HHS Public Access

Author manuscript

Nat Immunol. Author manuscript; available in PMC 2016 January 14.

Published in final edited form as:

Nat Immunol. 2015 August ; 16(8): 871–879. doi:10.1038/ni.3224.

Production of IL-10 by CD4+ regulatory T cells during the resolution of infection promotes the maturation of memory CD8+ T cells

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Abstract

Memory $CD8^+$ T cells are critical for host defense upon reexposure to intracellular pathogens. We found that interleukin 10 (IL-10) derived from $CD4^+$ regulatory T cells (T_{reg} cells) was necessary for the maturation of memory $CD8^+$ T cells following acute infection with lymphocytic choriomeningitis virus (LCMV). T_{reg} cell–derived IL-10 was most important during the resolution phase, calming inflammation and the activation state of dendritic cells. Adoptive transfer of IL-10-sufficient T_{reg} cells during the resolution phase 'restored' the maturation of memory $CD8^+$ T cells in IL-10-deficient mice. Our data indicate that T_{reg} cell–derived IL-10 is needed to insulate $CD8^+$ T cells from inflammatory signals, and reveal that the resolution phase of infection is a critical period that influences the quality and function of developing memory $CD8^+$ T cells.

Memory $CD8^+$ T cells are a principal component of immunity to intracellular pathogens such as viruses. They are distinguished by their ability to survive long term and to undergo

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Accession codes. GEO: RNA-seq data, SRP058713.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

AUTHOR CONTRIBUTIONS

B.J.L., J.C., S.M.K. conceived of and designed the experiments, analyzed the data and wrote the manuscript; and B.J.L., W.C., R.A.A., S.M.G., T.G., Y.L., Y.K., S.H.K. and R.A.F. performed the experiments.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

rapid and robust proliferation and acquisition of effector function upon reexposure to antigen[1]. Despite the utility of memory $CD8^+$ T cells in protection against pathogens (such as human immunodeficiency virus) that rapidly mutate to elude neutralizing antibodies, the development of T cell–based vaccines has proven problematic[2]. This failure has been largely due to an incomplete understanding of the signals and cell types that operate at different stages of the immune response to influence the quantity and quality of developing memory $CD8^+$ T cells.

The T cell response to an acute infection can typically be divided into the following three phases: expansion, contraction and memory. During the first phase, naive $CD8^+$ T cells divide and differentiate into effector cells that acquire the ability to produce the pro-inflammatory cytokines interferon- γ (IFN-γ) and tumor-necrosis factor (TNF), as well as cytotoxic proteins such as granzymes and perforin[3]. This process by which cytotoxic T lymphocytes (CTLs) undergo differentiation and clonal expansion is governed by signaling via antigens, costimulation and cytokine receptors (including the receptors for IL-2, IL-12, IL-27 and type I interferons) that induce the expression of transcription factors such as Eomes, T-bet and Id2 (ref. 4). However, the strength and duration of these signals, particularly signaling via receptors for inflammatory cytokines, also regulate the long-term fates of these effector cells by influencing whether they differentiate into terminal effector cells (TECs) or maintain memory-cell potential and develop into memory precursor cells (MPCs). These cell fates are controlled by a coordinated set of changes in the expression of the transcription factors Id2, T-bet and Blimp-1, which promote TEC differentiation, and Foxo1, TCF-1, Eomes and Bcl-6, which promote MPC development[5–10]. Activation of the kinases mTOR and Akt downstream of signaling via antigens, costimulation and cytokine receptors provide central regulation of the proliferation and function of CTLs by controlling anabolic metabolism, but they also regulate the differentiation of TECs and MPCs by enhancing T-bet expression and repressing Foxo1 activity[11,12].

Following clearance of the virus, the contraction and resolution phase ensues, in which the majority of the effector $CD8^+$ T cells die and ~5–10% of the cells survive. The surviving cells enter the third stage, the ‘memory’ phase, and develop into central memory T cells (T_{CM} cells), effector memory T cells and resident memory T cells that are maintained long term by IL-7 and IL-15 (ref. 4). Little is known about the signals that operate during the second stage (the contraction and resolution phase) to influence the types and protective capacity of developing memory $CD8^+$ T cells. Although virus is typically cleared by this time point during an acute infection, tissues remain inflamed, and repair processes are initiated to resolve inflammation and retain tissue homeostasis[13]. Sustained exposure of effector CTLs to bystander inflammation impairs the formation of mature memory cells and their precursors[14]. $CD4^+$ T cells are also required during the contraction phase for the formation of functional memory $CD8^+$ T cells, but the mechanisms of their actions are unknown[15]. Additionally, the anti-inflammatory cytokine IL-10 is important for the optimal maturation of memory $CD8^+$ T cells[5,16], but the relevant physiological source of IL-10, as well as the phase in which IL-10 acts to regulate the formation of memory $CD8^+$ T cells, remain ill defined.

Regulatory T cells (T_{reg} cells) are necessary for resolving inflammation and achieving tissue homeostasis following infection, through multiple mechanisms, including expression of inhibitory cytokines such as IL-10 and transforming growth factor-β, regulation of nutrient and cytokine availability, and inhibition of the maturation and function of dendritic cell (DCs) and macrophages[17]. However, the importance of T_{reg} cells in regulating the formation of memory $CD8^+$ T cells is unclear, with some studies identifying their negative role in the development of memory $CD8^+$ T cells[18] and others suggesting the opposite[19–21]. Given the connection between the requirement for $CD4^+$ T cells and that of IL-10 in promoting the formation of memory $CD8^+$ T cells, we investigated whether T_{reg} cells might be linked to this process. In doing so, we identified a previously unknown role for T_{reg} cells in promoting the development of memory $CD8^+$ T cells through their production of IL-10 during the resolution phase of an acute viral infection. This finding indicated that the resolution of inflammation mediated by T_{reg} cells is critical not only for tissue homeostasis but also for the formation of immunological memory.

RESULTS

IL-10 is required during the resolution phase of infection

To confirm and extend published studies investigating the role of IL-10 in the development of memory T cells, we infected IL-10-deficient ($Il10^{-/-}$) and wild-type ($Il10^{+/+}$) C57BL/6 mice with the acutely infectious (Armstrong) strain of lymphocytic choriomeningitis virus (LCMV) and assessed the virus-specific $CD8^+$ T cell response at memory time points (day 45–60 after infection)[5,16]. Although the magnitude of the LCMV-specific response to the immunodominant epitopes of LCMV glycoprotein residues 33–41 (gp(33–41)) and LCMV nucleoprotein residues 396–404 was similar for the two groups, there was a distinct difference in the composition of the splenic memory $CD8^+$ T cell pool (Fig. 1a,b and Supplementary Fig. 1a,b). The majority of the $CD8^+$ T cells from wild-type mice bore a $KLRG1^-CD127^+$ T_{CM} cell phenotype, whereas the $CD8^+$ T cells from $Il10^{-/-}$ mice were $KLRG1^+CD127^-$ and had higher expression of granzyme B than that of their wild-type counterparts and therefore resembled TECs (Fig. 1a,b and Supplementary Fig. 1a,b). Furthermore, the formation of $CXCR3^+CD27^+CD62L^+KLRG1^-$ T_{CM} cells with higher expression of TCF-1 was impaired in $Il10^{-/-}$ mice relative to their formation in wild-type mice (Fig. 1a,b and Supplementary Fig. 1a,b). Thus, consistent with published work[5,16], we found that IL-10 was important qualitatively for the development of mature memory $CD8^+$ T cells.

To determine when IL-10 was needed for the maturation of memory T cells, we infected wild-type mice with LCMV and treated them with neutralizing monoclonal antibody (mAb) to IL-10 during various phases of infection. As observed for $Il10^{-/-}$ mice, mice treated with mAb to IL-10 for the entirety of the infection displayed impaired formation of mature $KLRG1^{lo}CD127^{hi}$ and $CD62L^{hi}KLRG1^{lo}$ T_{CM} cells relative to that of their control counterparts treated with phosphate-buffered saline (PBS) (Fig. 1c and Supplementary Fig. 1c). This finding also suggested that alterations in the basal inflammatory state and/or the microbiota of $Il10^{-/-}$ mice were probably not responsible for the shift in the makeup of the memory $CD8^+$ T cell pool[22,23]. Mice treated with mAb to IL-10 during the priming phase of

infection (days 0–8) had little or no alteration in the composition of their memory $CD8^+$ T cell population relative to that of their counterparts treated for the entirety of infection (Fig. 1c). In contrast, $CD8^+$ T cells in mice in which IL-10 blockade was initiated at day 8 displayed a more TEC-like differentiation state than that of control (PBS-treated) mice (Fig. 1c), similar to mice treated with mAb to IL-10 for the entirety of infection. However, when the IL-10 blockade was begun at day 15, there was no defect in memory maturation (Fig. 1c). These data suggested that the presence of IL-10 during the resolution phase of infection (days 8–15) was critical for the maturation of memory $CD8^+$ T cells.

