Critical role for TRIM28 and HP1β/γ in the epigenetic control of T cell metabolic reprogramming and effector differentiation

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Naïve CD4+ T lymphocytes differentiate into different effector types, including helper and regulatory cells (Th and Treg, respectively). Heritable gene expression programs that define these effector types are established during differentiation, but little is known about the epigenetic mechanisms that install and maintain these programs. Here, we use mice defective for different components of heterogeneous-chromatin-dependent gene silencing to investigate the epigenetic control of CD4+ T cell plasticity. We show that, upon T cell receptor (TCR) engagement, naive and regulatory T cells defective for TRIM28 (an epigenetic adapter for histone binding modules) or for the heterochromatin protein 1 β and γ isoforms (HP1β/γ), 2 histone-binding factors involved in gene silencing, fail to effectively signal through the P35-AKT–mTOR axis and switch to glycolysis. While differentiation of naive TRIM28β/γ T cells into cytokine-producing effector T cells is impaired, resulting in reduced induction of autoimmunity, TRIM28β/γ regulatory T cells also fail to expand in vivo and to suppress autoimmunity effectively. Using a combination of transcriptome and chromatin immunoprecipitation-sequencing (ChIP-seq) analyses for H3K9me3, H3K9Ac, and RNA polymerase II, we show that reduced effector differentiation correlates with impaired transcriptional silencing at distal regulatory regions of a defined set of Treg-associated genes, including, for example, NRP1 or Snai3. We conclude that TRIM28 and HP1β/γ control metabolic reprogramming through epigenetic silencing of a defined set of Treg-characteristic genes, thus allowing effective T cell expansion and differentiation into helper and regulatory prototypes.

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Data deposition: The microarray and sequencing data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE140448).

Significance

CD4 T cells are major regulators of immune responses against both self and pathogens. Understanding pathways that govern CD4 T cell differentiation and regulation are thus key for the discovery of new immunoregulatory drug targets. Here, we have identified an epigenetic pathway that regulates the expression of a set of proteins that determine T cell responsiveness. By silencing enhancers distal to a set of genes known to be involved in regulatory T cell function, the epigenetic modifiers TRIM28 and HP1β/γ regulate T cell receptor signaling. This leads to defective metabolic reprogramming and inefficient effector differentiation of naive T cells. This mechanism provides an exciting opportunity to regulate T cell responsivity in both autoimmunity and T cell–based immunodeficiencies.

T cells | immunology | epigenetics | autoimmunity | TRIM28

Cells participate and control the vast majority of adaptive immune responses. While CD8+ T cells mediate killing of virus-infected and tumor cells, CD4+ T cells positively (helper T cells, Th) and negatively (regulatory T cells, Tregs) regulate both cytotoxic and antibody responses. Similar to pluripotent stem cells, which retain the ability to differentiate into tissue-specific cell types, naive cells leaving the thymus have the capacity to differentiate into distinct subsets of effector or regulatory cells upon activation that differ in their transcriptional programs and metabolic needs. For example, while all T cells rely on glucose metabolism for clonal expansion, only Th effector functions are linked to glycolysis, while the suppressive capacity of Tregs has been linked to β-oxidation of fatty acids (1, 2).
Typically, these differentiation programs are supported by a series of heritable gene expression programs under the control of specific transcription factors. Epigenetic modifications, including histone acetylation and methylation, or DNA methylation, determine the accessibility of genomic regions to transcription factors. For T cell differentiation into effector T cells, for example, trimethylation of lysine 4 on histone 3 (H3K4me3) is a permissive mark for transcription of lineage specific cytokines IFNγ (Th1), IL4 and IL13 (Th2), and IL17 (Th17), while H3K27me3 is associated with their transcriptional repression (3–7). DNA methylation, in turn, is critical for regulating the expression of Foxp3, the hallmark transcription factor of Tregs (8).

Recently, we showed that H3K9 trimethylation, characteristic for transcriptionally inactive heterochromatin domains, at the Ifnγ locus is decisive for Th2 lineage commitment (9). Differentiated Th2 cells from mice lacking the histone methyltransferase Suv39h1 or the heterochromatin protein 1a (HP1α), readily converted to IFNγ-expressing Th1-like cells, while wild-type (WT) cells did not. HP1α and its 2 other isoforms, HP1β or HP1γ, are recruited to chromatin by a tripartite motif containing, scaffolding protein, TRIM28 (also known as Kap1 or Tif1β) (10). TRIM28 interacts with KRAB-zinc finger proteins to target specific genomic regions and modulates transcription through interactions with HP1 isoforms (11, 12). Moreover, recent studies suggest that TRIM28 could interact with both polycomb and trithorax complexes (13, 14). Taken together, these findings indicate that TRIM28 regulates initiation and/or elongation of RNA polymerase II (Pol II)-dependent transcription (13–15).

