. 8 weeks later mice were infected with LCMV Cl 13. Serum IL-6 levels (C) and viremia (D) were determined. **(E)** *Il6* expression relative to *gapdh* was determined in FACS isolated $PI^{-}CD45^{-}$, $PI^{-}CD45^{-}FDCM1^{-}CD21/35^{-}$ and $PI^{-}CD45^{-}FDCM1^{+}CD21/35^{+}$ splenocytes from either WT mice either naïve, at day 1 or day 25 post Cl 13 infection. (A–D) are the mean ± SEM of ≥4 mice per group representative of >2 independent experiments and statistical comparison were performed by two-way ANNOVA. *WT>WT *versus* WT>IL- 6ko or $^{\#}$WT>WT *versus* IL-6ko>IL-6ko. (E) Data is pool of >2 mice per group and representative of 3 independent experiments. * P<0.05, ** P<0.01 and ***P<0.001.

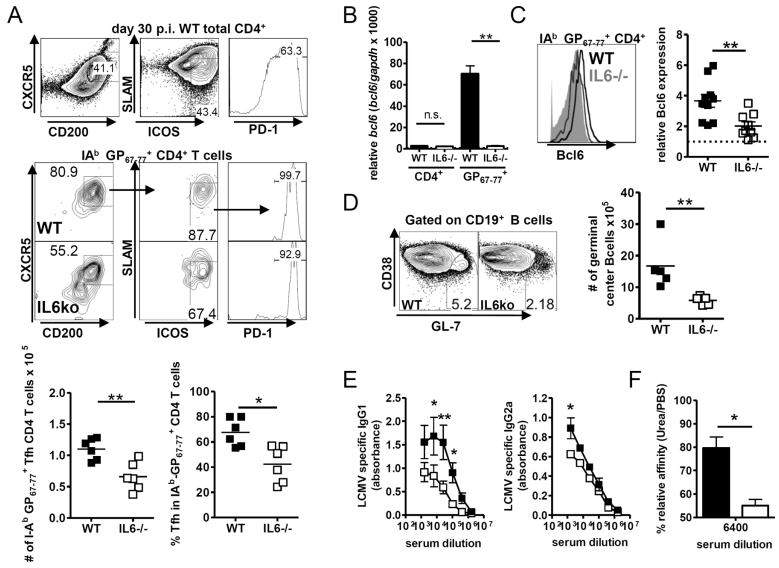

Fig. 2.

T follicular helper cell and germinal center responses are increased in an IL-6 dependent fashion at late stages of chronic LCMV infection. WT or IL-6 ko mice were infected with LCMV Cl 13 and splenocytes analyzed at day 30 p.i. **(A)** The number and percentages of $CD4^+$ $I\text{-}A^b$ $GP_{66–77}$ $tetramer^+$ $CXCR5^+$ $ICOS^+$ $SLAM^–$ $CD200^+$ PD1 T follicular helper cells (Tfh) was determined by flow cytometry. **(B)** Sorted WT and IL-6ko $I\text{-}A^b$ $GP_{67–77}$ $tetramer^+$ and total $CD4^+$ T cells were isolated and *bcl6* expression, relative to *gapdh*, determined by qPCR. **(C&D)** BCL6 protein levels within WT and IL-6ko $I\text{-}A^b$ $GP_{67–77}$ $tetramer^+$ $CD4^+$ T cells (C) and germinal center B cell ($CD19^+GL7^+CD38^–$) formation (D) were determined by flow cytometry. **(E&F)** LCMV specific IgG1 and IgG2a (E) and Ig avidity (F) were determined by ELISA in serum from WT and IL-6ko mice at day 30 p.i.. Data are presented as individual mice or as mean ± SEM of ≥ 4 mice/group and representative of ≥ 2 experiments, with indicative FACS plots and % gated population shown where necessary. * P<0.05, ** P<0.01 and ***P<0.001.

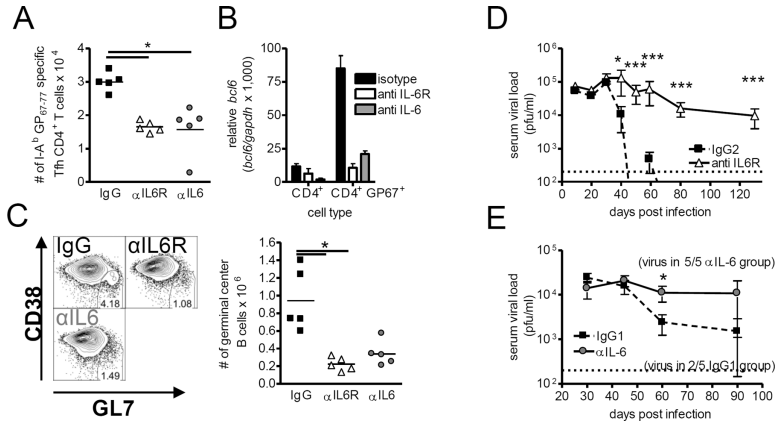

Fig. 3.