Signaling through STAT3 is important in promoting the maturation of memory $CD8^+$ T cells, which suggests that IL-10 might signal via this transcription factor and signal transducer[5]. To test this hypothesis, we generated P14 mice (which have transgenic expression of an LCMV-specific T cell antigen receptor) with T cell–specific deletion of the gene encoding the IL-10 receptor (*Il10ra*)(generating '*Il10ra*$^{f/f}$*Cd4*-Cre' mice, with *loxP*-flanked *Il10ra* alleles (*Il10ra*$^{f/f}$) deleted by Cre recombinase expressed from the T cell–specific gene encoding the coreceptor CD4 (*Cd4*-Cre)). We obtained T cells from those mice and transferred small numbers of these cells into congenically mismatched donors, followed by infection of the recipients with LCMV the following day. Although there was a substantially lower percentage and number of *Il10ra*$^{f/f}$*Cd4*-Cre P14 cells than *Il10ra*$^{f/f}$ P14 cells that survived to a memory time point (Supplementary Fig. 2a), consistent with published work examining *Stat3*-deficient $CD8^+$ T cells[5], *Il10ra*$^{f/f}$*Cd4*-Cre P14 cells displayed no gross impairment in the formation of mature $KLRG1^-CD127^+$ or $CD62L^+KLRG1^-$ populations, although they did display slightly more granzyme B protein and less TCF-1 protein than did *Il10ra*$^{f/f}$ P14 cells (Supplementary Fig. 2a,b). Thus, although *Il10ra* expression seemed to be important for the survival of $CD8^+$ T cells in a competitive setting, the phenotypes of the *Il10ra*-deficient memory CD8 T cells did not fully recapitulate those observed in the IL-10-deficient mouse. These experiments suggested that IL-10 signaling acted in both an intrinsic manner and an extrinsic manner to support the generation and maturation of a phenotypically mature memory $CD8^+$ T cell population.

It is possible that IL-10 acts in a $CD8^+$ T cell–extrinsic manner to support memory maturation by insulating the effector $CD8^+$ T cells from exposure to pro-inflammatory signals, such as IFN-α, IFN-β, IL-2 and IL-12, which drive terminal effector differentiation[8,24,25]. To investigate this hypothesis, we infected wild-type and *Il10*$^{-/-}$ mice with LCMV and administered the dinucleotide CpG B, which is an agonist of Toll-like receptor 9, at day 8 or day 15 to induce further inflammation[26]. Wild-type mice were largely insulated from the excess inflammation, with negligible differences between the CpG B–treated group and the control (PBS-treated) group in memory $CD8^+$ T cell composition (Fig. 2). However, *Il10*$^{-/-}$ mice treated with CpG B at day 8 displayed significantly greater loss of $KLRG1^-CD127^+$ and $CD62L^+KLRG1^-$ T_{CM} cells than did control (PBS-treated) *Il10*$^{-/-}$ mice. In contrast, $CD8^+$ T cells in *Il10*$^{-/-}$ mice treated with CpG B at day 15 were indistinguishable from those in control (PBS-treated) *Il10*$^{-/-}$ mice. Thus, IL-10 seemed to be important for insulating effector $CD8^+$ T cells from inflammatory cytokines present during the resolution of infection that could sustain the differentiation or survival of TECs.

T_{reg} cell–derived IL-10 is critical for $CD8^+$ T cell memory

Multiple cell types, including DCs, monocytes-macrophages and T cells, secrete IL-10 following infection with LCMV[27,28]. To distinguish the physiologically relevant source of IL-10 necessary for the memory maturation of $CD8^+$ T cells, we generated mice in which *Il10* was deleted specifically in DCs (*Il10*$^{f/f}$*Cd11c*-Cre mice), myeloid cells (*Il10*$^{f/f}$Lyz2-Cre mice) or T cells (*Il10*$^{f/f}$*Cd4*-Cre mice). We then infected these mice with LCMV and killed them at memory time points (days 45–60). Although the deletion of *Il10* in DCs or myeloid cells had little effect on the maturation of memory $CD8^+$ T cells, deletion of *Il10* in T cells resulted in the loss of mature $KLRG1^-CD127^+$ and $CD62L^+KLRG1^-$ T_{CM} cells, comparable to the phenotype seen in *Il10*$^{-/-}$ mice (Fig. 3a and Supplementary Fig. 3a).

The findings reported above suggested that effector $CD8^+$ T cells, $CD4^+$ T cells, and/or T_{reg} cells might act as the physiological source of IL-10 needed for the memory maturation of $CD8^+$ T cells. We assessed IL-10 expression in these populations in 10BiT reporter mice, following infection with LCMV. 10BiT reporter mice have a bacterial artificial chromosome transgene containing *Il10*, with replacement of the endogenous coding segment of *Il10* exon 1 with cDNA encoding the alloantigen Thy-1.1 (containing a stop codon), such that cells transcribing mRNA from the transgene express Thy-1.1 on their surface[29]. Published work has confirmed that Thy-1.1^+ cells produce IL-10 during infection with LCMV[27]. Although virus-specific $CD4^+$ T cells and $CD8^+$ T cells, as well as T_{reg} cells, had high expression of Thy-1.1 at day 5 after infection, as reported before[27], only T_{reg} cells continued to express *Il10* during the resolution phase of infection. The substantial increase in the number of IL-10-competent T_{reg} cells occurring between day 8 and day 15 is likely due in part to the increase in total T_{reg} cell numbers as type I interferons wane[30] (Supplementary Fig. 3b). Furthermore, T_{reg} cells present during this phase displayed a more activated phenotype than that of T_{reg} cells present at earlier time points, as shown by their loss of expression of the memory marker CD62L and progressive acquisition of expression of the activation markers CD69 and KLRG1 (ref. 31) (Fig. 3b). The expression of *Il10* (as inferred from the expression of Thy-1.1 protein) correlated strongly with the activation state of T_{reg} cells, with $KLRG1^+$ terminally differentiated T_{reg} cells having higher Thy-1.1 expression than that of less-differentiated T_{reg} cell subsets, both on a population basis and a per-cell basis (Fig. 3c). IL-10-competent T_{reg} cells were largely protected from *in vivo* labeling with antibody to CD4 (Supplementary Fig. 3c), which suggested that these cells were located mainly in the white pulp of the spleen, a site that also contains lymphoid DCs and MPCs[32,33].

The data above suggested that T_{reg} cell–derived IL-10 might be important for the maturation of memory $CD8^+$ T cells. To test this hypothesis directly, we generated mice with deletion of *Il10* specifically in T_{reg} cells expressing the transcription factor Foxp3 (*Il10*$^{f/f}$*Foxp3*-Cre mice), as described[34], infected these mice with LCMV and evaluated the formation of virus-specific $CD8^+$ T cells at memory time points (days 45–60 after infection). *Il10*$^{f/f}$*Foxp3*-Cre mice displayed considerable alteration in the maturation of memory $CD8^+$ T cells but not the number of these cells, relative to that of their *Il10*$^{f/f}$ counterparts, as indicated by the large fraction of $KLRG1^+CD127^-$ TEC-like $CD8^+$ T cells (Fig. 3d and Supplementary Fig. 3d). Furthermore, *Il10*$^{f/f}$*Foxp3*-Cre mice had significantly fewer $CD62L^+KLRG1^-$ T_{CM} cells, along with lower expression of the memory-associated markers CXCR3, CD27 and TCF-1

and higher expression of the effector-associated cytolytic factor granzyme B, relative to that of their *Il10*$^{f/f}$ counterparts (Fig. 3d,e). These results demonstrated that T_{reg} cell–derived IL-10 was critical for the maturation of memory CD8$^+$ T cells.