Several studies revealed an important role for TRIM28 in T cell development and activation. Mice with T cell-specific deletion of TRIM28 (TRIM28−/−) lack invariant natural killer T cells due to altered use of T cell receptor α (TCRα) chains and develop autoimmune diabetes due to TGFβ overexpression and deregulation of the Th17 pathway (16–18). TRIM28−/− mice also have increased Tregs numbers and a defect in IFNγ production in Th1 cells, but the mechanism for this imbalance remains unclear (17). Although it was shown that TRIM28 could interact with both polycomb and trithorax family proteins in other cell types, it is still unclear which of these 2 epigenetic effector pathways mediates the effects of TRIM28 in T cells (19).

We now show that TRIM28−/− and HP1β/γ−/− T cells fail to differentiate into helper and Tregs due to an early defect in metabolic reprogramming to glycolysis. Defective metabolic reprogramming results from impaired TCR/CD28 signaling through the P13K–Akt–mammalian target of rapamycin (mTOR) axis. These defects correlate with impaired epigenetic silencing by TRIM28 and HP1β/γ at distal regulatory regions to a defined set of Treg-characteristic genes, whose silencing is critical to proper T cell expansion and differentiation into helper and regulatory phenotypes.

**Results**

TRIM28 Is Necessary for Efficient T Cell Differentiation and Function In Vivo. As shown previously by others, TRIM28lox/lox × CD4Cre mice (TRIM28−/−) develop phenotypically normal primary and secondary lymphoid organs and display minor alterations in thymocyte development (17, 18) (SI Appendix, Fig. S1 G–I). TRIM28 expression is normal in double-negative thymocytes and decreases by about 50% in double-positive thymocytes and by almost 100% in CD4+ and CD8+ peripheral T cells. Lack of TRIM28 expression in peripheral T cells is also visible by Western blot (SI Appendix, Fig. S1A).

Bone marrow (BM) from TRIM28−/− mice contains significantly fewer central and effector memory T cells (SI Appendix, Fig. S1B) and fewer CD44+ memory CD4+ T cells expressing the proliferation marker Ki67 in peripheral lymph nodes (SI Appendix, Fig. SIC). Upon sorting of effector memory T cells from spleen and mesenteric lymph nodes of aged mice, we also detected less effector cytokines (IFNγ, IL13, and IL17) after ex vivo restimulation with PMA/ionomycin (SI Appendix, Fig. S1D). Adoptively transferred TRIM28-deficient OT-II naive T cells proliferated less and produced less effector cytokines after immunization with ovalbumin (OVA) in complete Freund’s adjuvant (CFA) (SI Appendix, Fig. S1E). CFA-OVA immunization of TRIM28−/− resulted in significantly fewer IL4-producing OVA-specific CD4+ T cells compared to WT controls (SI Appendix, Fig. S1F).

To test whether this defect in effector T cell function translates to decreased T cell-mediated immunopathology, we studied a T cell transfer colitis model (20). Rag2−/− mice transferred with TRIM28−/−× RorcGFP+ naive CD4+ T cells survived longer and developed significantly less colitis, as evidenced by the delay in weight loss, decreased T cell infiltration in colonic mucosa, increased colon length, and lower immunohistopathology scores of colonic tissue (Fig. 1 A–E). Time course analysis of CD4+ T cells in the blood revealed a strong defect in T cell expansion and development into Rorγt-expressing cells that translated into fewer IFNγ- and IL17-producing splenic CD4+ T cells (Fig. 1 F–I). We conclude that TRIM28 is necessary for efficient effector
Therefore, TRIM28−/− T cells bear an intrinsic defect that impairs effector differentiation. To identify the transcriptional pathways deregulated in TRIM28−/− Th1 cells, we analyzed the transcriptomes of in vitro differentiated Th1 cells using Affymetrix microarrays. Genes involved in cell cycle progression and in metabolism were significantly down-regulated in TRIM28−/− T cells, compared to WT littermates (Fig. 3C). Anabolic pathways, such as “Cholesterol Biosynthesis” or “Glucose Transport and Metabolism,” were down-regulated in TRIM28−/− Th1 cells, compared to control littermates (SI Appendix, Fig. S3I). The only metabolic RNA category up-regulated in TRIM28−/− over WT Th1 cells was mitochondrial β-oxidation (SI Appendix, Fig. S3I). The switch to glucose metabolism is a hallmark of effector T cell differentiation, while naive T cells and Tregs generate their energy mainly through β-oxidation of fatty acids (1). These results suggest that the defect in effector function observed in TRIM28−/− T cells results from an impaired switch from oxidation to glycolysis.

To investigate whether TRIM28 interferes with effector function in already differentiated effector T cells or early during T cell differentiation, we used naive T cells from TRIM28flox/flox or TRIM28−/− mice crossed with tamoxifen-inducible RosaCreERT2 mice. Addition of tamoxifen in vitro, at the time of naive T cell activation, resulted in strong TRIM28 depletion in effector T cells (after 5 d of differentiation). TRIM28-depleted effector T cells, however, showed no defect in cytokine production while Foxp3 expression was still increased in Th1 cells (Fig. 3E). Therefore, impaired effector cytokine production in T cells requires early TRIM28 depletion, and cannot be obtained by delayed depletion, while the effect on Foxp3 follows TRIM28 depletion, even if it occurs at later stages (suggesting a more direct effect of TRIM28 on silencing of Foxp3 during effector differentiation).

