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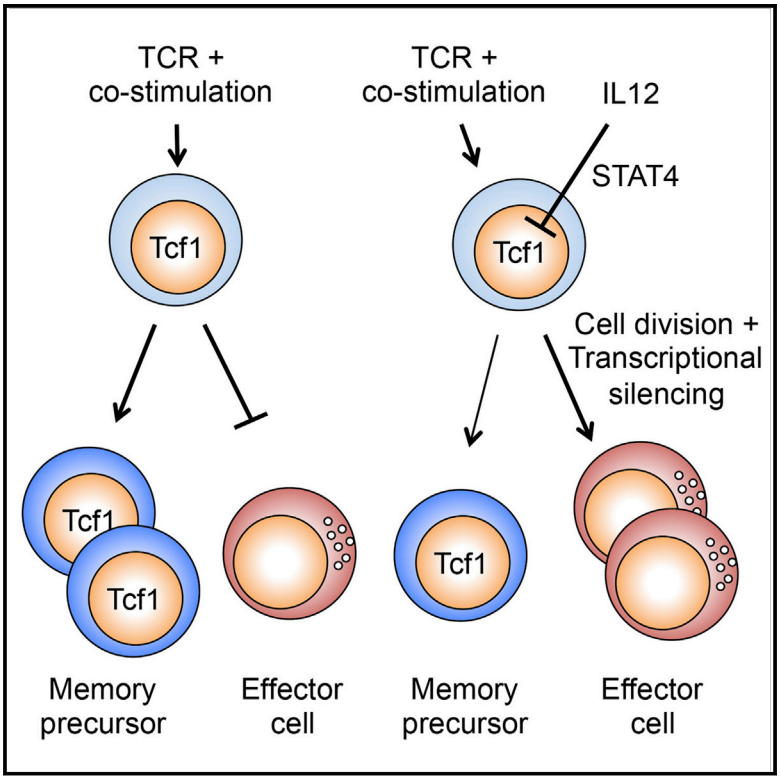
Suppression of Tcf1 by Inflammatory Cytokines Facilitates Effector CD8 T Cell Differentiation

Graphical Abstract



Highlights

- Systemic inflammatory signals suppress Tcf1 expression in primed CD8 T cells
- IL-12 downregulates Tcf1 via IL-12R/STAT4 signaling in CD8 T cells
- Tcf1 suppression requires cell cycling and is explained by reduced transcription
- Inflammation-induced Tcf1 suppression facilitates effector differentiation

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In Brief

The transcription factor Tcf1 is downregulated during CD8 effector differentiation. Danilo et al. show that IL-12 suppresses Tcf1 in primed CD8 T cells. Tcf1 suppression requires cell cycling, is transcriptional, and is mediated in part by DNA methylation. Inflammation-induced Tcf1 downregulation facilitates effector differentiation by de-repressing IL-12Rβ2 and BLIMP1.

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Suppression of Tcf1 by Inflammatory Cytokines Facilitates Effector CD8 T Cell Differentiation

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SUMMARY

The formation of central CD8 T cell memory in response to infection depends on the transcription factor Tcf1 (*Tcf7*). Tcf1 is expressed at high levels in naive CD8 T cells but downregulated in most CD8 T cells during effector differentiation. The relevance of Tcf1 downregulation for effector differentiation and the signals controlling Tcf1 expression have not been elucidated. Here, we show that systemic inflammatory signals downregulated Tcf1 in CD8 T cells during dendritic cell vaccination and bacterial infections. The suppressive effect was mediated by the inflammatory cytokine interleukin 12 (IL-12), which acted via STAT4 in CD8 T cells. IL-12-induced Tcf1 downregulation required cell cycling, occurred at the transcriptional level, and was prevented in part by inhibiting DNA methyltransferases. Absence of Tcf1 during T cell priming circumvented the need of systemic inflammation for effector differentiation. We conclude that silencing of Tcf1 by systemic inflammation facilitates effector CD8 T cell differentiation.

INTRODUCTION

Pathogen recognition usually generates a large number of specific CD8 T cells that have acquired effector functions and are needed to control intracellular pathogens (Kaech and Cui, 2012). These CD8 T cells are heterogeneous and have distinct fates, which can be followed using cell-surface markers, at least in response to certain infections. $CD127^-$ ($IL7R\alpha$) $KLRG1^+$ CD8 T cells express high levels of cytotoxic effector molecules (including granzyme B), but most of these cells die once the pathogen is cleared (termed short-lived effector T cells [SLECs]). On the other hand, $CD127^+$ $KLRG1^-$ CD8 T cells (referred to as memory precursor effector T cells [MPECs]) can produce interleukin 2 (IL-2), are more likely to survive, and give rise to long-lived memory cells. Memory cells, which provide long-term protection from infection (Kaech and Cui, 2012), are also heterogeneous. Among recirculating memory cells, central memory cells (T_{CM}: $CD62L^+$ $CD127^+$) are generally better mediators of host protection than effector memory cells (T_{EM}: $CD62L^-$ $CD127^+$), as the former display enhanced proliferative capacity in response to antigen re-challenge (Sallusto et al., 1999).

Effector and memory CD8 T cell differentiation is directed by multiple cell-extrinsic cues, which include antigen recognition, co-stimulation, and cytokines (Chang et al., 2014; Kaech and Cui, 2012). While antigen recognition and co-stimulation impact CD8 T cell expansion and persistence, inflammatory cytokines, such as IL-12 and type I interferon (IFN), or high levels of IL-2 promote effector differentiation (Kim and Harty, 2014). IL-12 chiefly activates STAT4, which induces T-bet (*Tbx21*) expression in *Listeria monocytogenes* infection (Joshi et al., 2007; Xiao et al., 2009). IL-12 also induces *Blimp*1 (*Prdm1*) in a *STAT4*-dependent manner in Th1 cells (Neumann et al., 2014). T-bet (*Tbx21*) and Blimp1 (*Prdm1*), as well as Id2 and Zeb2, represent key transcription factors mediating terminal CD8 effector T cell differentiation (Dominguez et al., 2015; Intlekofer et al., 2007; Joshi et al., 2007; Rutishauser et al., 2009; Takemoto et al., 2006; Yang et al., 2011). Conversely, memory T cell formation is promoted by a distinct set of transcription factors, including Eomes, Id3, Bcl6, and Tcf1 (encoded by the *Tcf7* gene) (Chang et al., 2014; Ichii et al., 2004; Jeannet et al., 2010; Ji et al., 2011; Kaech and Cui, 2012; Masson et al., 2013; Yang et al., 2011; Zhou et al., 2010). The expression of *Eomes* and *Bcl6* can be induced by IL-21 and IL-10 in a STAT3-dependent fashion (Cui et al., 2011). Thus, effector versus memory CD8 T cell differentiation is regulated by distinct cytokines and signaling pathways, which induce the expression of specific transcription factors that promote or enforce specific cell fates.

While transcription factors promoting memory, such as *Eomes* and *Bcl6*, are induced during T cell differentiation, Tcf1 is expressed at high levels in naive CD8 T cells and downregulated in most, but not all, cells during the primary CD8 T cell response to viral or bacterial infection (Boudousquié et al., 2014; Jeannet et al., 2010; Lin et al., 2016; Zhou et al., 2010). Similarly, most CD8 T cells in chronic viral infection have downregulated Tcf1 and display an effector gene expression program (Utzschneider et al., 2016; Im et al., 2016). Downregulation of Tcf1, which has been associated with asymmetric partitioning of Tcf1 in dividing CD8 T cells (Lin et al., 2016), thus correlates with effector differentiation. However, whether Tcf1 downregulation is cause for or a consequence of effector differentiation, and which signals suppress Tcf1 *in vivo*, are not known.