To further evaluate the importance of T_{reg} cell–derived IL-10 in the maturation and function of memory CD8$^+$ T cells, we infected *Il10*$^{f/f}$ and *Il10*$^{f/f}$*Foxp3*-Cre mice with LCMV (Armstrong strain), isolated gp(33–41)-specific CD8$^+$ T cells from these mice at a memory time point, and transferred an equal number of each genotype into congenically mismatched mice. We then challenged the recipient mice the next day with a recombinant strain of *Listeria monocytogenes* expressing the gp(33–41) epitope and, 4 d later, assessed the ability of the transferred cells to expand their populations and mediate bacterial control. Although the *Il10*$^{f/f}$ cells were able to robustly increase in number and diminish the bacterial burden, the cells transferred from *Il10*$^{f/f}$*Foxp3*-Cre mice demonstrated substantial impairment in proliferation relative to the *Il10*$^{f/f}$ cells and failed to reduce bacterial loads from the levels seen in control mice not given cell transfer (Fig. 4). Therefore, T_{reg} cell–derived IL-10 was critical for the development of protective memory CD8$^+$ T cells in the circulation.

T_{reg} cell–derived IL-10 regulates exposure to inflammation

We next performed high-throughput sequencing for cDNA (RNA-seq) to evaluate the transcriptional profiles of virus-specific CD8$^+$ T cells isolated from *Il10*$^{f/f}$*Foxp3*-Cre and *Il10*$^{f/f}$ mice at day 15 after infection with LCMV (Fig. 5a and Supplementary Fig. 4a). Although the composition of virus-specific CD8$^+$ T cells at this early time point seemed to be similar between the groups (Supplementary Fig. 4b), we hypothesized that CD8$^+$ T cells might be already programmed to adopt a more terminally differentiated state as further development occurs. Indeed many of the 81 genes differentially expressed by *Il10*$^{f/f}$*Foxp3*-Cre cells relative to their expression by *Il10*$^{f/f}$ cells (adjusted P value, <0.2) encoded products associated with the formation and function of memory T cells (Fig. 5a and Supplementary Fig. 5). A panel of select genes encoding biologically relevant products also showed dysregulation in CD8$^+$ T cells from *Il10*$^{f/f}$*Foxp3*-Cre mice relative to their regulation in CD8$^+$ T cells from *Il10*$^{f/f}$ mice, with many of these genes being associated with pro- or anti-inflammatory gene sets (Fig. 5a). We used gene-set–enrichment analysis (GSEA) to detect genome-wide changes in expression and identified significant enrichment in the effector signature in CD8$^+$ T cells isolated from *Il10*$^{f/f}$*Foxp3*-Cre mice relative to results obtained for CD8$^+$ T cells isolated from *Il10*$^{f/f}$ mice, which displayed robust enrichment for genes with high expression in the memory signature[35,36] (Fig. 5b and Supplementary Fig. 6a). The transcriptional profile of CD8$^+$ T cells isolated from *Il10*$^{f/f}$*Foxp3*-Cre mice also showed significant enrichment for genes encoding products in pro-inflammatory pathways, with their transcriptional signature resembling that of cells exposed to pro-inflammatory cytokines such as IL-12 or type I interferons, or Toll-like receptor ligands such as the synthetic RNA duplex poly(I:C) or CpG[37,38] (Fig. 5c,d and Supplementary Fig. 6b–d). Therefore, the progenitors of memory cells generated in *Il10*$^{f/f}$*Foxp3*-Cre mice displayed a more TEC-like differentiation state than that of their counterparts generated in *Il10*$^{f/f}$ mice and a genetic signature suggestive of enhanced exposure to inflammatory signals.

Transfer of T_{reg} cells 'rescues' memory maturation of $CD8^+$ T cells

The findings reported above indicated that T_{reg} cell–derived IL-10 might be important during the resolution phase of infection in controlling the degree of inflammatory exposure to effector $CD8^+$ T cells as they develop into memory T cells. IL-10 restricts the maturation of DCs and their production of the pro-inflammatory cytokines IL-6, IL-1β and TNF[39]. Therefore, we assessed the maturation status of $CD45^+CD11c^{hi}MHCII^+$ DCs in *Il10*$^{f/f}$*Foxp3*-Cre mice at day 15 after infection with LCMV and found that the surface expression of markers associated with mature DCs, including CD86, CD80, PD-L1 and PD-L2, was significantly higher in those cells than in their counterparts from LCMV-infected *Il10*$^{f/f}$ mice (Fig. 6a). Additionally, at day 15 after infection, higher concentrations of pro-inflammatory cytokines associated with mature DCs, including IL-6, IL-1β, TNF and IL-12p70, were present in the serum of *Il10*$^{f/f}$*Foxp3*-Cre mice than in that of *Il10*$^{f/f}$ mice (Fig. 6b). There was no difference between uninfected *Il10*$^{f/f}$*Foxp3*-Cre mice and uninfected *Il10*$^{f/f}$ mice in their serum cytokines (Fig. 6b), which indicated that these systemic alterations in the inflammatory milieu did not occur until after infection.

We hypothesized that the production of IL-10 by T_{reg} cells during the resolution phase might be critical for the suppression of inflammation and promotion of the memory maturation of $CD8^+$ T cells. To further test this hypothesis, we used *Foxp3*$^{GFP\text{-}DTR}$ mice (which have cDNA encoding the human diphtheria toxin receptor (DTR) fused to sequence encoding green fluorescent protein (GFP) inserted into *Foxp3*) to ensure depletion of T_{reg} cells (via treatment with diphtheria toxin) at various time points after infection[40]. Consistent with published work[19,20], depletion of T_{reg} cells at the time of infection with LCMV led to impairment in the maturation of $CD8^+$ T cells at memory time points (days 45–60) (Fig. 7a and Supplementary Fig. 7a). However, when the depletion of T_{reg} cells began at day 8 after infection, there was a similar defect in the formation of mature $KLRG1^-CD127^+$ memory $CD8^+$ T cells and that of $CD62L^+KLRG1^-$ memory $CD8^+$ T cells. Mice that underwent depletion of T_{reg} cells at day 15 after infection were indistinguishable from mice not treated with diphtheria toxin, which lent additional support to the model in which T_{reg} cell–derived IL-10 was most critical during the immediate resolution phase in promoting the maturation of memory $CD8^+$ T cells.

To further test that model, we purified IL-10-sufficient *Foxp3*$^+$ $CD4^+$ T_{reg} cells from donor mice 8 d after infection with LCMV and transferred the cells into 'infection-matched' *Il10*$^{-/-}$ host mice. Transfer of $Foxp3^+$ $CD4^+$ T_{reg} cells into *Il10*$^{-/-}$ mice was sufficient to restore the maturation state of $CD45^+CD11c^{hi}MHCII^+$ DCs at day 15 after infection to that found in *Il10*$^{-/-}$ mice that did not receive T_{reg} cells (Fig. 7b). Additionally, *Il10*$^{-/-}$ mice that received IL-10-sufficient T_{reg} cells displayed a significantly greater proportion of mature memory $CD8^+$ T cells 45–60 d after infection and had higher expression of CD127 and TCF-1 along with lower expression of KLRG1 and granzyme B relative to that of *Il10*$^{-/-}$ mice that did not receive T_{reg} cells (Fig. 7c,d and Supplementary Fig. 7b). Therefore, the presence of IL-10-sufficient T_{reg} cells during the resolution phase of infection was sufficient to at least partially 'rescue' many of the defects in memory $CD8^+$ T cell maturation observed in the absence of IL-10.

DISCUSSION

Understanding the signals that promote the formation of a protective memory $CD8^+$ T cell population following viral infection is of crucial importance to furthering efforts in the design of T cell–based vaccines. Here we have described a process by which signals present during the resolution phase of infection promoted the formation of a mature memory $CD8^+$ T cell pool. Early suppression of T_{reg} cell function following infection allowed the activation and population expansion of effector T cells, but following eradication of the virus, IL-10-producing T_{reg} cells accumulated to resolve inflammation and initiate tissue repair and simultaneously promoted the development and maturation of protective memory $CD8^+$ T cells. Our results highlight the need to consider, in the design of T cell–based vaccines, not only the initial signals T cells encounter following vaccination but also the inflammatory environment present during the resolution phase.

Our findings indicated an important role for T_{reg} cells in promoting the maturation of memory $CD8^+$ T cells through calming of the activity of DCs (and probably other pro-inflammatory cells) via the production of IL-10. Our work is consistent with findings showing that DCs that cannot sense IL-10 secrete enhanced amounts of pro-inflammatory cytokines following activation[39]. T_{reg} cells can also suppress DC activation through other mechanisms. For example, T_{reg} cells can induce downregulation of the expression of CD80 and CD86 on DCs through a process dependent on the integrin LFA-1 ($\alpha_L\beta_2$) and the immunomodulatory receptor CTLA-4 (CD152)[41]. Additionally, T_{reg} cells can inhibit the activation of DCs through the hydrolysis of extracellular ATP via expression of the ectoenzyme CD39, or through the induction of immunosuppressive tryptophan metabolism in DCs[42,43]. Thus, IL-10 production is probably one of multiple mechanisms by which T_{reg} cells can promote the development of memory $CD8^+$ T cells.