Loss of TRIM28 Leads to Defective mTOR Signaling and Glycolysis in Autoreactive BM T Cells. To investigate whether the defect in glycolytic switch is due to defective T cell activation or TCR engagement, we first measured proliferation and blasting after stimulation of naive CD4+ T cells with αCD3/αCD28. TRIM28−/− cells displayed a significant delay in proliferation and blasting starting 24 h after stimulation (as defined by forward-scatter acquisition) (Fig. 4A and B). Transcriptomic analysis and 13C-glucose tracing experiments revealed deregulation of glucose (and lipid) metabolisms only in activated, not naive TRIM28−/− CD4 T cells (Fig. 4C and SI Appendix, Fig. S4 A–C). In activated TRIM28−/− T cells, Tricarboxylic acid cycle, β-oxidation, and fatty acid oxidation metabolisms were down-regulated in TRIM28−/− T cells. Of note, the glycolytic switch is due to a significantly smaller fraction of glycolysis-dependent metabolites such as lactate, serine, methionine, citrate, and succinate, compared to WT T cells (Fig. 4C). Bioenergetics measurements revealed a significant defect in glycolysis in TRIM28−/− T cells after 24 h (but not 4 or 0 h) of activation, compared to cells from control littersmates, while mitochondrial respiration remained unchanged (Fig. 4D and SI Appendix, Fig. S4 D and E). Defective glycolysis at 24 h was not due to increased presence of Foxp3+ cells (SI Appendix, Fig. S4F). Since glucose metabolism is mainly regulated via the mTOR pathway, we measured 5′-EFP1 phosphorylation as surrogate markers of mTOR activity. As expected, activated TRIM28−/− T cells exhibited lower levels of 4′-EFP1 and S6 phosphorylation, both in Foxp3+ and Foxp3− T cells (Fig. 4 E and F and SI Appendix, Fig. S4 G and H).

In recent years, mTOR has emerged as a central signaling hub that distinguishes effector from regulatory T cell differentiation by regulating key metabolic pathways, such as glycolysis (21). Importantly, while the absence of mTOR activity is important for Foxp3 expression and iTreg differentiation, its activity is necessary for the expansion of Tregs and maintenance of immune homeostasis in vivo. Tregs from TRIM28−/− mice exhibited reduced glycolysis and lactate production 24 h after αCD3/αCD28 activation, coinciding with a decrease in αCD3/αCD28 and IL2-dependent S6 phosphorylation and proliferation (Fig. 4 G and H). Together, these results suggest that the absence of TRIM28 impacts
Defective Signaling through CD28 in TRIM28−/− T Cells. To explain the decreased mTOR activity in TRIM28−/− T cells, we investigated signaling events proximal to TCR/CD28 stimulation. Metabolic switch to glycolysis in effector T cells is mainly driven by CD28–PI3K–AKT–mTOR signaling axis (22). Expression of CD28, but not CD3, was slightly, but significantly, reduced in TRIM28−/−, compared to control T cells (SI Appendix, Fig. S5A). To test whether defective signaling through CD28 may explain the mTOR defect, we measured the response of WT and TRIM28−/− CD4+ T cells to increasing concentrations of αCD28 (leaving the concentration of αCD3 unchanged). While S6 phosphorylation, T cell blasting (FSC), and IFNγ production were similar in WT and TRIM28−/− cells stimulated with αCD3 alone, increasing concentrations of αCD28 led to a significant increase in pS6, FSC, and IFNγ production in activated CD4+ T cells from WT, but not from TRIM28−/− mice (Fig. 5 A–C). In contrast, increased CD28 engagement in TRIM28−/−, but not in WT Th1 cells, resulted in up-regulation of Foxp3 expression (Fig. 5 D, Left). Foxp3 expression was further increased by inhibition of PI3K by Ly294002 (Fig. 5 D, Right), suggesting that CD28-dependent Foxp3 induction is achieved through an alternative, PI3K-independent pathway. In contrast, we did not observe any differences in Ca2+ signaling between WT and TRIM28−/− T cells following activation with αCD3/αCD28 or ionomycin (SI Appendix, Fig. S5B). The signaling defect was limited to stimulation through CD28, as T cell activation, S6 phosphorylation, and blasting were rescued by stimulation with PMA/ionomycin (SI Appendix, Fig. SSC). Western blot analysis of downstream signaling molecules revealed that phosphorylation of the Akt substrate Foxo1/3a, but not that of ERK1/2, downstream of CD3e signaling, was impaired in TRIM28−/−, compared to WT T cells, also consistent with a defect in signaling through the CD28–PI3K–AKT–mTOR axis (Fig. 5 E and F). PKCθ recruitment is a hallmark difference in CD28 signaling between Tregs and Tconv (23). Strikingly, αCD3/αCD28 stimulation led to decreased recruitment of PKCθ to the immune synapse in TRIM28−/− compared to WT cells (SI Appendix, Fig. S5 D and E).