Using dendritic cell vaccination, we showed that systemic inflammatory signals downregulated Tcf1 in primed CD8 T cells. Tcf1 suppression was chiefly mediated by inflammatory cytokine IL-12, which acted via IL-12Rβ2 and STAT4 expressed in CD8

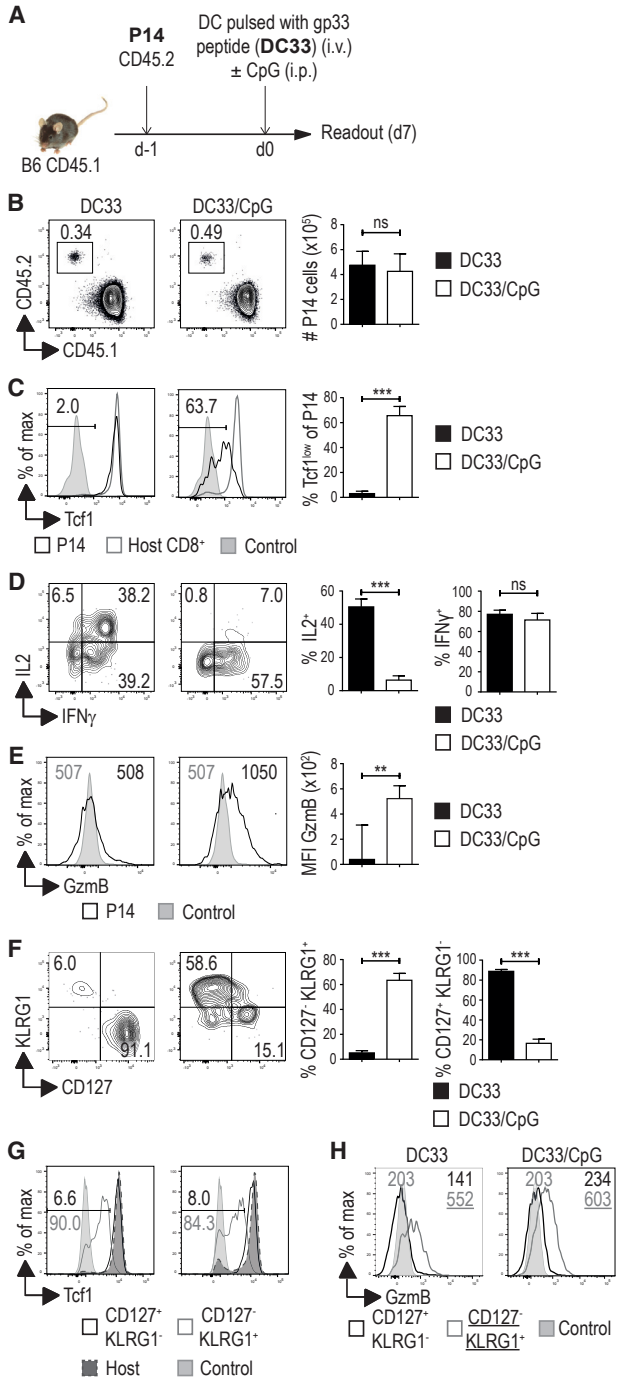


Figure 1. Tcf1 Downregulation Is Mediated by Systemic Inflammation

(A) Experimental approach. P14 chimeric mice (i.e., B6.CD45.1 mice adoptively transferred with 10^4 naive P14 cells [CD45.2$^+$]) were vaccinated with LPS-matured, LCMV gp_{33-41} (gp33) peptide-pulsed dendritic cells (DC33) without or in combination with CpG (i.p.) (DC33/CpG).

(B) Abundance of P14 cells (CD45.2$^+$) in the spleen 7 days post-vaccination of P14 chimeric mice with DC33 or DC33/CpG.

(C) Histogram overlays show Tcf1 expression in P14 cells (black open) relative to host CD8 T cells (gray open) and to no antibody control (gray fill). Numbers and bar graph depict the fraction of Tcf1^{low} P14 cells.

T cells. IL-12-mediated Tcf1 downregulation depended on cell cycling and occurred at the transcriptional level. Finally, Tcf1 downregulation in CD8 T cells was found to facilitate effector differentiation.

RESULTS

Systemic Inflammation Downregulates Tcf1 in Primed CD8 T Cells

While virtually all naive CD8 T cells express Tcf1, most CD8 T cells downregulate Tcf1 during the primary immune response to infection, and this coincides with effector differentiation (Boudousquié et al., 2014; Jeannet et al., 2010; Lin et al., 2016; Zhou et al., 2010). However, it is not known how Tcf1 expression is controlled and whether downregulation is cause for, or a consequence of, effector differentiation.

We first addressed whether Tcf1 downregulation depended on the T cell receptor (TCR) signal strength. We followed OT-1 TCR transgenic CD8 T cells responding to *L. monocytogenes* expressing the high-affinity ligand SIINFEKL (N4) or the low-affinity ligand SIITFEKL (T4) (Zehn et al., 2009). 7 days after *L. monocytogenes* N4 infection, OT-1 cells had massively expanded, while expansion in *L. monocytogenes* T4 infections was 20-fold reduced. In contrast, effector differentiation was not very different (Figures S1A–S1C), in agreement with published data (Oberle et al., 2016; Zehn et al., 2009). Further, compared to naive OT-1 cells (not shown) or host CD8 T cells, Tcf1 downregulation in OT-1 cells following *L. monocytogenes* N4 and *L. monocytogenes* T4 infection was not very different, albeit it was more prominent with *L. monocytogenes.* T4 (Figure S1D). Corresponding results were obtained when analyzing the expression of the Tcf1 target *Axin2* (Figure S1E). Thus, during a CD8 T cell response to infection, Tcf1 downregulation is only modestly impacted by the strength of the TCR-peptide MHC interaction and does not correlate with the extent of T cell expansion.

We next addressed whether Tcf1 expression was regulated by inflammation. To this end, we adopted a vaccination protocol using dendritic cell (DC) immunization as schematically shown in Figure 1A. We generated P14 chimeric mice; i.e., B6 (CD45.1$^+$)

(D) IL-2 and IFN-γ production by P14 cells following *in vitro* restimulation with gp33 peptide. The bar graphs depict the percentage of IL-2$^+$ (left) and IFN-γ$^+$ (right) P14 cells.

(E) Histogram overlays show granzyme B (GzmB) expression in P14 cells (black open) compared to isotype control (gray fill). Numbers indicate mean fluorescence intensity (MFI) of staining. The bar graph depicts the MFI of GzmB staining whereby the background was subtracted.

(F) CD127 versus KLRG1 expression among gated P14 cells. The bar graphs indicate the percentage of CD127$^-$ KLRG1$^+$ SLEC (left) and CD127$^+$ KLRG1$^-$ MPEC P14 cells (right).

(G and H) Histogram overlays show Tcf1 expression (G) or GzmB expression (H) in gated CD127$^+$ KLRG1$^-$ (black open) and CD127$^-$ KLRG1$^+$ P14 cells (gray open) compared to no antibody control (gray fill) and host CD8 T cells (dotted border, gray fill). Numbers depict the fraction of Tcf1^{low} P14 cells (G) or the MFI of GzmB staining (H).

All bar graphs show means (±SD, n = 5). Data are representative of 2 or more independent experiments. Statistical significance was determined using unpaired t tests (***p < 0.001; **p < 0.01; *p < 0.05; ns, not significant [p > 0.05]). See also Figure S1.

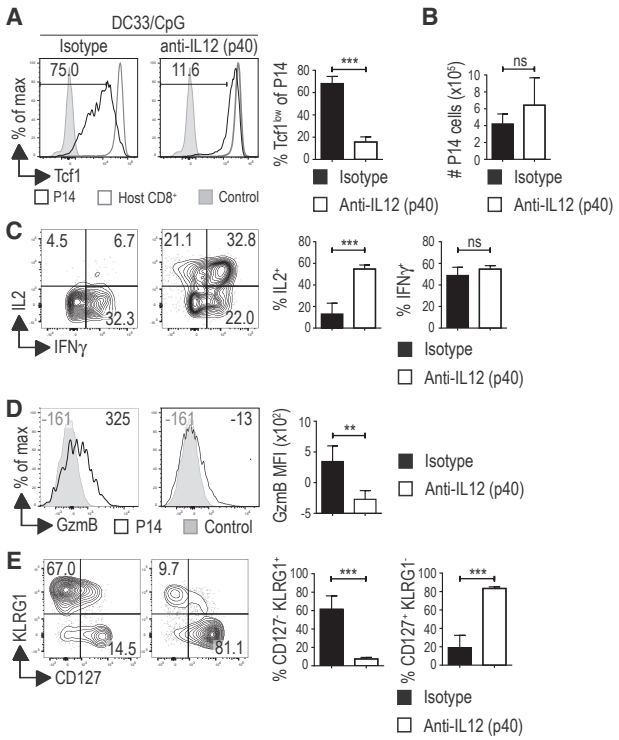


Figure 2. IL-12 (p40) Blockade Prevents Inflammation-Induced Tcf1 Downregulation

P14 chimeric mice were vaccinated with DC33/CpG and treated with isotype control antibody (Ab) or anti-IL-12 (p40) Ab.