DCs can be categorized into distinct subsets by their surface expression of CD8, with $CD8^+$ DCs (‘lymphoid DCs’) marked by enhanced expression of pro-inflammatory cytokines[44]. Lymphoid DCs are selectively located in the T cell zone in the spleen and are found mainly in the white pulp[32]. MPCs are also located mainly in the white pulp and, when they encounter large amounts of inflammatory signals, can further differentiate into TECs[24,33]. We found that IL-10-competent T_{reg} cells were present mostly in the white pulp, which would position them optimally to regulate the inflammatory state of DCs and thus insulate $CD8^+$ T cells from excess signals and preserve their MPC state. Our work suggests that tight regulation of the amount of inflammatory cytokines produced by DCs during the resolution phase is critical for the maturation of memory $CD8^+$ T cells.

T_{reg} cells are reported to have both negative roles[18] and positive roles[19–21] in regulating the development of $CD8^+$ T cell memory. Studies indicating a role for T_{reg} cells in the promotion of splenic $CD8^+$ T cell memory have focused on the function of T_{reg} cells during the priming phase of infection through regulation of T cell avidity or IL-2 availability. Here we have identified a previously unknown role for T_{reg} cells, during the resolution phase following viral infection, in promoting the memory maturation of $CD8^+$ T cells. The function of T_{reg} cells during the resolution phase is distinct from that shown in previous work[19–21], as T_{reg} cell–derived IL-10 was not necessary during the priming phase for the

optimal memory maturation of $CD8^+$ T cells. Collectively, this indicates that T_{reg} cells are capable of modulating T cells through discrete mechanisms at different time points and sites to allow the memory maturation of $CD8^+$ T cells while simultaneously suppressing immunopathology and promoting the reestablishment of tissue homeostasis[45,46].

Our study might also have implications for the ability to design vaccines against pathogens such as human immunodeficiency virus and influenza virus, which are intransigent to current vaccination approaches. Our work indicated that the presence of IL-10-competent T_{reg} cells during the resolution phase was necessary for the maturation of memory $CD8^+$ T cells. Thus, treatment with IL-10 during the resolution phase following vaccination might allow improved vaccine efficacy. Alternately, the administration of rapamycin during this phase might represent another approach, because rapamycin promotes the selective population expansion of T_{reg} cells *in vivo* and enhances the quantity and quality of $CD8^+$ T cell memory[11,47]. Treatment with rapamycin also enhances the formation of broadly neutralizing antibodies[48] and thus might operate through multiple mechanisms to promote vaccine efficacy. Additionally, our work suggests that the use of adjuvants that elicit more IL-10 production might be more effective in eliciting a protective $CD8^+$ T cell response than are adjuvants that induce small amounts of IL-10. Indeed, published work has found that the ability of adjuvants to elicit IL-10 positively correlates with their ability to generate memory $CD8^+$ T cell upon immunization[49]. Thus, our study suggests a critical period of time during which therapeutic intervention might allow considerable enhancement of the ability of vaccines to promote a productive immune response, leading to long-lasting and protective immunological memory.

METHODS

Methods and any associated references are available in the online version of the paper.

ONLINE METHODS

Mice, infections and bacterial titers

C57BL/6 mice were from the National Cancer Institute or The Jackson Laboratory. B6.129P2 $Il10^{tm1Cgn}$/J (*Il10*$^{-/-}$) mice, B6.129(cg)-$Foxp3^{tm3(DTR/GFP)Ayr}$/J (*Foxp3*$^{GFP–DTR}$) mice and B6.129 (Cg)-$Foxp3^{tm4(YFP/cre)}$Ayr/J (*Foxp3*-Cre) mice were from The Jackson Laboratory[51]. 10BiT mice[52], *Il10*$^{f/f}$ mice[53] and *Cd4*-Cre, *Lyz2*-Cre, *Cd11c*-Cre and P14 mice[54] have been described. *Il10ra*$^{f/f}$ mice were generated by the Flavell laboratory. Mouse genomic DNA of *Il10ra* was isolated from the C57BL/6 bacterial artificial chromosome clone RP23-329F21 (BACPAC Resources Center, Children's Hospital Oakland Research Institute). A targeting vector was constructed by cloning of three genomic fragments into the plasmid pEasy-Flirt. The construct was designed to target exon 3 of *Il10ra*, which contains the translation-initiation site of *Il10ra*, and thus allow abolishment of IL-10Rα protein after deletion by Cre recombinase. The vector pEasy-Flirt has both Flp recombinase for deletion of the neomycin cassette and Cre recombinase sites to target exon 3 of *Il10ra*. The linearized targeting vector was then transfected into mouse embryonic stem cells (JM8 line). Homologous recombinants were screened and identified by PCR analysis. Clones carrying the mutated alleles of *Il10ra* were injected into blastocycts that were then implanted into

foster mothers. Chimeric mice were bred to C57BL/6 mice, and the F_1 generation was screened for identification of germline transmission. All mice studied were 6–8 weeks of age.

For the generation of mice bearing LCMV-specific epitopes, splenocytes from P14 donor mice 'normalized' for 5×10^4 naive P14 $CD8^+$ T cells (with expected engraftment of 10%; thus, 5×10^3 cells) were transferred into C57BL/6 mice by intravenous injection. Bacterial titers were quantified by lysing of whole livers in 0.5% Triton-X100 and plating of tenfold serial dilutions of bacteria overnight on brain-heart–infusion agar plates. All animal experiments were done with approval of the Yale Institutional Animal Care and Use Committee.

Infection and treatment

For primary infection, mice were given intraperitoneal administration of 2×10^5 plaque-forming units of LCMV, Armstrong strain. For rechallenge experiments, mice were given 2×10^4 colony-forming units of recombinant *L. monocytogenes* expressing the LCMV gp(33–41) epitope. For blockade of IL-10, mAb to IL-10 (JES5-2A5; provided by J.M.M. den Haan) was administered at a dose of 0.25 mg/ml every other day for the appropriate period of time for the experiment. For treatment with CpG B, mice were given intraperitoneal administration of a dose of 3.75 μg CpG B per mouse every other day starting on the day appropriate for the experiment and ending after three doses had been administered. Bacterial titers were quantified by lysing of whole livers in 0.5% Triton-X100 and plating of tenfold serial dilutions of bacteria on brain-heart–infusion agar plates, followed by incubation overnight. Diphtheria toxin was reconstituted according to manufacturer's instructions (Sigma). Mice were given two intraperitoneal injections of diphtheria toxin at a dose of 50 μg per kg body weight on the appropriate day, as well as the following day, as described[55].

Antibodies for surface and intracellular staining

The isolation of lymphocytes, along with surface and intracellular staining, was performed as described[56]. Staining of transcription factors was performed after permabilization with the FoxP3 Fixation and Permeabilization Kit (eBioscience). The following antibodies were used for flow cytometry staining: Brilliant Violet 421–anti-CD44 (103039), allophycocyanin–anti-KLRG1 (138412), phycoerythrin (PE)–indotricarbocyanine (Cy7)–anti-CD69 (104512), peridinin chlorophyll protein (PerCP)–anti-Thy-1.1 (202512), fluorescein isothiocyanate (FITC)–anti-CD80 (104706), PE-Cy7–anti-PDL1 (124314), allophycocyanin–anti-Ly5.2 (109814), Alexa Fluor 780–anti-CD8 (100714), allophycocyanin–anti-CXCR3 (126512) and Pacific Blue–conjugated antibody to major histocompatibility complex class II (107619) (all from BioLegend); PE-Cy7–anti-CD127 (25-1271-82), FITC–anti-CD27 (11-0271-82), PE–anti-PDL2 (12-5986-81), PE–anti-CD25 (12-0251-83) and allophycocyanin–anti-CD11c (17-0114-81) (all from eBioscience); FITC–anti-CD62L (553150) and PE–anti-CD86 (553692) (both from BD Biosciences); rabbit mAb to TCF1 (C63D9; Cell Signaling); and allophycocyanin–anti-GzmB (GRB05; Invitrogen). Major histocompatibility complex class I tetramers were generated as described[54]. Flow cytometry data were acquired on a BD LSRII with FACSDiva software and were analyzed with FlowJo software (TreeStar).