Together, these results indicate that, while CD28 is active in both WT and TRIM28−/− T cells, CD28 signals through alternative pathways in TRIM28−/− T cells, leading to Foxp3 expression rather than mTOR activation. The results also show that TRIM28−/− naive T cells present differences in precocious events of activation, minutes after TCR engagement, when transcriptional or epigenetic regulation events are unlikely to have occurred. We therefore explored the possibility that the observed phenotype is due to epigenetic deregulation in naive T cells before they are activated.

TRIM28 Deficiency Reactivates Silent Regulatory Elements in Naive T Cells through H3K9 Histone Modifications. To investigate possible differences in gene expression between naive WT and TRIM28−/− cells, we analyzed their respective transcriptomes by means of Affymetrix microarrays. In total, 222 RNA species were significantly up-regulated, and 76 are down-regulated in naive TRIM28−/− T cells, compared to WT naive T cells (Fig. 6A). Differences in gene expression were statistically significant, but small (ranging between 1.2- and 10-fold). Three times more genes were up- than down-regulated, consistent with the known role of TRIM28 as a transcriptional corepressor (11).

TRIM28 functions as a molecular adaptor for gene silencing through both the polycomb and the HP1 pathways. HP1-mediated control of gene expression depends on H3K9 trimethylated (H3K9me3) and acetylated (H3K9Ac) epigenetic marks, which are associated with stable silencing and active mTOR signaling in naive and regulatory CD4 T cells and thereby interfere with effector T cell differentiation and Treg expansion.

mean ± SEM. Two-way ANOVA (A and E), one-way ANOVA (C), or Student’s t test (D) was used to calculate statistical significance. *P < 0.05; **P < 0.01; ***P < 0.001; and ****P < 0.0001.
transcription, respectively (24). To explore whether the observed transcriptional changes would be correlated to changes in H3K9 histone modifications, we performed chromatin immunoprecipitation sequencing (ChIP-seq) for H3K9me3, H3K9Ac on naive CD4+ T cells from TRIM28−/− and WT mice. We observed a significant correlation between increased acetylation and decreased trimethylation 20 to 50 kbp around differentially regulated genes (Fig. 6 B and C). Increase in acetylation and decrease in trimethylation positively correlated with changes in gene expression in TRIM28−/− T cells compared to WT littermates. H3K9Ac was significantly increased on 51 promoters and 347 distal regulatory elements (defined based on a classical H3K4me1/H3K27ac signature) around the differentially regulated genes (SI Appendix, Fig. S6A), indicating a more pronounced effect of TRIM28 depletion on distal regions and thus on potentially active enhancers, compared to promoters (Fig. 6C; P < 10−5; Fisher test).

RNA-seq and ChIP-seq for RNA Pol II revealed an increased transcription at H3K9-hyperacetylated distal regions in TRIM28−/− compared to WT CD4+ T cells (SI Appendix, Fig. S6B). This is exemplified at the TNFR1, SnaI3/Rosf166, and NR1P1 loci, which showed increased H3K9ac signals at a distal, regulatory upstream regions (E) that also correlated with decrease of the H3K9me3 signal (Fig. 6D and SI Appendix, Fig. S6C). Reanalysis of a published ChIP-seq dataset using TRIM28-specific antibodies on thymocytes revealed significant enrichment of TRIM28 peaks around transcriptionally deregulated genes (SI Appendix, Fig. S6D). We further confirmed the presence of TRIM28 at transcriptionally deregulated genes using TRIM28-specific ChIP-PCR and primers designed for TRIM28 peaks identified in S66C, negative and published positive controls (SI Appendix, Fig. S6E and refs. 17 and 18). Taken together, these results suggest that TRIM28 regulates the levels of acetylation vs. trimethylation of H3K9 at a selective set of distal regulatory elements (and promoters) of genes that are up-regulated in TRIM28-defective cells.

To investigate the nature of the link between this set of deregulated genes and the phenotype observed in TRIM28−/− T cells, we used pathway and transcription factor binding site analysis. While we did not detect any significant gene enrichment for published pathways and GO terms among differentially deregulated genes, we did detect a significant enrichment of binding sites for Foxo1, a transcription factor strongly associated with Tregs and metabolic regulation (25–28) (Fig. 6E and SI Appendix, Fig. S6F and G). Gene set enrichment analysis (GSEA) revealed that a significant proportion of differentially expressed genes overlap with a published Treg signature (Fig. 6E and F and ref. 29). Increased RNA levels of Tnfsf1b, Foh4, and Nrpl1 coincided with increased surface expression levels for TNFR II, FR4, or NR1P1 on naive CD4+ T cells (SI Appendix, Fig. S6H). Also, a similar increase in FR4 and NR1P1 was observed on naive TRIM28−/− T cells and CD4+ single- and double-positive thymocytes from mixed BM chimeras, indicating a direct, cell-intrinsic effect of TRIM28 on these genes during thymic development (SI Appendix, Fig. S6I). These results suggest that a defect in epigenetic silencing of a set of Treg-associated genes alters early T cell activation, causing defective downstream mTOR signaling and effector differentiation.