(A) Tcf1 expression in P14 cells (black open) compared to host CD8 T cells (gray open) and no antibody control (gray fill). Numbers and bar graph depict the percentage of Tcf1^{low} cells in P14 cells.

(B) The bar graph depicts the abundance of P14 cells in the spleen.

(C) Production of IL-2 and IFN-γ following *in vitro* restimulation. The bar graphs depict the percentage of IL-2$^+$ (left) and IFN-γ$^+$ (right) P14 cells.

(D) GzmB expression in P14 cells (black open) compared to isotype control (gray fill). The bar graph depicts the MFI of GzmB staining whereby background was subtracted.

(E) CD127 versus KLRG1 expression among gated P14 cells. The bar graphs show the percentage of CD127$^-$ KLRG1$^+$ SLECs (left) and CD127$^+$ KLRG1$^-$ MPECs (right).

Bar graphs show means (±SD, n = 5). Data are representative of at least 2 independent experiments. Statistical significance was determined using unpaired t tests (***p < 0.001; **p < 0.01; *p < 0.05; ns, not significant [p > 0.05]). See also Figure S2.

mice that were adoptively transferred with a small dose (10^4) of naive P14 T cells (CD45.2$^+$). The P14 TCR recognizes the lymphocytic choriomeningitis virus (LCMV)-derived gp33-41 epitope. Chimeric mice were vaccinated using bone-marrow-derived DCs that had been matured using the TLR4 ligand lipopolysaccharide (LPS) and pulsed with LCMV gp33-41 peptide (termed DC33). To address the role of systemic inflammation, DC vaccination was combined with systemic exposure to the TLR9 ligand CpG-B (termed DC33/CpG) (Badovinac et al., 2005; Cui et al., 2009).

P14 CD8 T cells were below detection in the absence of vaccination (data not shown) but were comparably abundant at day 7 post-DC33 and DC33/CpG vaccination (Figures 1B and S1F), in agreement with a previous study (Cui et al., 2009). Similar to naive P14 cells (data not shown) or host CD8 T cells, most P14 cells expressed high levels of Tcf1 following DC33 vaccination (Figure 1C). In contrast, DC33/CpG vaccination significantly reduced Tcf1 expression in P14 cells (Figure 1C). Tcf1 downregulation did not occur when CpG was administered in the absence of DC33 vaccination (data not shown). Thus, systemic inflammation suppressed Tcf1 in primed T cells. Corresponding results were obtained when analyzing the expression of the Tcf1 target Axin2 (Figure S1G).

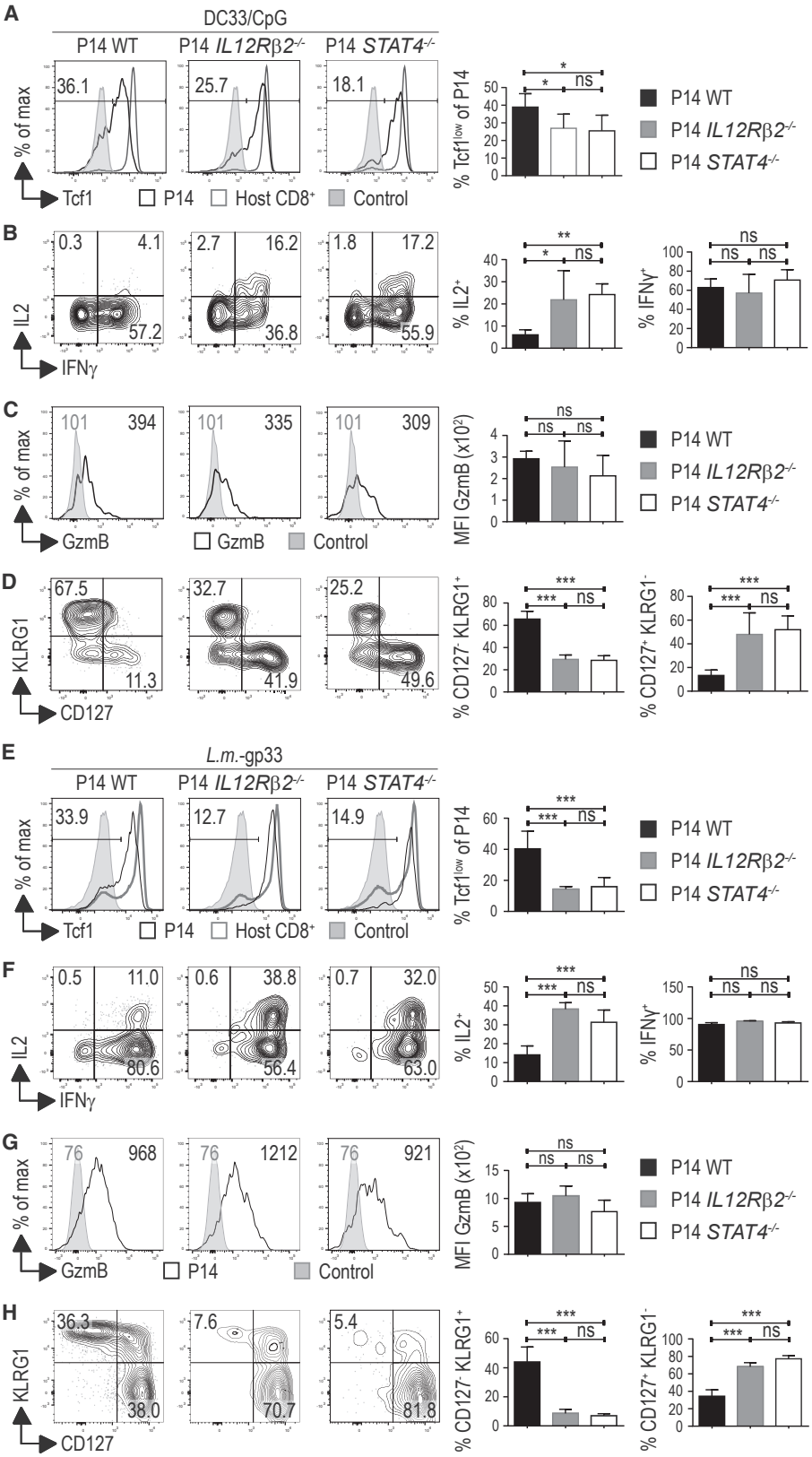
DC33-primed P14 cells efficiently produced IL-2 upon peptide restimulation *in vitro*, expressed low levels of granzyme B (GzmB), and were mostly CD127$^+$ KLRG1$^-$ MPECs (Figures 1D–1F). In contrast, P14 cells primed with DC33/CpG produced little IL-2, expressed higher levels of GzmB, and had a predominant CD127$^-$ KLRG1$^+$ SLEC phenotype (Figures 1D–1F). Nevertheless, Tcf1 expression was maintained in a subset of cells with an MPEC phenotype (Figure 1G), and these cells were undifferentiated based on a lack of granzyme B expression (Figure 1H). Thus, systemic inflammation repressed Tcf1 in most primed CD8 T cells and this correlated with effector differentiation.