In vivo CD4$^+$ T cell labeling

For intravascular staining of CD4$^+$ T cells, mice were given injection of 3 μg biotin-conjugated antibody to CD4 (RM4-5; eBioscience) diluted in 300 μl sterile DPBS (Life Technologies) as described[57] and were killed 5 min after this injection.

Isolation of lymphocytes from tissues

Spleens were homogenized with a cell strainer, and red blood cells were lysed with ACK lysing buffer (Quality Biologicals). Lymphocytes were then washed and counted.

Isolation of RNA and real-time quantitative PCR

For quantitative PCR, first, total RNA was isolated from sorted samples with the use of Qiazol and an RNeasy Mini kit (Qiagen), in which on-column treatment with DNase was included. Reverse transcription was then carried out with an iScript cDNA synthesis kit (Bio-Rad). Quantitative RT-PCR was performed on cDNA with iTaq Universal SYBR Green super mix (Bio-Rad) and the following primer sets: *Ccr7* (forward, GTGGTGGCTCTCCTTGTCAT; reverse, AGTTCCGCACATCCTTCTTG), *Pim1* (forward, GATCATCAAGGGCCAAGTGT; reverse, GATGGTTCCGGATTTCTTCA), *Cx3cr1* (forward, AAGTTCCCTTCCCATCTGCT; reverse, GGACAGGAAGATGGTTCCAA), *Zeb2* (forward, GAGCAGGTAACCGCAAGTTC; reverse, TGTTTCTCATTCGG) and *HPRT* (forward, GCTATAAATTCTTTGCTGACCTGCTG; reverse, AATTACTTTTATGTCCCCTGTTGACTGG). Differences in expression were calculated with expression of the control gene *Hprt* (encoding hypoxanthine guanine phosphoribosyl transferase) as a normalization constant.

RNA-seq library preparation and data analysis

Total RNA was purified with the use of a Qiazol and RNeasy Mini kit (Qiagen), in which on-column treatment with DNase was included. Purified RNA was submitted to the Yale Center for Genomic Analysis, where it was subjected to isolation of mRNA and library preparation. Libraries were pooled, six samples per lane, and were sequenced on an Illumina HISEQ 2500 (75–base pair paired-end reads), followed by alignment with TopHat2 software. A ‘count-based’ differential-expression protocol was adapted for this analysis[58]; data that could be mapped were ‘counted’ with the HTSeq package that provides infrastructure for data processing, then were imported into software of the R project for analysis of differential expression with DESeq2 software. A multi-factor design was used to take into the two conditions (*Il10*$^{f/f}$*Foxp3*-Cre and *Il10*$^{f/f}$) and account for pairwise groupings of the six samples. For the generation of the heat map, genes with a difference in expression ($\log_2$) of 1.5-fold or greater and a false-discovery rate of <0.2 were chosen for visualization of the overall consensus of rankings (via column *z*-score). Select biologically relevant genes were chosen for a subsequent presentation on a heat map, with genes in pro- and anti-inflammatory or effector-memory signatures designated as such according to gene-set definitions of the Molecular Signatures Database.

GSEA

Gene sets (1,911) from the immunological signatures collection (C7) of the Molecular Signatures Database were chosen for assessment of enrichment in the RNA-seq analysis of *Il10*$^{f/f}$*Foxp3*-Cre cells versus *Il10*$^{f/f}$ cells. R-GSEA code was used for analysis of the data, with the signal-to-noise ranking metric and 1×10^5 permutations of the gene labels for testing of statistical significance. Results were visualized with software of the R project, with the running enrichment score, member position by the signal-to-noise ranking metric ('bar code'), and ranking metric scale presented. Values in gene-set plots showing individual differences in expression ($\log_2$) ± standard error were derived from analysis of the RNA-seq data with DESeq2 software as described[59].

Cytokine analysis

Serum was collected from mice at the appropriate time points and was stored at −80 °C until analysis. The expression of IL-6, IL-12p70, IL-1β and TNF was determined with Milliplex Cytokine kits according to the manufacturer's instructions (Millipore) and results were 'read' on a Bio Plex System (Bio-Rad).

Statistical analysis

Statistical significance was determined by the unpaired two-tailed Student's *t*-test. Statistical analyses were performed using Prism GraphPad software, version 6.0.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank J.M.M. den Haan (UV University Medical Center, Amsterdam) for mAb ES5-2A5; and all members of the Kaech and Craft laboratories for discussions and critical reading of the manuscript. Supported by the US National Institutes of Health (RO1AI066232 and R01AI074699 to S.M.K.; R01AR40072, P30AR053495 and R21AR063942 to J.C.; T32AI07019 and F31AG07777 to B.J.L.; and T32GM07205 to S.M.G.) and the Howard Hughes Medical Institute (S.M.K., B.J.L., R.A.F. and T.G.).

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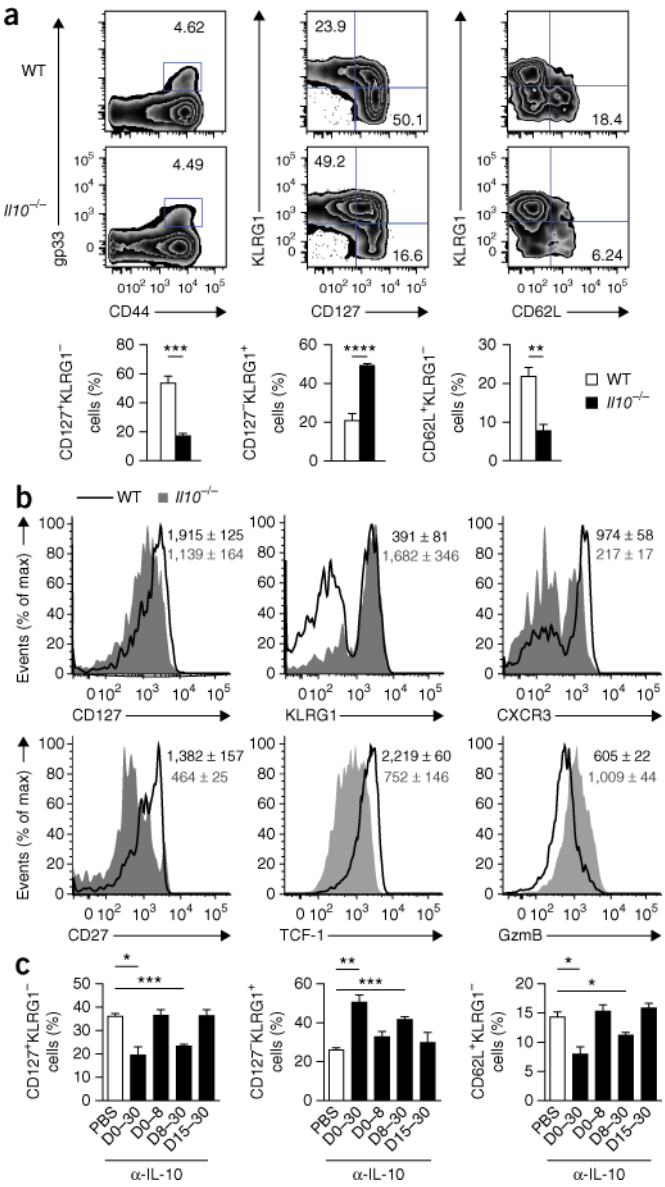

Figure 1.

IL-10 is required during the resolution phase of infection to allow optimal maturation of memory CD8$^+$ T cells. (**a**) Flow cytometry of cells from the spleen of wild-type (WT) and *Il10*$^{-/-}$ mice 60 d after infection with LCMV (Armstrong strain), assessing the response of T cells positive for the epitope gp(33–41) (gp33). Numbers in plots indicate percent gp33$^+$ T cells (far left) or percent KLRG1$^-$CD127$^+$ cells (top left quadrant) or KLRG1$^+$CD127$^-$ cells (bottom right quadrant) in the gp33$^+$ T cell population (middle) or KLRG1$^-$CD62L$^+$ cells in the gp33$^+$ T cell population (far right). Below, frequency of CD127$^+$KLRG1$^-$ cells (left), CD127$^-$KLRG1$^+$ cells (middle) or CD62L$^+$KLRG1$^-$ cells (right) among gp33$^+$ T cells (assessed on days 45–60). (**b**) Expression of CD127, KLRG1, CXCR3, CD27, TCF-1 and granzyme B (GzmB) in wild-type and *Il10*$^{-/-}$ gp33$^+$ T cells from mice as in **a**. Numbers in plots indicate mean fluorescence intensity (MFI) of marker (horizontal axis) in wild-type cells (top number) or *Il10*$^{-/-}$ cells (number below). (**c**) Frequency of CD127$^+$KLRG1$^-$,

$CD127^{-}KLRG1^{+}$ and $CD62L^{+}KLRG1^{-}$ cells among $gp33^{+}$ T cells from mice infected with LCMV (Armstrong strain) (day 0) and given mock injection of PBS or injection of antibody to IL-10 (α-IL-10) on days (D) 0–30, 0–8, 8–30 or 15–30 (horizontal axis), assessed at day 30. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ (unpaired two-tailed Student's *t*-test). Data are from one experiment representative of five experiments with at least four mice per group (**a,b**) or three experiments (**c**; mean and s.e.m.).