TRIM28 Regulates T Cell Activation and Differentiation through Treg-Associated Genes and Akt. NRPl1 has been shown to interfere with T cell activation by recruiting the phosphatase PTEN to the immunological synapse (30). To test whether thymic epigenetic silencing of NRPl1 is required for activation and proliferation of peripheral T cells, we overexpressed NRPl1 along with Ametrine, a fluorescent reporter gene in an OVA-specific T cell hybridoma and activated the transduced cells via OVA-pulsed dendritic cells. NRPl1-overexpressing cells proliferated less in response to dendritic cell-mediated activation compared to the Ametrine reporter transfection control, as evidenced both by less CFSE dilution and lower cell numbers up to 12 d posttransfection, relative to the Ametrine-only control (Fig. 7A–C). To further explore the contribution of overexpressed Treg genes to TRIM28-associated defects, we first sought to block Nrpl1 function in TRIM28−/− naive T cells. Using blocking antibodies against Nrpl1 and its ligand semaphorin 4A or CRISP/RCas9-mediated gene knockdown, we restored IL17, but not IFNγ production was partially restored by blocking the Nrpl1-signaling axis (Fig. 7D and E). In contrast, CRISP/RCas9 targeting of SnaI3 increased both IFNγ and IL17 production. CRISP/RCas9-mediated knockdown led to a 40 to 50% decrease in Nrpl1 surface levels as assessed by flow cytometry (SI Appendix, Fig. S7A) and 30 to 50% SnaI3 frameshift mutations (SI Appendix, Fig. S7B).
TRIM28 regulates the metabolic switch to glycolysis in naive and regulatory CD4+ T cells following TCR activation. (A and B) Histogram plots of Cell Trace Violet (CTV) dilution (A), division index (B, Left), or FSC (B, Right; used as a measure of T cell blastng), 48 h (A) or at different time points (B) after stimulating TRIM28Δ/Δ or littermate control naive CD4+ T cells with αCD3/αCD28. (C) Graphic representation of glucose-dependent metabolic pathways and scatter plots of 13C incorporation in indicated metabolites in TRIM28Δ/Δ or littermate control CD4+ T cells activated for 24 h using αCD3/αCD28. (D) Quantification of the extracellular acidification rate (ECAR) of TRIM28Δ/Δ or littermate control naive CD4+ (D) or Tregs (G) activated for 24 h using αCD3/αCD28 (D), in presence or absence of IL2 (G) as measured by the Seahorse XF96 Analyzer. (E and F) Quantification of ribosomal S6 phosphorylation at 24 h and division index 48 h after activation of naive CD4+ T cell from TRIM28Δ/Δ or littermate control mice with αCD3/αCD28 in total (E) or Foxp3+ vs. Foxp3− cells from cells stimulated in Th1 conditions (F). (H) Quantification of ribosomal S6 phosphorylation at 24 h and division index 48 h after activation of CD4+ Tregs from TRIM28Δ/Δ or littermate control mice with αCD3/αCD28 in presence of absence of IL2. (A, B, D, and H) Data are from 2 independent experiments. Two-way ANOVA (B, D, and G), Student’s t test (C and E), or paired t test (F and H) was used to determine statistical significance. *P < 0.05; **P < 0.01; ***P < 0.001; and ****P < 0.0001.

Fig. S7B) as assessed by tracking of indels by decomposition, as described previously (31).

Since NRP1 has been shown to recruit PTEN, an antagonist of the PI3K–Akt pathway, we asked whether overexpression of constitutively active Akt in TRIM28Δ/Δ CD4 T cells would rescue effector T cell differentiation. Following transduction of activated naive CD4 T cells with a construct coding for myristoylated Akt and subsequent differentiation into Th1, Th2, or Th17 effector cells, TRIM28Δ/Δ Th1 cells produced significantly more IFNγ (Th1) and IL17 (Th17) than the respective transduction control (Fig. 7 F and G and SI Appendix, Fig. S7C). Cytokine levels reached those of control vector transduced WT Th1 and Th17 cells (Fig. 7 F and G and SI Appendix, Fig. S7C). Similar findings were made for Th2 cells, although differences did not reach statistical significance (SI Appendix, Fig. S7D).

Increased cytokine responses in Th1 and Th17 cells were dependent on glucose concentrations in the medium suggesting that myr-Akt overexpression rescues effector T cell differentiation by restoring glycolysis (SI Appendix, Fig. S7E). We conclude that TRIM28 permits effector T cell differentiation due to epigenetic silencing of genes that interfere with the Akt signaling pathway. In the absence of TRIM28, restoring the Akt pathway can overcome the TRIM28-induced hyposensitiveness in a glucose-dependent manner.