Tcf1 Downregulation Is Mediated by IL-12 (p40)

The data so far showed that TLR9 signaling played a key role for suppressing Tcf1 and promoting effector differentiation of primed CD8 T cells. TLR9 expression by the immunizing DCs or the responding CD8 T cells are reportedly not essential for effector differentiation (Pham et al., 2009), indicating a role for soluble factors produced by host cells expressing TLR9. DC/CpG vaccination increases serum levels of multiple cytokines (Cui et al., 2014; Pham et al., 2009), including IL-12 and type I IFN, as well as IL-2, which promotes effector CD8 T cell differentiation (Joshi et al., 2007; Kalia et al., 2010; Keppler et al., 2012; Williams et al., 2006; Xiao et al., 2009). We thus tested whether these cytokines downregulated Tcf1 in the context of DC33/CpG vaccination. Indeed, blockade of IL-12 (p40) almost completely prevented Tcf1 downregulation in CD8 T cells (Figure 2A). IL-12 (p40) blockade did not significantly impact P14 expansion (Figure 2B) but prevented effector differentiation as judged by enhanced IL-2 production, reduced GzmB expression, and reduced SLEC differentiation (Figures 2C–2E). In contrast, blockade of interferon-α/β receptor 1 (*IFNAR1*) or IL-2 had essentially no effect on Tcf1 expression or effector differentiation (Figure S2). Thus, Tcf1 repression in primed CD8 T cells chiefly depends on IL-12 (p40).

Tcf1 Downregulation Is Mediated by IL-12Rβ2–STAT4 Signaling in CD8 T Cells

Since IL-12 (p40) is a subunit of IL-12 and IL-23, we next addressed whether both cytokines suppressed Tcf1 and whether they acted directly on CD8 T cells. When CD8 T cells were activated with anti-CD3 plus anti-CD28 monoclonal antibody (mAb) *in vitro*, Tcf1 expression remained comparable to nonactivated cells but was reduced when IL-12 was added (Figure S3A). In contrast, IL-23 had no effect (Figure S3B). Corresponding data were obtained when P14 cells were stimulated with gp33 peptide-pulsed splenocytes (Figure S3C). We tested additional



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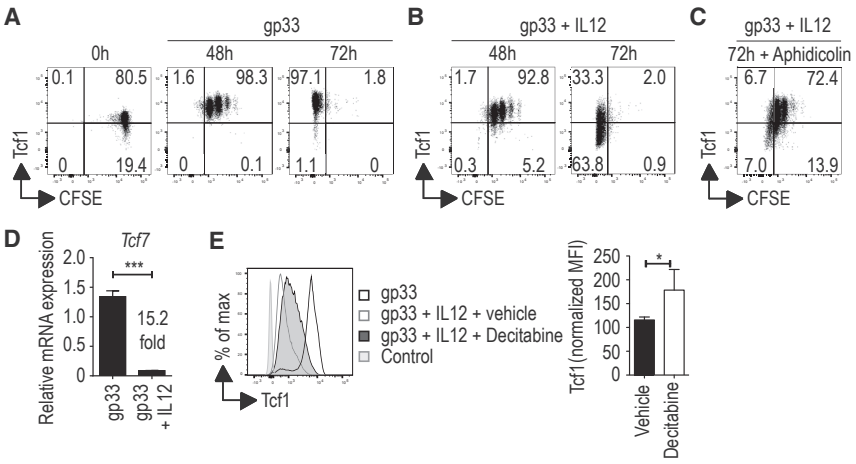

Figure 4. IL-12 Represses *Tcf7* Transcription and Establishes Asymmetric Tcf1 Abundance during Cell Division

(A–C) Carboxyfluorescein succinimidyl ester (CFSE)-labeled P14 cells (CD45.2$^+$) were activated *in vitro* with gp33 peptide-pulsed splenocytes (CD45.1$^+$ or CD45.1$^+$/2$^+$). Representative flow cytometry plots show CFSE dilution versus Tcf1 expression at the indicated time point post stimulation in the absence of IL-12 (A), in the presence of IL-12 (B), and upon addition of IL-12 and the cell-cycle inhibitor aphidicolin at 48 hr post-stimulation (C). Numbers indicate the fraction cells in the respective quadrants. Note that CFSE was gated according to residual CFSE expression observed at 48 hr post-stimulation. The data shown are representative of 3 independent experiments.

(D) P14 cells were activated as above for 72 hr, and *Tcf7* mRNA levels were determined using qRT-PCR analysis. Data shown represent quadruplicate determinations and are representative of 2 independent experiments.

(E) P14 cells were activated as above, and the DNA methyltransferase inhibitor decitabine (black border, gray fill) or vehicle (DMSO) (gray border, open) was added at 48 hr post stimulation. Tcf1 expression was measured at 72 hr post-stimulation. The bar graph depicts the mean MFI (±SD) of Tcf1 expression normalized to P14 cells activated with gp33 + IL-12 (100%) from n = 4 (vehicle) or n = 8 (decitabine) independent determinations.

Statistical significance was determined using an unpaired t test (***p < 0.001; *p < 0.05; ns, not significant [p > 0.05]). See also Figure S3.

inflammatory cytokines and found that IFN-β also suppressed Tcf1, albeit weakly as compared to IL-12 (Figure S3C).

We next used *IL12Rβ2*$^{-/-}$ CD8 T cells to address whether IL-12 acted directly on CD8 T cells during DC vaccination. Since IL-12Rβ2 chiefly signals via STAT4 (Jacobson et al., 1995; Nguyen et al., 2002), we further tested whether STAT4 played a role. Following DC33/CpG vaccination, Tcf1 downregulation was significantly less efficient when P14 cells lacked *IL12Rβ2* or *STAT4* (Figure 3A). This was associated with increased IL-2 production and reduced SLEC differentiation (Figures 3B and 3D), in agreement with Keppler et al. (2009). Thus, IL-12Rβ2-STAT4 signaling in CD8 T cells downregulates Tcf1 and induces effector differentiation. However, the effect of IL-12Rβ2-STAT4 deficiency was reduced compared to IL-12 (p40) blockade, indicating that IL-12 acts in part indirectly. The reduced effect of *IL12Rβ2* or *STAT4*-deficiency likely explains why there was no significant change in GzmB expression (Figure 3C). Irrespectively, IL-12Rβ2-STAT4 signaling in CD8 T cells contributes to DC33/CpG-vaccine-induced Tcf1 downregulation.

We further determined the importance of the IL-12Rβ2-STAT4 axis for Tcf1 expression in CD8 T cells responding to an acute infection. Infection with *L. monocytogenes* expressing gp33 (*L.m.*-gp33) resulted in a large fraction of Tcf1^{low} cells when P14 cells were wild-type, but not when they lacked *IL12Rβ2* or *STAT4* (Figure 3E). The latter was associated with reduced effector differentiation (Figures 3F and 3H), in agreement with Keppler et al. (2009). Again, GzmB expression was not different (Figure 3G). Thus, Tcf1 downregulation and effector differentiation in response to DC vaccination and bacterial infection depends on IL-12Rβ2-STAT4 signaling in CD8 T cells.

IL-12-Mediated Tcf1 Downregulation Depends on Cell Cycling and Occurs at the Level of Gene Expression

We further addressed the mechanisms of IL-12-mediated Tcf1 downregulation. P14 cells stimulated *in vitro* using gp33 peptide-pulsed splenocytes maintained high Tcf1 expression through multiple cell divisions (Figure 4A), in agreement with Lin et al. (2016). IL-12 induced precipitous Tcf1 downregulation after 48 hr of activation (Figure 4B). To address whether this drop in Tcf1 expression required cell division, we blocked cell-cycle progression after 48 hr of stimulation using aphidicolin. Cell-cycle blockade prevented Tcf1 downregulation (Figure 4C), demonstrating that IL-12-induced Tcf1 loss was cell-cycle dependent.

We next investigated whether IL-12 regulated Tcf1 at the level of gene expression. The addition of IL-12 during activation of P14

Figure 3. Absence of *IL12Rβ2* or *STAT4* from CD8 T Cells Prevents Tcf1 Downregulation during Vaccination and Bacterial Infection

(A–H) WT, *IL12Rβ2*$^{-/-}$ and *STAT4*$^{-/-}$ P14 chimeric mice were vaccinated with DC33 or DC33/CpG (A–D) or infected with *L.m.*-gp33 (E–H).