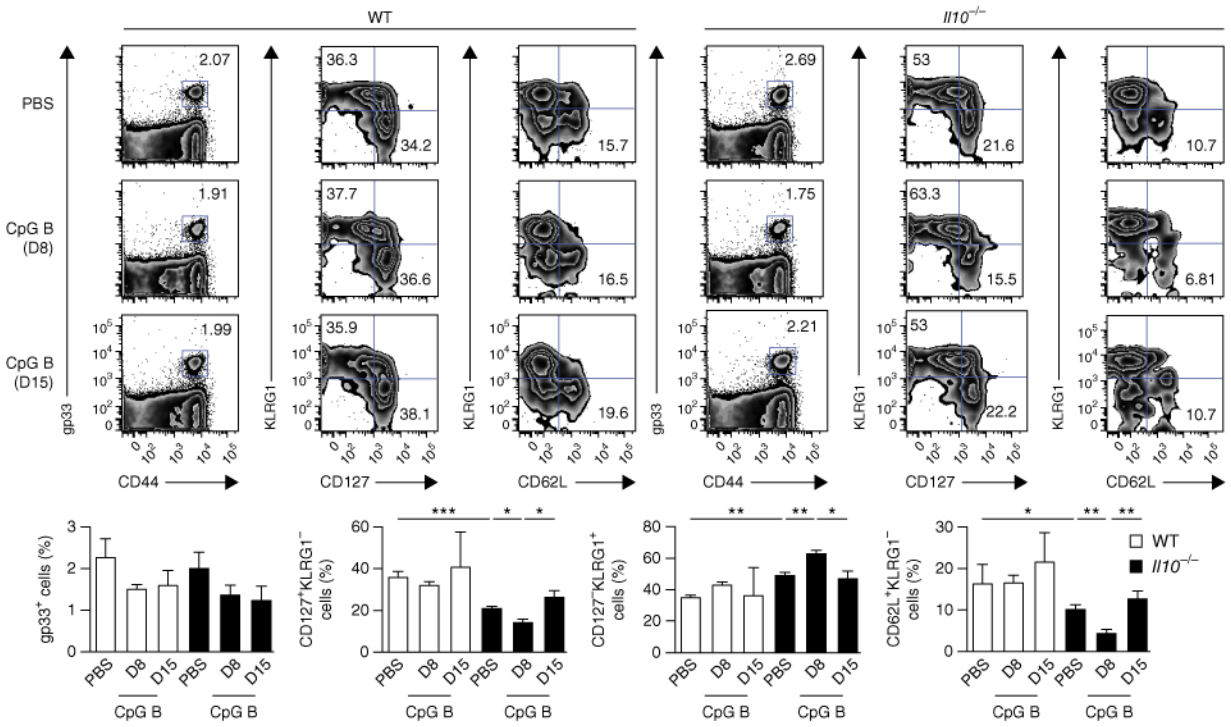

Figure 2.

IL-10 is important in insulating $CD8^+$ T cells from inflammatory signals following viral infection. Flow cytometry of cells from wild-type and $Il10^{-/-}$ mice infected with LCMV (Armstrong strain) and given mock injection of PBS or injection of CpG B at day 8 (D8) or day 15 (D15) after infection, assessed 60 d after infection (numbers in plots (top) as in Fig. 1a, top). Below, summary of results above ($gp33^+$ cells among $CD8^+$ T cells (far left) or as in Fig. 1a, bottom). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (unpaired two-tailed Student's *t*-test). Data are from one experiment representative of three experiments with at least four mice per group (mean and s.e.m.).

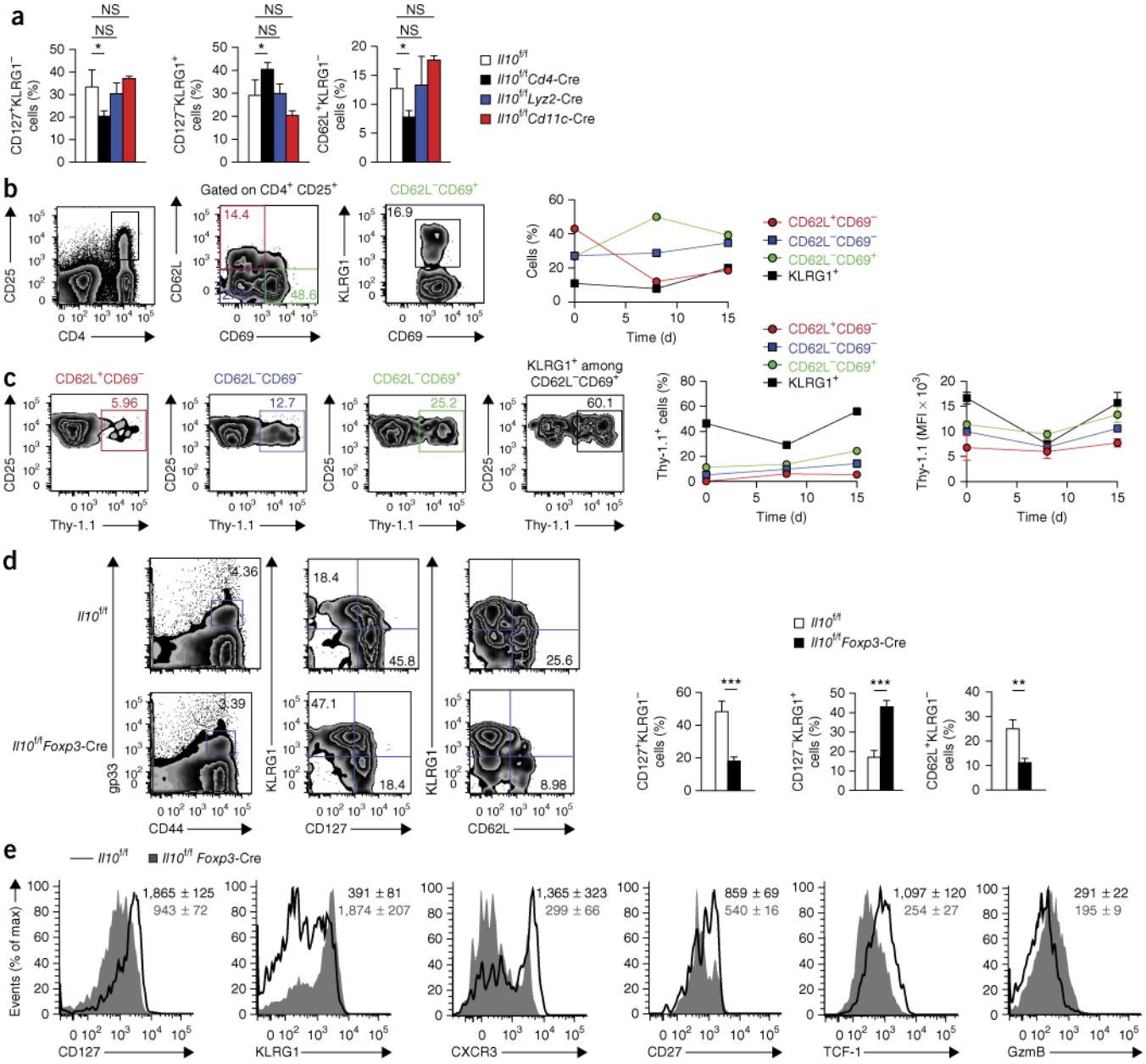

Figure 3.