TRIM28 Requirement for Effector T Cell Differentiation Is Dependent on HP1. Since transcriptional changes in TRIM28Δ/Δ T cells correlated with changes in H3K9 histone modifications, we investigated whether TRIM28 needs to interact with the H3K9me3-binding HP1 isoforms (α, β, and γ) to control the induction of glycolysis and effector/regulatory fates. We first analyzed T cell effector differentiation in mice harboring a mutated version of TRIM28 (V488L490/AA), which impairs interactions with all HP1 isoforms (32). TRIM28ΔHP1box/− mice express normal levels of the mutated TRIM28 protein (SI Appendix, Fig. S8A). While TRIM28ΔHP1box/− mice had phenotypically normal spleens, thymus, and T cell compartments (SI Appendix, Fig. S8 B–D, Left), CD4+ T cells from TRIM28ΔHP1box/− mice...
SIMULTANEOUS DISRUPTION OF HP1β AND HP1γ, however, reproduced all of the observed phenotypes of TRIM28−/− or TRIM28ΔHP1box−/− T cells (Fig. 8 A, Right). An impaired glycolytic switch, reduced blasting, and decreased mTOR activity were all observed in HP1β/γ−/−, but not HP1α−KO T cells (Fig. S8 B and SI Appendix, Fig. S8 E and F). We also detected a similar increase in Treg signature genes, in both TRIM28ΔHP1box−/− and HP1β/γ−/− but not HP1α−/− T cells, both at the RNA and protein levels (Fig. 8 C and D and SI Appendix, Fig. S8 G and H). Finally, HP1β/γ−/− or TRIM28ΔHP1box−/− BM chimeras developed autoimmune symptoms (similar to TRIM28−/− chimeras), as indicated by severe weight loss and a lack of splenic Tregs (Fig. 8 E and F). We conclude that effector T cell differentiation and Treg expansion both require the interaction of TRIM28 with HP1β/γ isoforms, establishing a direct link between the observed phenotypes and heterochromatin dynamics.

Discussion

Over the last few years, it has become clear that T cell functions are linked to specific modes of cell metabolism. While naive and memory T cells, similar to Tregs, rely mainly on oxidative respiration, differentiation to effector helper T cells, capable of active expansion and secreting high levels of cytokines, requires a “switch” to glycolysis. This glycolytic switch depends on gene expression reprogramming that occurs after TCR/CD28-mediated activation of the mTOR pathway. In this study, we unravel an unexpected level of epigenetic control of metabolic reprogramming by heterochromatin-dependent gene silencing. TRIM28 or HP1β/γ expression in early differentiating T cells represents an absolute requirement for effective switch of CD4+ T cells to glycolysis. In the absence of the heterochromatin regulators, the balance between H3K9me3 and H3K9ac is modified at regulatory enhancers at a series of genes, causing their overexpression and inhibiting glycolytic reprogramming.

It is unclear at this point which genes are direct targets of TRIM28-HP1β/γ regulation, and which genes are overexpressed as a consequence of indirect effects, as TRIM28 ChIP-seq proved to be irreproducible (in our hands) and unconvincing (in published datasets). It is also unclear whether the defective glycolytic switch is due to deregulation of a complex combination of genes, or to one individual gene among this list. While decreased H3K9me3 levels at some gene regulatory regions were decreased in TRIM28−/− cells, neither TRIM28 nor HP1 possess H3K9 trimethylation activity, suggesting that the TRIM28-HP1β/γ complex recruits a histone-methyltransferase (HMT) of H3K9. HP1 proteins can recruit the HMTs Suv39h1 or Suv39h2, while TRIM28 can recruit another H3K9 HMT, SETDB1 (33–35). Tregs from TRIM28−/− mice were equally suppressive in vitro (33–35). Our preliminary results indicate that neither Suv39h1- nor Suv39h2-defective T cells phenocopy the observed defect in effector T cell differentiation or the increased expression of the Treg signature. It is therefore most likely that other H3K9 trimethylases, such as SETDB1, are involved.

If TRIM28 is necessary for the full development of effector T cell responses, how then can TRIM28-deficient mice develop autoimmunity [as reported by others (18)]? The authors suggested a defect in the suppressive activity of TRIM28-defective Tregs, even though they did not detect differences in Treg-suppressive activity in vitro. Our results indicate that Tregs from WT and TRIM28−/− mice were equally suppressive in vitro (SI Appendix, Fig. S2F). Tregs from TRIM28−/− mice, however, did not respond to IL2 and failed to expand in vivo to suppress colitis as well as in pure and mixed BM chimeras (Fig. 2C and SI Appendix, Figs. S2 B, D, and E and S4F). Our data further suggest that this expansion defect is due to a defect in mTOR signaling and glycolytic switch (Fig. 4 E and F), which was recently suggested to be necessary for Treg expansion and thereby for their suppressive capacity in vivo (36). Thus, in addition to its role in regulating effector T cell responses, TRIM28 also regulates the expansion of regulatory T cells. A partial defect in Teff development, associated to a strong defect in Treg expansion, most likely accounts for the autoimmune
phenotype observed by others in TRIM28-deficient mice, and by us in BM chimeras (17, 18).