(A and E) Tcf1 expression in P14 cells (black open) compared to host CD8 T cells (gray open) and *Tcf7*$^{-/-}$ P14 cells (control) (gray fill). The bar graph depicts the percentage of Tcf1^{low} P14 cells.

(B and F) IL-2 and IFN-γ production by P14 cells following *in vitro* restimulation with gp33 peptide. The bar graphs depict the percentage of IL-2$^+$ (left) and IFN-γ$^+$ (right) in P14 cells.

(C and G) GzmB expression in P14 cells (black open) compared to isotype control (gray fill). The bar graph depicts the MFI of GzmB staining whereby background was subtracted.

(D and H) CD127 versus KLRG1 expression among gated P14 cells. The bar graphs indicate the percentage of CD127$^-$ KLRG1$^+$ SLECs (left) and CD127$^+$ KLRG1$^-$ MPECs (right).

Bar graphs depict means (±SD, n = 5). Data are representative of at least 2 independent experiments. Statistical significance was determined with one-way ANOVA tests (***p < 0.001; **p < 0.01; *p < 0.05; ns, not significant [p > 0.05]). See also Figure S3.

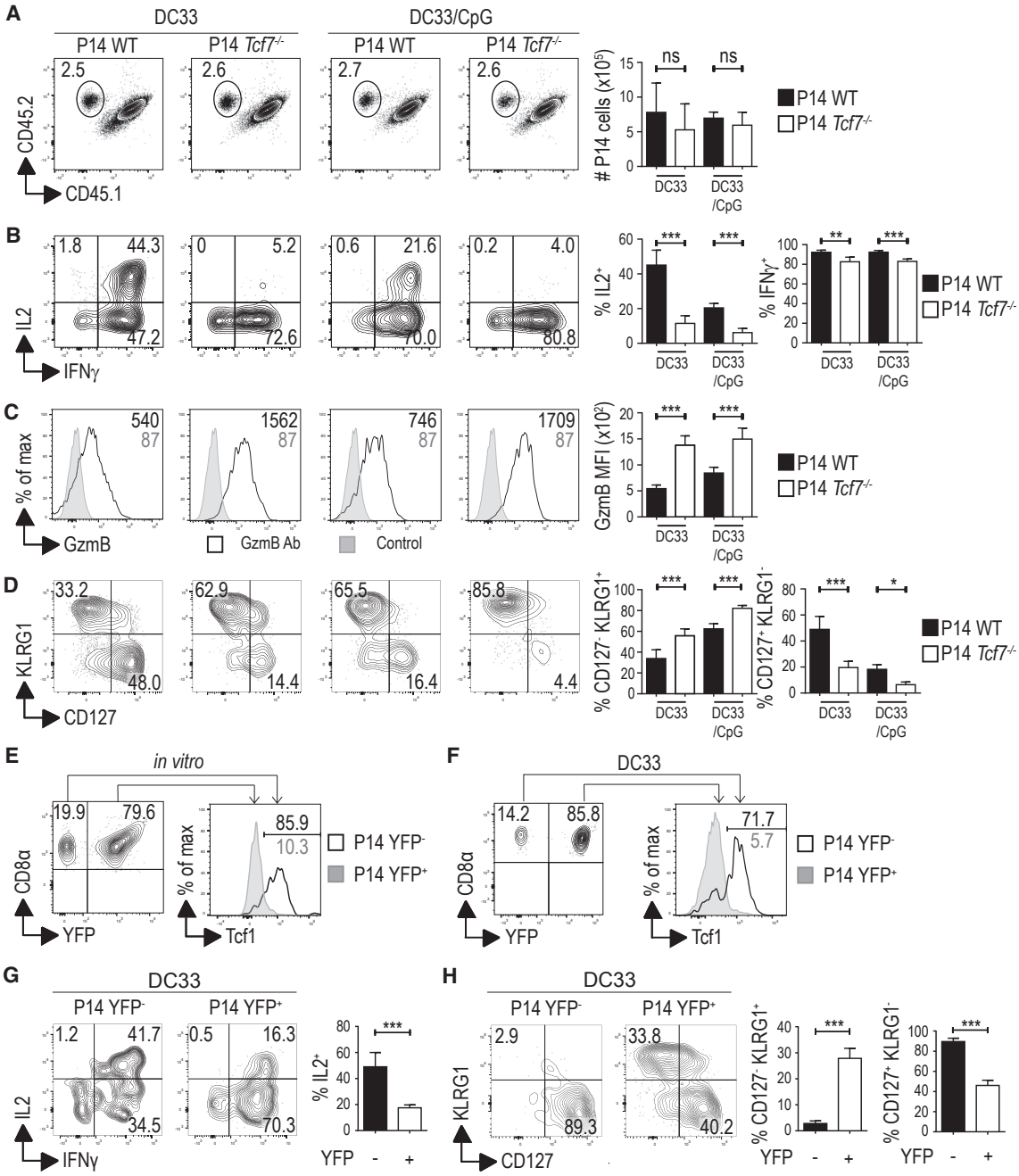

Figure 5. *Tcf7*$^{-/-}$ CD8 T Cells Undergo Effector Differentiation Even in the Absence of Systemic Inflammation

WT or *Tcf7*$^{-/-}$ P14 chimeric mice were vaccinated with DC33 or DC33/CpG.

(A) Abundance of P14 cells (CD45.2^{+}) in the spleen at day 7 post-vaccination.

(B) IL-2 and IFN-γ production by P14 cells following *in vitro* restimulation with gp33 peptide. The bar graphs show the percentage of P14 cells producing IL-2 (left) and IFN-γ (right).

(C) GzmB expression (black open) compared to isotype control (gray fill). The bar graph shows the MFI of GzmB staining whereby background was subtracted.

(D) CD127 versus KLRG1 expression among gated P14 cells. The bar graphs show the percentage of CD127^{-} KLRG1^{+} SLECs (left) and CD127^{+} KLRG1^{-} MPECs (right).

(E) *Tcf7*$^{lox/lox}$ *R26-lox-YFP* P14 cells (CD45.2^{+}) were treated with Tat-Cre *in vitro*, and the frequency of YFP^{+} P14 cells (*Tcf7* deleted) was determined after 48 hr of culture *in vitro* (left). Histogram overlay shows Tcf1 expression by flow-sorted YFP^{+} (gray fill) and YFP^{-} P14 cells (black open) (right). Numbers indicate the percentage of cells in the respective quadrants (left) and the percentage of Tcf1^{hi} cells (right).

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cells with gp33 peptide-pulsed splenocytes strongly reduced *Tcf7* mRNA levels (Figure 4D), indicating that IL-12 silenced *Tcf7*. The magnitude of the transcriptional effect (19.6- and 15.2-fold in two independent experiments) even exceeded the reduction in Tcf1 protein expression (9.0±1.7-fold, n = 6) (Figure S3C). A role for IL-12-induced transcriptional silencing was further addressed using the DNA methyltransferase inhibitor 5-aza-2-deoxycytidine (decitabine). Decitabine addition (at 48 hr post-stimulation) prevented IL-12-mediated Tcf1 suppression in part (Figure 4E). Reduced Tcf1 suppression in the presence of decitabine was not due to reduced cell cycling (data not shown). The data show that IL-12-induced Tcf1 suppression depended on cell division, was explained by reduced gene expression, and depended in part on DNA methylation, collectively suggesting a role for epigenetic *Tcf7* silencing.

Primed CD8 T Cells Lacking Tcf1 Undergo Default Effector Differentiation

The observation that inflammation suppressed Tcf1 and promoted effector differentiation raised the question regarding the causal relationship between these two events. Inflammation-dependent Tcf1 suppression may be a prerequisite for effector differentiation. Conversely, inflammation may induce effector differentiation and that leads to Tcf1 suppression. Finally, inflammation may independently promote effector differentiation and inhibit Tcf1 expression. If Tcf1 suppressed effector differentiation, *Tcf7*$^{-/-}$ CD8 T cells should differentiate more efficiently into effector cells than WT cells.