CD4$^+$ T_{reg} cell–derived IL-10 is critical for the maturation of memory CD8$^+$ T cells. (**a**) Frequency of CD127$^+$KLRG1$^-$, CD127$^-$KLRG1$^+$ and CD62L$^+$KLRG1$^-$ cells in the gp33$^+$ T cell population from *Il10*$^{f/f}$, *Il10*$^{f/f}$*Cd4*-Cre, *Il10*$^{f/f}$*Lyz2*-Cre, and *Il10*$^{f/f}$*Cd11c*-Cre mice 60 d after infection with LCMV (Armstrong strain). (**b**) Flow cytometry (top) of T cells from the spleen of 10BiT IL-10 reporter mice 15 d after infection with LCMV (Armstrong strain). Numbers in quadrants (middle) indicate percent CD62L$^+$CD69$^-$ cells (top left), CD62L$^-$CD69$^+$ cells (bottom right) or CD62L$^-$CD69$^+$ cells (bottom left) among CD25$^+$CD4$^+$ cells gated at left (outlined area); number adjacent to outlined area (right) indicates percent KLRG1$^+$CD69$^+$ cells among CD62L$^-$CD69$^+$ cells gated at left (green outlined area). Right, frequency of CD62L$^+$CD69$^-$, CD62L$^-$CD69$^-$ or CD62L$^-$CD69$^+$ cells

among $CD25^+CD4^+$ cells (colors match middle plot at left), or $KLRG1^+$ cells among $CD62L^-CD69^+$ cells, at days 0, 8 and 15 after infection as above. (**c**) Expression of IL-10 (assessed as Thy-1.1) by subsets (above plots) of cells from mice as in **b** (top). Right, frequency of Thy-1.1^+ cells (left) and MFI of Thy-1.1 (right) among the Thy-1.1^+ cells in those subsets (as in **b**) at days 0, 8 and 15 after infection as above. (**d**) Flow cytometry (left) of cells from *Il10*$^{f/f}$ and *Il10*$^{f/f}$*Foxp3*-Cre mice at 60 d after infection with LCMV (Armstrong strain) (numbers in plots as in Fig. 1a, top). Right, summary of results at left (as in Fig. 1a, bottom). (**e**) Expression of CD127, KLRG1, CXCR3, CD27, TCF-1 and granzyme B in *Il10*$^{f/f}$ and *Il10*$^{f/f}$*Foxp3*-Cre gp33^+ T cells. Numbers in plots indicate MFI of marker (horizontal axis) in *Il10*$^{f/f}$ cells (top number) or *Il10*$^{f/f}$*Foxp3*-Cre cells (number below). **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 (unpaired two-tailed Student's *t*-test). Data are from one experiment representative of three experiments with three to six mice per group (mean and s.e.m. in **a**–**d**).

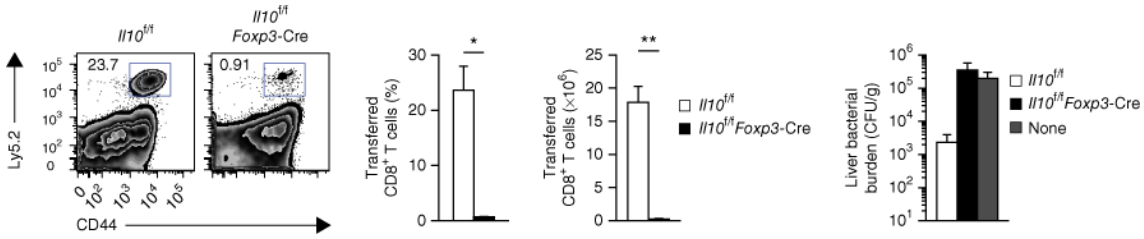

Figure 4.

$CD4^+$ T_{reg} cell–derived IL-10 is necessary for the formation of protective memory $CD8^+$ T cells. Flow cytometry (left) of splenic T cells from host mice ($Ly5.1^+$) given no cells (None) or 1.5 × 105 $gp33^+$ $CD8^+$ T cells pooled from congenically mismatched ($Ly5.2^+$) $Il10^{f/f}$ and $Il10^{f/f}$*Foxp3*-Cre mice 45 d after infection of donor mice with LCMV (Armstrong strain), followed by challenge of the recipient mice with recombinant *L. monocytogenes* expressing the gp(33–41) epitope 1 d after cell transfer and analysis 4 d after challenge. Numbers adjacent to outlined areas (far left) indicate percent $Ly5.2^+$ (donor) $CD44^+$ cells. Middle, frequency (middle left) and abundance (middle right) of donor $CD8^+$ T cells in the spleen of host mice. Far right, bacterial burden in the liver of host mice, presented as colony-forming units (CFU) per gram of liver. **P* < 0.001 and ***P* < 0.0001 (unpaired two-tailed Student's *t*-test). Data are from one experiment representative of two experiments with at least four mice per group (mean and s.e.m.).

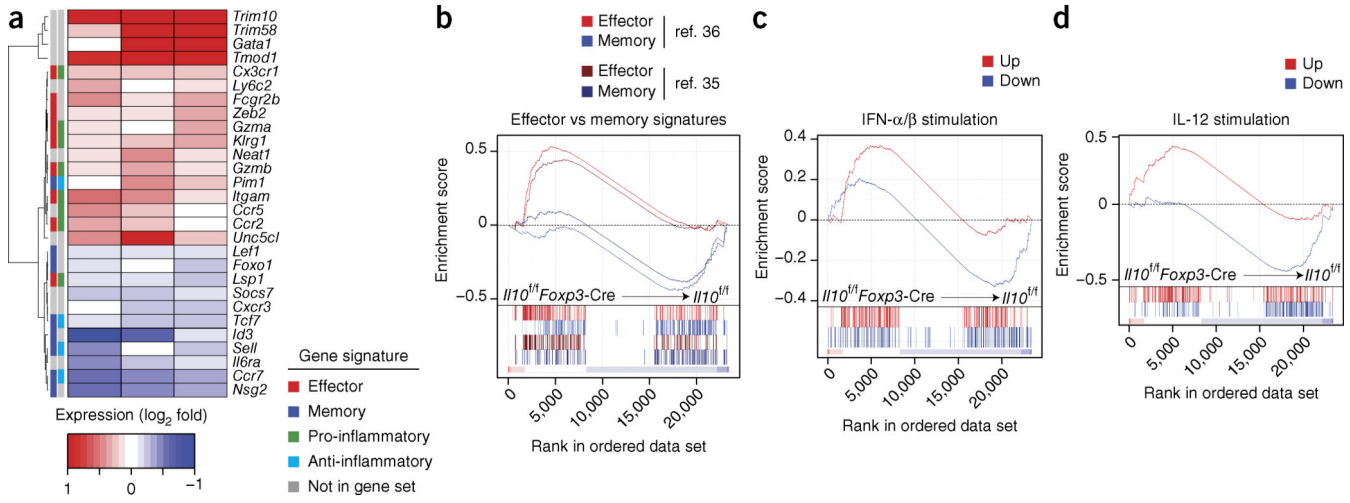

Figure 5.

Virus-specific $CD8^+$ T cells from mice lacking $CD4^+$ T_{reg} cell–derived IL-10 display a robust inflammatory gene signature. (**a**) RNA-seq analysis of selected biologically relevant genes among mRNA isolated from $gp33^+$ $CD8^+$ T cells and $CD8^+$ T cells positive for the epitope nucleoprotein residues 396–404, pooled from *Il10*$^{f/f}$ and *Il10*$^{f/f}$*Foxp3*-Cre mice 15 d after infection with LCMV (Armstrong strain), presented as expression (log_2) in *Il10*$^{f/f}$*Foxp3*-Cre cells relative to that in *Il10*$^{f/f}$ cells (key below; columns indicate paired replicates); left margin, grouping of genes by signature (key, bottom right), as determined by published gene sets8,35–38,50. (**b**) GSEA of gene sets from the Molecular Signatures Database of the Broad Institute, showing gene sets with significant enrichment (false-discovery rate, $<10^{-5}$) and their enrichment score (where a positive score indicates 'enrichment' (higher expression) in the *Il10*$^{f/f}$*Foxp3*-Cre sample relative to that in the *Il10*$^{f/f}$ sample), with members of the gene set presented in the ranked list of genes ('bar code' below) and the signal-to-noise ranking metric (bar at bottom), assessing effector signatures versus (vs) memory signatures in $CD8^+$ T cells, based on published gene sets35,36. (**c,d**) GSEA of genes upregulated (Up) or downregulated (Down) after stimulation with IFN-α and IFN-β (collectively called 'IFN-α/β')) (**c**) or IL-12 (**d**), based on published gene sets37; presented as in **b**. Data are from three independent experiments with three mice per group pooled for each sample.