One of the most striking differences between WT and TRIM28 KO T cells is signaling downstream of CD28. While stimulation with αCD28 induced efficient mTOR phosphorylation and Th1 differentiation in WT T cells, it resulted in increased Foxp3 induction in TRIM28−/− T cells, even under Th1 priming conditions (Fig. 4 A). Foxp3 induction was antagonized by PI3K, as pharmacological inhibition of this kinase further increased Foxp3 expression in KO T cells, while inhibiting IFNγ expression in WT Th1 cells (Fig. 4F). Therefore, CD28 activation can lead to at least 2 different signaling pathways: one inducing PI3K–Akt–mTOR activity and favoring effector T cell differentiation, the second one leading to Foxp3 expression independently of PI3K. It is interesting to notice that TRIM28 and even HPI have been implicated as targets for phosphorylation after TCR or IL2 activation (16, 18, 37, 38). TRIM28 can also be phosphorylated downstream of Grb2, an adaptor for the intracellular domain of CD28 (39). Thus, TRIM28 acts proximal to TCR signaling, possibly through chromatin remodeling. Interestingly, Sauer et al. (40) described a TGFβ-independent pathway of Foxp3 induction that involved chromatin remodeling. The authors demonstrated that premature termination of CD3/CD28 signaling leads to Foxp3 expression in around 10% of activated cells and could further be augmented by inhibition of mTOR and PI3K. Premature termination of signaling also led to increased levels of H3K4me2/3 marks and chromatin accessibility at the promoter and 5′-untranslated region of Foxp3 and other Treg-associated genes (40). Altogether, the results suggest that, in addition to controlling the expression of critical genes in naive T cells, TRIM28 is also necessary for chromatin remodeling at enhancers of Treg-associated genes upon TCR engagement.

Previous studies suggested that hyperresponsivity to TGFβ-derived signals causes the bias toward Treg cell differentiation (17). While we confirm the overexpression of TGFβ3 mRNA, especially in Th17 cells, we found that deficient effector cell differentiation is a T cell-intrinsic defect and could not be restored by blocking TGFβ present in the culture (SI Appendix, Fig. S1 D and E). Furthermore, the transcriptional Treg signature was enriched in TCR signaling- or Foxp3-dependent genes, but not in genes related to TGFβ signaling. Interestingly, we could not detect any changes in H3K9me3, H3K9Ac, or Pol II proximal to the Foxp3 gene (SI Appendix, Fig. S7C), and its expression was not significantly changed between WT and KO naive T cells up to 24 h postactivation (SI Appendix, Fig. S4F).

These results also suggest that TRIM28 is an important epigenetic regulator during thymic development. Zhou et al. (16) showed that TRIM28 intervenes in TCR rearrangement, and Chikuma et al. (18) found severe defects in thymus development when TRIM28 was deleted at the CD4+/CD8+ stage. Disruption of TRIM28 at the CD4+/CD8+ stage of thymocyte differentiation (in our case) does not lead to abnormal T cell development. However, silencing of Treg-associated genes, such as Nrp1, already occurs in the thymus and might be a prerequisite for the development of naive CD4+ T cells with the full potential to differentiate into Treg or Teff in the periphery. Supporting this notion, overexpression of NRPI in a T cell hybridoma strongly reduced the proliferative response to antigenic stimulation by dendritic cells (Fig. 7 A–C), while Nrp1 inactivation in naive TRIM28−/− T cells partially restored cytokine secretion. Interestingly, genetic ablation of the Treg-associated genes Nrp1 or Snai3 had different outcomes. Indeed, Snai3 deletion partially restores cytokine secretion both in Th1 and Th17 conditions, while Nrp1 inactivation only had an effect on IL17 production. This suggests that the different Treg-associated genes overexpressed in TRIM28−/− T cells contribute by various mechanisms to the observed defects. Full phenotype restoration is thus likely to depend on targeting multiple genes simultaneously.
Since the overexpression of a constitutively active form of Akt in TRIM28−/− CD4 T cells rescued their ability to differentiate into cytokine-expressing effectors, and since NRP1 has been shown to interfere with the PI3K–Akt pathway by recruiting the phosphatase PTEN (30), we believe that TRIM28 regulates peripheral T cell activation by epigenetically silencing antagonists of the PI3K–Akt–mTOR pathway during thymic development.

Altogether, this work unravels an unexpected, mechanism of control of effector T cell responses through epigenetic imprinting in thymocytes and/or naive T cells. Whether this process of “imprinting” is physiologically controlled during infections, chronic inflammation or aging will be a fascinating subject for future studies.

Methods

For detailed information and protocols, see SI Appendix.

In Vitro T Cell Activation and Differentiation. To isolate naive T cells for in vitro assays, secondary lymphoid organs were collected, pooled, and homogenized into single-cell suspensions. Naive T cells were enriched using magnetic and fluorescence-activated cell sorting (MACS and FACS) of naive CD4+CD62L+CD25−CD44− and CD8+CD62L+CD44− T cells using a FACSAria II (BD Biosciences).

Sorted, naive T cells were stained with Cell Trace Violet (Thermo Fisher Scientific) according to the manufacturer’s instructions, stimulated on αCD3 (BD Biosciences)-coated flat-bottomed 96-well plates (Corning) with soluble αCD28 (BD Biosciences) in the absence or presence of T cell differentiation mixtures for Th1, Th2, Th17, or Treg.