In response to DC33/CpG vaccination, WT and *Tcf7*$^{-/-}$ P14 cells expanded comparably (Figure 5A). However, *Tcf7*$^{-/-}$ P14 cells expressed less IL-2 and more GzmB and differentiated more efficiently into SLECs than WT P14 cells (Figures 5B–5D). Interestingly, considerable effector differentiation of *Tcf7*$^{-/-}$ P14 cells was also observed in response to DC33 in the absence of systemic inflammation (Figures 5B–5D). Thus, lack of Tcf1 resulted in default effector differentiation.

The above experiments were performed with P14 cells from mice with germline *Tcf7* deletion, in which T cell development is significantly impaired (Ioannidis et al., 2001; Verbeek et al., 1995). It was thus possible that increased effector differentiation was somehow the consequence of altered T cell development. To rule this out, we generated *Tcf7*$^{lox/lox}$ mice harboring a Rosa26 lox stop lox EYFP cassette (R26-stop-YFP) and a P14 TCR transgene (schematically shown in Figure S4A). Exposure of naive P14 *Tcf7*$^{lox/lox}$ R26-stop-YFP cells to Tat-Cre fusion protein *in vitro* resulted in YFP expression in a considerable fraction of P14 cells. Importantly, the vast majority of the YFP$^+$ cells lacked Tcf1 protein expression, while most YFP$^-$ cells were Tcf1$^+$ (Figure 5E). Thus, YFP induction could be used to track *Tcf7*-deleted cells at the single-cell level.

Next, we adoptively transferred Tat-Cre-treated P14 *Tcf7*$^{lox/lox}$ R26-stop-YFP cells (CD45.2$^+$) into CD45.1$^+$ recipient mice, which were then vaccinated with DC33. The fraction of YFP$^+$ P14 cells (*Tcf7* deleted) at day 8 corresponded to that of input (Figures 5E and 5F), indicating comparable expansion of YFP$^+$ (*Tcf7* deleted) and YFP$^-$ cells (nondeleted). We confirmed that YFP$^+$ cells were mostly Tcf1$^-$ and that YFP$^-$ cells were predominantly Tcf1$^+$ (Figure 5F). As expected, YFP$^-$ P14 cells (nondeleted) were IL-2^{hi}, GzmB^{low} and predominantly CD127$^+$ KLRG1$^-$ MPECs. In contrast, YFP$^+$ P14 cells (*Tcf7*-deleted) were IL-2^{low}, GzmB^{hi} and predominantly CD127$^-$ KLRG1$^+$ SLECs (Figures 5G and 5H). Thus, default effector differentiation of T cells lacking *Tcf7* was observed when Tcf1 was deleted in naive CD8 T cells and was thus entirely independent of a developmental defect. Altogether, these data showed that Tcf1 counteracted effector differentiation and that inflammation-induced Tcf1 suppression was needed for effector differentiation.

Lack of Tcf1 Prevents Default Central Memory Formation in Response to Vaccination

Exposure to antigen in the context of DCs but in the absence of systemic inflammation results in default memory formation (i.e., accelerates the rate at which CD8 T cells acquire late-memory characteristics) (Badovinac et al., 2005). As we observed default effector differentiation of *Tcf7*$^{-/-}$ CD8 T cells during DC vaccination, we next asked whether this impacted memory formation. WT and *Tcf7*$^{-/-}$ P14 cells were readily detected >40 days after DC33 vaccination, although *Tcf7*$^{-/-}$ P14 memory cells were slightly reduced (p = 0.08) (Figure 6A). While CD127$^+$ CD62L$^-$ effector memory cells were present normally, CD127$^+$ CD62L$^+$ central memory (T_{CM}) cells were severely (>10-fold) reduced among *Tcf7*$^{-/-}$ as compared to WT P14 cells (Figure 6B). In agreement with reduced default central memory formation, *Tcf7*$^{-/-}$ memory cells produced less IL-2 but expressed more GzmB (Figures 6C and 6D). Finally, equal numbers of flow-sorted memory P14 cells were transferred to naive secondary recipients, which were then challenged with LCMV cl13. While WT P14 memory cells expanded efficiently, the recall expansion of *Tcf7*$^{-/-}$ P14 memory cells was greatly reduced (Figure 6E). Corresponding results were obtained when P14 chimeric mice had been vaccinated with DC33/CpG (Figures S4B–S4F). We conclude that the formation of default central memory in response to vaccination depends on Tcf1 and that deficient central memory differentiation in the absence of Tcf1 is associated with default effector differentiation during the primary response.

Tcf1 Suppresses *IL12Rβ2* and Transcription Factors Promoting Effector Differentiation

While naive T cells express low levels of IL-12R, both IL-12R chains are upregulated following T cell activation (Trinchieri,

(F) Tat-Cre-treated *Tcf7*$^{lox/lox}$ *R26-lox-YFP* P14 cells (CD45.2$^+$) were transferred into CD45.1$^+$ mice, which were then vaccinated with DC33. The frequency of YFP$^+$ P14 cells (*Tcf7* deleted) was determined 7 days later (left). Tcf1 expression by flow sorted YFP$^+$ (gray fill) and YFP$^-$ P14 cells (black open) (right). Numbers indicate the percentage of cells in the respective quadrants (left) and the percentage of Tcf1^{hi} cells (right).

(G and H) Gated YFP$^+$ (*Tcf7*-deleted) and YFP$^-$ P14 cells (non-deleted) were analyzed for the percentage of cells producing IL-2 (G) and the percentage of CD127$^-$ KLRG1$^+$ SLECs and CD127$^+$ KLRG1$^-$ MPECs (H). Bar graphs depict means (±SD, n = 5).

Data are representative of at least two independent experiments. Statistical significance was determined with unpaired t tests (***p < 0.001; **p < 0.01; *p < 0.05; ns, not significant [p > 0.05]). See also Figure S4.

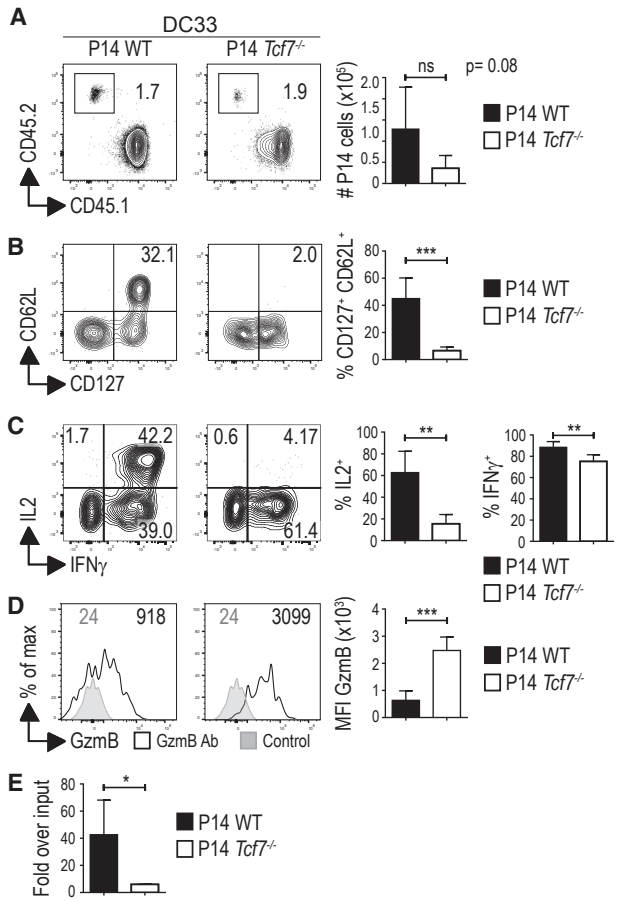

Figure 6. Tcf1 Is Essential for Default Central Memory Formation in Response to DC33 Vaccination

WT or *Tcf7*$^{-/-}$ P14 chimeric mice were vaccinated with DC33.

(A) Abundance of P14 cells in the spleen 40 days post-vaccination.