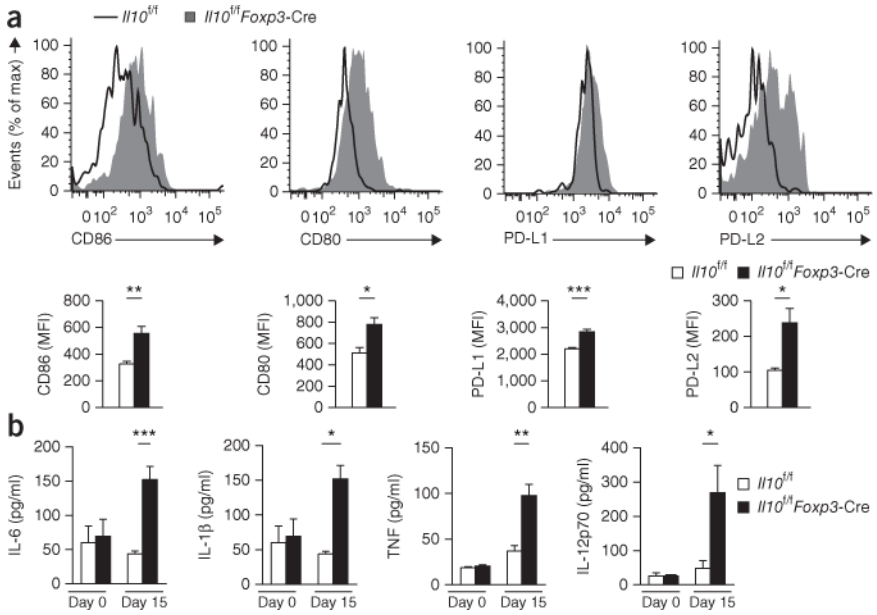

Figure 6.

Enhanced maturation of DCs and inflammation in the absence of T_{reg} cell–derived IL-10. (**a**) Flow cytometry (top) analyzing the expression of CD86, CD80, PD-L1 and PD-L2 in $CD45^{+}CD11c^{hi}MHCII^{+}$ DCs from *Il10*$^{f/f}$ and *Il10*$^{f/f}$*Foxp3*-Cre mice 15 d after infection with LCMV (Armstrong strain). Bottom, summary of results above. (**b**) Immunoassay of IL-6, IL-1β, TNF and IL-12p70 in the serum of *Il10*$^{f/f}$ and *Il10*$^{f/f}$*Foxp3*-Cre mice left uninfected (day 0) or at day 15 after infection with LCMV. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 (unpaired two-tailed Student's *t*-test). Data are from one experiment representative of three experiments with at three to five mice per group (mean and s.e.m.).

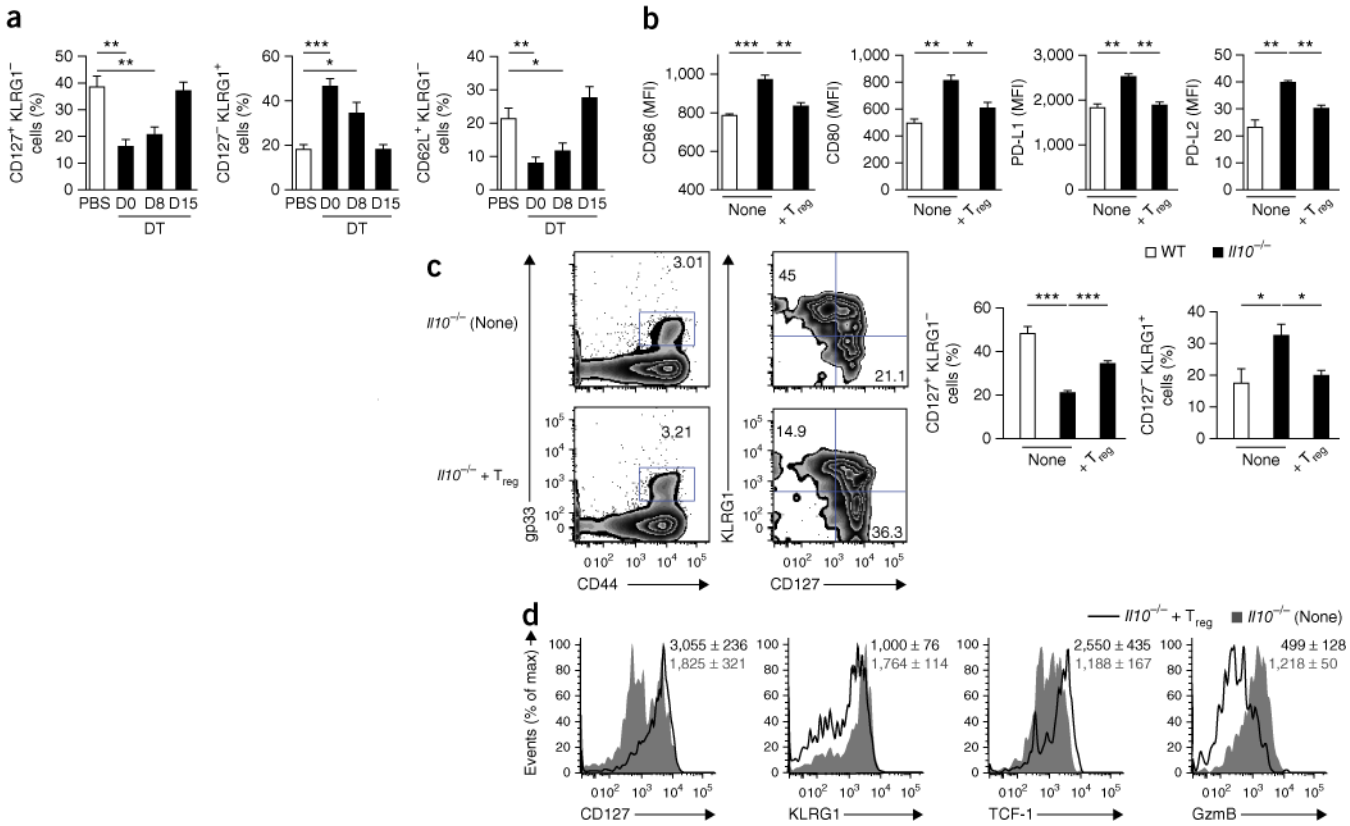

Figure 7.

Transfer of IL-10-competent CD4$^+$ T_{reg} cells during the resolution phase of LCMV infection is sufficient to 'rescue' the defect in the maturation of memory CD8$^+$ T cells in *Il10*$^{-/-}$ mice. (**a**) Frequency of CD127$^+$KLRG1$^-$, CD127$^-$KLRG1$^+$ and CD62L$^+$KLRG1$^-$ cells in the gp33$^+$ T cell population of *Foxp3*$^{GFP\text{-}DTR}$ mice infected with LCMV (Armstrong strain) and give mock injection of PBS or injection of diphtheria toxin (DT) on day 0 (D0), day 8 (D8) or day 15 (D15) after infection, followed by analysis 60 d after infection. (**b**) Expression of CD86, CD80, PD-L1 and PD-L2 in DCs from wild-type and *Il10*$^{-/-}$ host mice infected with LCMV (Armstrong strain) and, 8 d later, given no cells (None) or 3 × 105 Foxp3$^+$ CD4$^+$ T_{reg} cells isolated from 'infection-matched' *Foxp3*$^{GFP\text{-}DTR}$ donor mice ($^+T_{reg}$), followed by analysis 15 d after infection of host mice. (**c**) Flow cytometry (left) of cells from *Il10*$^{-/-}$ mice as in **b**, analyzed 60 d after quadrants (near left) indicate percent KLRG1$^-$CD127$^+$ cells (bottom right) or KLRG1$^+$CD127$^-$ cells (top left) in the gp33$^+$ T cell population. Right, frequency of CD127$^+$KLRG1$^-$cells or CD127$^-$KLRG1$^+$ cells among gp33$^+$ T cells. (**d**) Expression of CD127, KLRG1, TCF-1 and granzyme B in gp33$^+$ T cells from *Il10*$^{-/-}$ mice as in **b**. Numbers in plots indicate MFI of marker (horizontal axis) in cells from *Il10*$^{-/-}$ mice given no cells (top number) or given CD4$^+$ T_{reg} cells (number below). *P < 0.05; **P < 0.01 and ***P < 0.001 (unpaired two-tailed Student's t-test). Data are from one experiment representative of three experiments (**a**) or two experiments (**b**–**d**) with three to seven mice per group (mean and s.e.m.).