In Vivo Differentiation Experiments. Naive WT or TRIM28-deficient CD4+ OT-II-GFP cells were isolated using a CD4+ T cell enrichment kits (Thermo Fisher Scientific) according to the manufacturer’s instructions, stimulated on αCD3 (BD Biosciences)-coated flat-bottomed 96-well plates (Corning) with soluble αCD28 (BD Biosciences) in the absence or presence of T cell differentiation mixtures for Th1, Th2, Th17, or Treg.

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In Vivo Differentiation Experiments. Naive WT or TRIM28-deficient CD4+ OT-II-GFP cells were isolated using a CD4+ T cell enrichment kits (Thermo Fisher Scientific) as described above, followed by FACS sorting and staining with Cell Trace Violet as described above. On day −1, 5 × 106 sorted naive T cells were injected i.v. into C57BL/6 recipient mice followed by s.c. immunization with 50 μg of OVA in CFA (Thermo Fisher Scientific) into the left flank on day 0. Four and 8 d later, mice were killed and draining lymph nodes and spleen were removed for analysis of OT-II proliferation (division index based on Cell Trace Violet dilution) and cytokine production. Animal care and use for this study were performed in accordance with the recommendations of the
European Community (2010/63/UE) for the care and use of laboratory animals. Experimental procedures were specifically approved by the ethics committee of the Institut Curie CEEA-IC #118 (CEEA-IC 2014-03 and CEEA-IC 2017-006) in compliance with the international guidelines.

**T Cell Transfer Colitis.** Transfer colitis was performed similar as reported by Powrie et al. (20).

**BM Chimeras.** BM was prepared from tibia and femur of TRIM28−/−, littermate control, or CD45.1 congenic mice, as described previously (41).

**Seahorse Assay.** Sorted, naive CD4+ and CD8+ T cells were activated in vitro using plate-bound αCD3 and soluble αCD28 for 24 h as described above. Activated T cells were harvested, counted, and washed, and resuspended in assay medium (Seahorse Biosciences). Assay plates (Seahorse Biosciences) were coated with Cell-Tak (Corning) at 22.4 μg/mL in PBS for 1 h before cells were plated at a density of 2 × 10^5 cells per well according to the manufacturer's instructions. Mitochondrial respiration and glycolysis were measured using Cell Mito Stress Kit and Glycolysis Stress Kit (both Seahorse Biosciences) according to the manufacturer's instructions.

**Immunohistochemistry and Microscopy.** Immunohistochemistry of colon samples. Swiss roll samples were fixed in 4% formaldehyde, and 4-μm-thick slices were cut and embedded in paraffin for further immunohistochemical analysis. CD3 (clone 145-2C11) staining was performed at pH9 (Dako S2367) using a 1:100 dilution for 1 h at room temperature. Staining was revealed using an Elite ABC kit (Vector Laboratories) using diaminobenzidine for 10 min. Nuclei were stained using diluted Harris hematoxylin for 1 min. All immunostainings were performed on a LabVision Autostainer (Thermo Fisher Scientific).

**RNA Isolation and Analysis by qPCR or NanoString nCounter.** RNA was isolated from naive, activated, or differentiated effector or regulatory cells using the miRNeasy isolation kit (Qiagen) according to the manufacturer's instructions. For qPCR, 200 ng of RNA was transcribed into cDNA using reverse transcriptase (Thermo Fisher Scientific) according to the manufacturer's instructions. qPCR was performed using 5 ng of template cDNA, H2O, Sybr Green (Roche), and specific primers on a LightCycler 480 (Roche) (45 cycles, 60 °C annealing temperature). The following primer sequences were used for not normally distributed data. For paired samples (e.g., data from mixed BM chimera), paired t test or Wilcoxon test was used, depending on the type of distribution. Data from kinetic experiments were analyzed using 2-way ANOVA using Bonferroni’s multiple-test correction. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Statistical analysis was performed using GraphPad software (GraphPad Software).

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**Transduction and Protein Overexpression in CD4 T Cells.** T cell transduction protocol was performed similar to Singh et al. (45). Cytokine production was measured 2 d after transduction. See SI Appendix for detailed information.

**Statistical Analysis.** Student’s t test and one-way ANOVA were used for normally distributed data, while Mann–Whitney U or Kruskal–Wallis tests were used for not normally distributed data. For paired samples (e.g., data from mixed BM chimeras), paired t test or Wilcoxon test was used, depending on the type of distribution. Data from kinetic experiments were analyzed using 2-way ANOVA using Bonferroni’s multiple-test correction. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Statistical analysis was performed using GraphPad software (GraphPad Software).

**Chromatin Immunoprecipitation.** Chromatin immunoprecipitation was performed similar as previously published (9). Data is deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE140448).

**CRISPR/Cas9 Deletion in Naive CD4 T Cells.** CRISPR/Cas9-based deletion of NRP1 and Snai3 in naive CD4 T cells was performed as described in ref. 44.
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