(B) CD127 versus CD62L expression among gated P14 cells. The bar graphs show the percentage of CD127^{+} CD62L^{+} (central memory phenotype) cells.

(C) IL-2 and IFN-γ production by P14 cell following *in vitro* restimulation. The bar graphs depict the percentage of IL-2^{+} (left) and IFN-γ^{+} (right) P14 cells.

(D) GzmB expression in P14 cells (black open) compared to isotype control (gray fill). Numbers indicate the MFI of staining, and the bar graph depicts the MFI of GzmB staining whereby background was subtracted.

(E) WT and *Tcf7*$^{-/-}$ P14 memory cells (CD45.2^{+}) were flow sorted, and equal numbers (10^4 cells) were transferred into secondary recipients (CD45.1^{+}), which were infected with LCMV cl13. The abundance of P14 cells was determined 8 days later. The bar graph shows fold expansion of P14 cells compared to input, assuming 10% take of input cells.

Bar graphs depict means (±SD, n = 3–5). Data are representative of two independent experiments. Statistical significance was determined with unpaired t tests (***p < 0.001; **p < 0.01; *p < 0.05; ns, not significant [p > 0.05]). See also Figure S4.

2003). This improves IL-12 responsiveness, enhances STAT4 activation and further increases the expression of the STAT4 target IL-12Rβ2, thus generating an IL-12-dependent feedback loop (Becskei and Grusby, 2007), which promotes effector differentiation. Here, we find that IL-12Rβ2-STAT4 signaling suppressed Tcf1 and that Tcf1 expression counteracted effector differentiation. To do the latter efficiently, we hypothesized that Tcf1 negatively regulated *IL12Rβ2*. Indeed, following DC33 vaccination, *IL12Rβ2* was significantly overexpressed in *Tcf7*$^{-/-}$ as compared to WT MPECs (Figure 7A). *IL12Rβ2* expression in *Tcf7*$^{-/-}$ MPECs was as high as in WT SLECs. Inspection of available chromatin immunoprecipitation sequencing (ChIP-seq) data (Xing et al., 2016) revealed that Tcf1 is associated with the *IL12Rβ2* locus in CD8^{+} thymocytes (Figure S5A). Thus, Tcf1 expression limited effector differentiation at least in part by counteracting *IL12Rβ2* upregulation and consequently by reducing IL-12 responsiveness.

To further address the basis for default effector and deficient central memory differentiation, we investigated the expression of key transcription factors involved in effector and memory differentiation. Among transcription factors involved in memory differentiation, *Tcf7*$^{-/-}$ and WT MPECs expressed comparable levels of *Bcl6*, while *Eomes* was increased in *Tcf7*$^{-/-}$ MPECs (Figure 7B). These expression patterns did not explain deficient memory formation in the absence of Tcf1. On the other hand, compared to WT, *Tcf7*$^{-/-}$ MPECs overexpressed several transcription factors mediating effector differentiation (i.e., *Prdm1, Tbx21, Id2*, and *Zeb2*). Except for Zeb2, *Tcf7*$^{-/-}$ MPECs expressed these transcription factors at equal or even higher levels as compared to WT SLECs (Figure 7C). Tcf1 was associated with the *Prdm1*, *Tbx21*, and *Id2* loci, but not the *Zeb2* locus, in CD8^{+} thymocytes (Figure S5B), indicating both direct and indirect regulatory mechanisms. Thus, upregulation of *IL12Rβ2* and several transcription factors promoting effector differentiation in MPECs can account for the default effector differentiation of primed CD8 T cells lacking Tcf1.

DISCUSSION

Unlike other transcription factors required for memory formation, Tcf1 is expressed at high levels in naive CD8 T cells and is downregulated in most CD8 T cells during the primary response to viral or bacterial infection (Boudousquié et al., 2014; Jeannet et al., 2010; Lin et al., 2016; Zhou et al., 2010), which correlates with effector differentiation. The signals mediating Tcf1 downregulation *in vivo* and the hierarchical relationship between Tcf1 downregulation and effector differentiation have not been elucidated. Here, we show that IL-12-mediated systemic inflammatory signals induce Tcf1 downregulation in primed CD8 T cells. Importantly, CD8 T cells lacking Tcf1 efficiently differentiated into effector cells even in the absence of systemic inflammation, indicating that Tcf1 expression counteracted effector differentiation. Thus, IL-12-induced Tcf1 downregulation in wild-type CD8 T cells facilitated effector differentiation. Tcf1 is also downregulated during certain viral infections (Boudousquié et al., 2014; Jeannet et al., 2010; Tiemessen et al., 2014; Wu et al., 2016; Zhou et al., 2010), which are rich in type I IFN rather than IL-12. We did obtain evidence that also IFN-β had the capacity to downregulate Tcf1 *in vitro*. However, the effect of type I IFN was surprisingly weak, indicating that additional factors, perhaps induced by type I IFN, are involved in Tcf1 downregulation during certain viral infections.

Downstream of IL-12R, we find that STAT4 plays an essential role for Tcf1 suppression. While STATs were originally identified as transcriptional activators, there is growing evidence that they

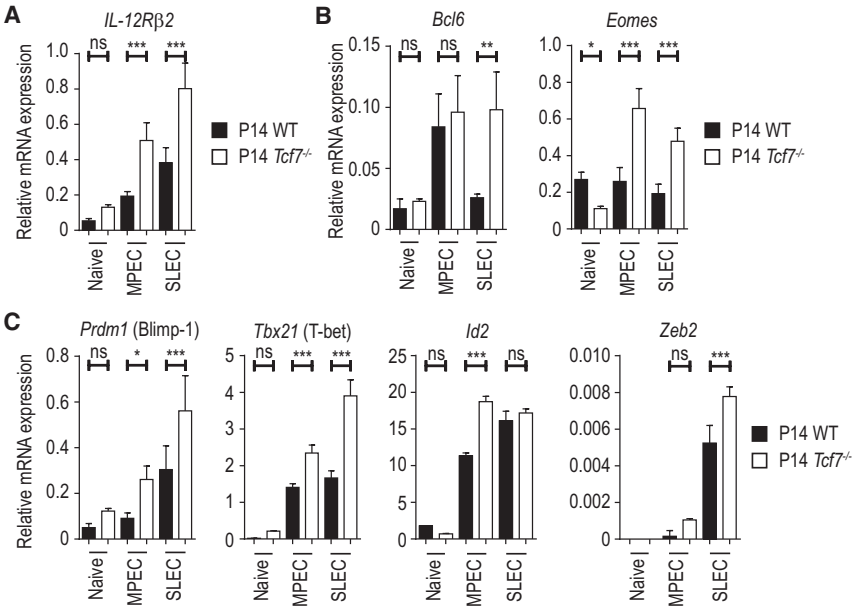

Figure 7. Memory Precursor Cells Lacking Tcf1 Overexpress *IL12Rβ2* and Transcription Factors Promoting Effector Differentiation

(A–C) WT or *Tcf7*$^{-/-}$ P14 chimeric mice were vaccinated with DC33. 7 days later, P14 MPECs and SLECs, as well as naive P14 cells, were flow sorted and analyzed for the expression of *IL12Rβ2*$^{-/-}$ (A), *Bcl6* and *Eomes* (B), and *Prdm1* (Blimp1), *Tbx21* (T-bet), *Id2*, and *Zeb2* (C). Gene expression is shown relative to *HPRT*. Bar graphs depict means (±SD) from quadruplicate determinations. Data are from a single experiment. Equivalent results were obtained using DC33/CpG vaccination. Statistical significance was determined with unpaired t tests (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns, not significant [$p > 0.05$]). See also Figure S5.

also mediate transcriptional repression (Wei et al., 2010). Consistent with these latter findings, we show that IL-12 reduced *Tcf7* transcription and that the DNA methyltransferase inhibitor decitabine partially rescued Tcf1 from IL-12-mediated silencing. These data are in agreement with an increase of repressive histone marks (Crompton et al., 2016) and of DNA methylation at the *Tcf7* locus in effector CD8 T cells (Scharer et al., 2013). A relative absence of methylated CpG at the *Tcf7* locus may explain why Tcf1 remains highly expressed during the first 2–3 cell divisions, an observation similarly reported by (Lin et al., 2016). The eventual drop in Tcf1 expression may be due to cell cycle and IL-12-dependent accumulation of CpG methylation at the *Tcf7* locus. During an immune response *in vivo*, both Tcf1^{+} and Tcf1^{-} CD8 T cells are present. Maintenance versus downregulation of Tcf1 may be determined by unequal responsiveness of CD8 T cells to inflammatory signals. This could be based on differential expression of IL-12R, or components of the respective signaling pathway, by responding T cells. Indeed, *IL12Rb2* is unequally expressed in CD8 T cells that have undergone a single division (Kakaradov et al., 2017). Together with the data shown herein, it is tempting to speculate that the pool of responding CD8 T cells diversifies during initial cell divisions and that inflammatory signals promote stable effector cell differentiation in part by silencing Tcf1.