Late blockade of IL-6 or IL-6R reduces T follicular helper responses, B cell responses and delays viral clearance. **(A–E)** WT mice were infected with LCMV Cl 13. Mice received either 150μg of IL6R mAb i.p. every 5 days between days 20 and 45 p.i. or an initial dose of 0.5 mg followed by 0.25 mg i.p. every 2 days of IL6 mAb between days 20 and 35 p.i. Control groups were given the equivalent dose, isotype, and regime of Ab treatment. **(A–C)** At day 30 p.i. splenocytes were analyzed for Tfh virus specific $CD4^+$ T cells by flow cytometry (A), *Bcl6* expression in sorted I-A^b $GP_{67–77}$ tetramer$^+$ $CD4^+$ T cells by qPCR (B), and germinal center B cell ($CD19^+GL7^+CD38^-$) formation by flow cytometry (C). **(D&E)** Viremia was determined in mAb treated mice at indicated timepoints p.i. by plaque assay. Representative of 2 experiments with n = 5 mice per group each. * P<0.05, ** P<0.01 and ***P<0.001.

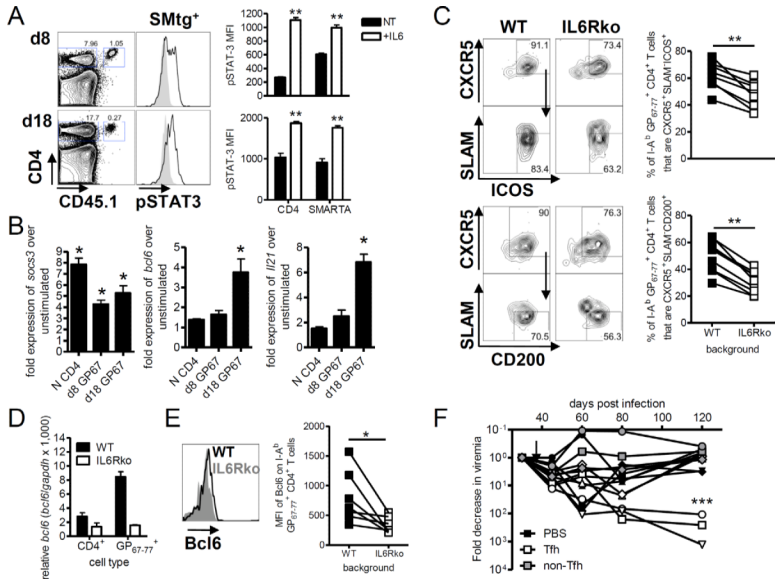

Fig. 4.

Cell-intrinsic IL-6 signaling on virus-specific CD4 T cells upregulates BCL-6 and Tfh responses during chronic LCMV infection. **(A&B)** Adoptively transferred CD45.1+ SMARTA CD4 T cells (A) or LCMV specific I-A^b $GP_{67–77}$ tetramer$^+$ $CD4^+$ (B) were FACS isolated from WT Cl 13 infected mice at day 8 and 18 p.i. and stimulated with rmIL-6 ex-vivo. Phosphorylation of STAT3 was determined by flow cytometry 1h post-stimulation (A). Levels of *socs3*, *bcl6* (12 hrs post stimulation) and *Il21* (6 hrs post stimulation) were determined by qPCR and data shown as fold increase over unstimulated (B). **(C–E)** CD45.1 WT mice were lethality irradiated and reconstituted with matched BM from CD45.1 and IL6-Rko. 8 weeks later mixed chimeras were infected with LCMV Cl13 and analyzed at day 30 p.i. The proportion of splenic CD45.1 and CD45.2 IA^b $GP_{67–77}$ tetramer$^+$ CD4 Tfh cells was determined by flow cytometry (C). IA^b $GP_{67–77}$ tetramer$^+$ CD4 T cells were FACS isolated and *bcl6* expression, relative to *gapdh*, determined by qPCR (D). Bcl6 protein levels were determined in IA^b $GP_{67–77}$ tetramer$^+$ by flow cytometry (E). **(F)** 2×10^6 $CD4^+SLAM^-CD62L^-$ (Tfh enriched) or $CD4^+SLAM^+$ (non-Tfh) cells from day 30 post LCMV Cl13 infected WT mice were transferred i.v. into infection matched IL6ko mice (day 30 p.i.), control IL6ko mice received PBS. Serum viremia was determined by plaque assay and significance determined by 2-way ANNOVA. Changes in the CD4 T cell compartment were assessed by paired t tests between the two populations in individual mice. (A&B) are representative of 2 experiments with ≥4 mice/group. (C–E) are representative of 2 experiments of ≥7 mice/group each. (F) represents 1 experiment with 5 mice per group. * P<0.05, ** P<0.01 and *** P<0.001.