The significance of Tcf1 downregulation emerged from our finding that CD8 T cells that lacked Tcf1 underwent effector differentiation even in the absence of systemic inflammation. This "default" effector differentiation was associated with impaired central memory formation. The latter was independent of changes in the expression of memory promoting transcription factors such as Eomes or Bcl6. However, we noted an inability of DC33-primed *Tcf7*$^{-/-}$ CD8 T cells to produce IL-2, which is needed for the secondary expansion of memory CD8 T cells (Feau et al., 2011; Williams et al., 2006). Naive *Tcf7*$^{-/-}$ CD8 T cells stimulated *in vitro* readily produced IL-2 (data not shown), indicating that IL-2 production was not directly dependent on Tcf1 but was lost during priming. Consistent with aberrant differentiation, *Tcf7*$^{-/-}$ memory precursor cells overexpress factors associated with effector differentiation such as IL-12Rβ2, *Prdm1* (Blimp-1), *Tbx21* (T-bet), Id2, and Zeb2, which likely explains "default" effector differentiation and impaired central memory formation

The data included in this paper identify a key role of the IL-12-STAT4 axis to suppress Tcf1 and consequently to modify memory versus effector differentiation of CD8 T cells. Interventions that prevent Tcf1 downregulation may thus be useful to dampen effector differentiation and reduce autoimmune or allograft responses. Conversely, approaches to maintain Tcf1 expression may improve memory formation during vaccination.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 (B6) (CD45.2^{+}) mice were purchased from Charles River, and B6.*Cd45.1* (CD45.1^{+}), B6.*IL12Rβ2*$^{-/-}$, B6.*ROSA26Sor*$^{tm1(EYFP)}$ (B6.*R26-stop-YFP*) mice were obtained from The Jackson Laboratory. B6 P14 TCR Tg (line 237) (Pircher et al., 1989) mice were provided by H.P. Pircher (Freiburg), *Tcf7*$^{-/-}$ mice (Verbeek et al., 1995) were provided by H. Clevers (Utrecht) and backcrossed >12 times to B6, and B6.*STAT4*$^{-/-}$ mice (Kaplan et al., 1996) were provided by M. Löhning (Berlin). *Tcf7*-GFP mice have been described previously (Utzschneider et al., 2016). B6.*Tcf7*$^{tm1aWtsi/+}$ mice were obtained from the International Knockout Mouse Consortium. Founder mice were crossed to B6 ROSA26:FLPe mice (Jackson Laboratory) to delete the frt-flanked LacZ-neo cassette and to generate B6.*Tcf7*^{tm1c} mice, in which exon 4 of *Tcf7* is flanked with loxP sites (B6.*Tcf7*^{lox}). P14 *Tcf7*$^{-/-}$, P14 *Axin2*$^{LacZ/+}$, OT1 *Axin2*$^{LacZ/+}$, P14 *Tcf7*$^{-/-}$, P14 Tcf7-GFP, P14 *IL-12Rβ2*$^{-/-}$, P14 *STAT4*$^{-/-}$, and P14 *Tcf7*$^{lox/lox}$ *R26-stop-YFP* strains were generated by breeding. As controls for *Tcf7*$^{-/-}$ mice, we used wild-type (WT) *Tcf7*$^{+/+}$ littermate or B6 mice. Mice were bred and maintained in a specific-pathogen-free (SPF) environment, vaccinated in a conventional environment, and infected in the P2 animal facility of the University of Lausanne. Experiments were performed in 6- to 12-week-old male and female mice in compliance with the University of Lausanne Institutional regulations and were approved by the veterinarian authorities of the Canton de Vaud.

DC Vaccination and *L. monocytogenes* Infection

Bone marrow cells derived from B6 (CD45.2^{+}) mice were cultured for 6 days in granulocyte-macrophage colony-stimulating factor (GM-CSF) (10 ng/mL) and

IL-4 (20 ng/mL) (Peprotech). Lipopolysaccharide (100 ng/mL; Sigma) was added overnight and $gp_{33–41}$ (gp33) peptide (KAVYNFATM) (10 μg/mL) for the last 2 hr. The resulting cells (termed DC33) consisted of 50%–80% $CD11c^+$ cells. One million $CD11c^+$ cells were injected intravenously (i.v.) into $CD45.1^+$ or $CD45.1^+2^+$ recipients with or without 50 μg CpG-B 1826 oligodeoxynucleotides (ODNs) administered intraperitoneally (i.p.) (TriLink Biotechnologies). 1–2 × 10^4 purified TCR Tg CD8 T cells ($CD45.2^+$) were adoptively transferred one day prior to DC injection. Mice were analyzed on day 7 or day 50 post-vaccination.

L. monocytogenes expressing LCMV gp33-41 (KAVYNFATC) together with the ovalbumin ($OVA_{257–264}$)-derived epitope SIINFEKL (N4) or the altered peptide-ligand SIITFEKL (T4) (Oberle et al., 2016) were kindly provided by D. Zehn (Munich). 2 × 10^3 colony-forming units (CFUs) were injected i.v. into naive $CD45.1^+$ mice one day after the adoptive transfer of P14 or OT1 cells. Mice were analyzed on day 7 post-infection.

Antibody Treatment

Mice were injected i.p. with 500 μg rat anti-mouse IL-12 p40 (C17.8) or with rat immunoglobulin G2a (IgG2a) isotype control antibody (2A3) (BioXCell) once at the time of DC vaccination and a second time 5–6 hr later. Mice were analyzed on day 7 post-vaccination.

Flow Cytometry

Splenocyte suspensions were incubated with anti-CD16/32 (2.4G2) hybridoma supernatant before staining for 15 min at 4°C with fluorescent mAbs (Table S1). Zombie Aqua Fixable Viability Kit (BioLegend) was used to exclude dead cells.

For intracellular cytokine staining, splenocytes were restimulated *in vitro* with gp33-41 peptide (KAVYNFATM) (1 μM) for 5 hr in the presence of brefeldin A (7 μg/mL) for the last 4.5 hr. Cells were then fixed and permeabilized using the intracellular fixation and permeabilization buffer kit (eBioscience) and stained with mAbs against IFN-γ, tumor necrosis factor α (TNF-α), and IL-2 (Table S1). The FoxP3 transcription factor staining kit (eBioscience) was used to detect granzyme B and Tcf1 using specific mAbs (Table S1) followed by anti-rabbit IgG PE (eBioscience). Flow cytometry analysis was performed on an LSR-II or LSRFortessa II flow cytometer (BD).

Data Analyses and Statistics

Flow cytometry data were analyzed using FlowJo (TreeStar). Graphics were prepared with GraphPad Prism. Bar graphs depict the mean ± SEM or SD as indicated. Statistical analyses were performed using Prism 6.0 (GraphPad Software) using unpaired t tests (two tailed) or one-way ANOVA as indicated. p values < 0.05 were considered significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, nonsignificant [i.e., p values > 0.05]).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.01.072.

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AUTHOR CONTRIBUTIONS

M.D. designed experiments, performed experimental work, analyzed results, and prepared figures. V.C. and J.G.S. performed experimental work and analyzed results, and S.S. analyzed results. W.H. conceived the study, supervised the project, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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