Immunity

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Previews

Foxp3 Re-distributes Its Heavy Lifting

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https://doi.org/10.1016/j.immuni.2020.10.021

Understanding the mechanisms that establish regulatory T (Treg) cell identity is central to understanding Treg cell function. van der Veeken et al. now show that the lineage-determining transcription factor Foxp3 establishes Treg-cell-specific chromatin architecture indirectly, mostly by decreasing the expression of other transcriptional regulators, including TCF1.

Regulatory T (Treg) cells are characterized by the expression of the forkhead family transcription factor Foxp3, and loss of Foxp3 leads to non-functional Treg cells and autoimmunity (Josefowicz et al., 2012). Despite the definite role of Foxp3 in Treg cell function, the mechanisms through which this transcription factor controls Treg cells remain unclear. In most developmental programs, lineage-determining transcription factors pervasively bind to thousands of genomic regions, creating accessible chromatin regions and enabling the biological activity of other transcription factors. But Foxp3 does not follow this strategy. Instead, Foxp3 binds predominantly to genomic regions that have pre-established chromatin accessibility in conventional CD4^{+} T cells (Samstein et al., 2012). Although a small portion of Foxp3 targets acquire Treg-specific chromatin re-modeling, thousands of *de novo* open chromatin regions are not directly bound by Foxp3. Thus, Foxp3 is special among lineage-determining transcription factors, as it may indirectly determine cell fate. A better understanding of the direct versus indirect effect of Foxp3 on transcriptional and epigenomic landscapes of Treg cells can help us define how Treg-specific genes, which are also mostly dependent on Foxp3 expression, are regulated. Insight into mechanisms determining Treg identity may also shed light into Treg cell function. In this issue of *Immunity*, van der Veeken et al. (2020) reveal that Foxp3 establishes Treg-cell-specific chromatin landscape indirectly by modulating levels of TCF1 and other chromatin remodeling factors.

To rigorously dissect the direct versus indirect effects of Foxp3, van der Veeken et al. elegantly designed their study on the genetically heterozygous F1 (C57BL/6$^{\text{Foxp3-GFP-DTR/GFP-KO}}$ X Cast/EiJ$^{\text{GFP-WT/Y}}$) hybrid mice, which include around 20 million allelic variants derived from the parental strains. One can think of these genomic experiments as performing 20 million allele-specific genome-editing experiments in Treg cells at the same time! Relying on natural genetic variations can disentangle the *cis* versus *trans* effect of sequence polymorphisms on chromatin landscape and gene expression profiles if measurements are performed in cells of F1 offspring. By accurately aligning sequences of F1 cells back to parental genomes, van der Veeken et al. were able to assess how sequence variation between two alleles affected Foxp3 binding, chromatin accessibility, and gene expression. These genetic strategies allowed the authors to simultaneously determine how gain or loss of transcription factor binding due to natural genetic variation within cognate motifs can alter the chromatin landscape in presence or absence of Foxp3. When allele-specific chromatin accessibility was measured experimentally and linked to disruption of Foxp3 binding and Foxp3 motif due to nucleotide differences between two alleles, it was suggested that Foxp3 had a direct effect. However, when allele-specific chromatin accessibility was not linked to disruption of Foxp3 binding, it was suggested that Foxp3 had an indirect effect.

To identify Treg-cell-specific chromatin accessibility and gene expression signatures, the authors first used ATAC sequencing (ATAC-seq) and RNA sequencing (RNA-seq) on resting CD44$^{\text{lo}}$ CD62L$^{\text{hi}}$ and activated CD44$^{\text{hi}}$CD62L$^{\text{lo}}$ GFP^{+} Treg and GFP^{-} conventional CD4 T (Tcon) cells. These efforts led to the identification of a common set of T cell activation genes and a smaller subset of Treg-cell-specific deregulated genes, including *Samhd1*, *Ikzf4*, *Gpr83*, *Tcf7*, *Il7r*, *Themis*, *Cd40lg*, and *Pde3b*. Next, they leveraged known difference in C57BL/6 and Cast genomes and examined disruption in which transcription factor binding sites, due to nucleotide differences between alleles, can be linked to gain or loss in chromatin accessibility. A comprehensive and unbiased analysis of the effects of known transcription factor binding motifs on chromatin accessibility suggested that most changes in recognition motifs influencing local chromatin states acted as positive regulators: i.e., disruption in recognition site due to nucleotide difference in one allele led to loss of chromatin accessibility in the allele. Motifs with a negative effect on accessibility were the exception and included motifs for specific zinc finger transcription factor, such as Ikzf1 and YY1. This large-scale mutagenesis approach revealed that variations within Forkhead motif did not have significant effects on Treg-cell-specific accessible chromatin regions. Strikingly, variants within binding sites of high-mobility group (HMG) proteins, also referred to as Sox motif, affected Treg-cell-specific chromatin accessibility. Furthermore, protein levels of the HMG transcription factor TCF1 correlated with changes in Treg-cell-specific

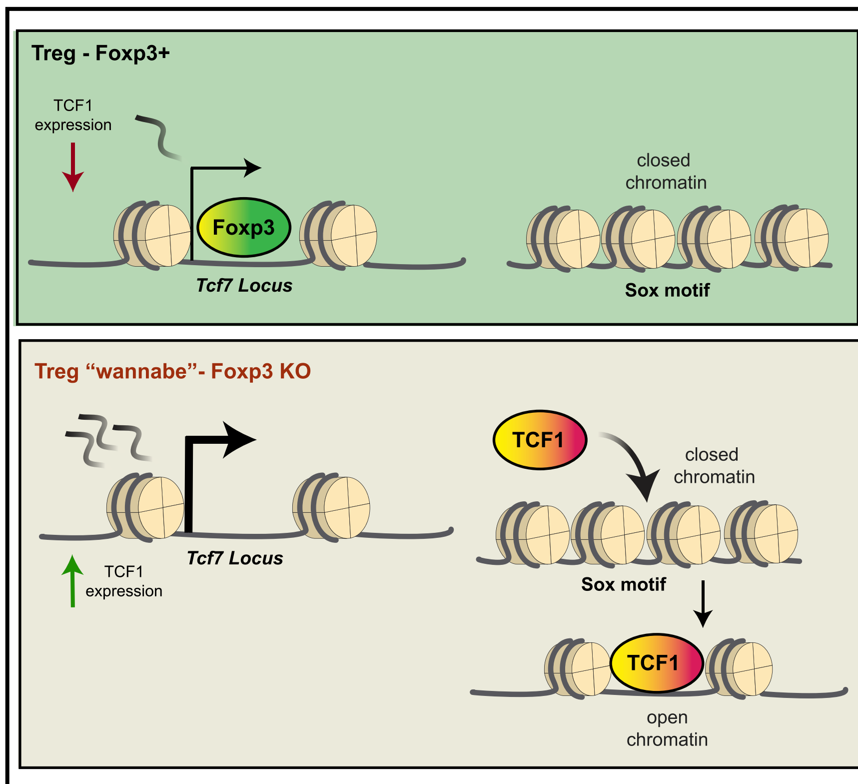

Figure 1. Foxp3 Indirect Modulation of Treg Cell Chromatin via Alteration of other Transcriptional Regulators, Including TCF1
In normal Tregs (top panel) Foxp3 binds to the *Tcf7* locus and lowers TCF1 expression; as a result, chromatin regions enriched for TCF1's recognition motif (sox motif) remain closed. This results in Foxp3 indirectly regulating the Treg cell chromatin landscape. In Foxp3 deficient "wannabe" Treg cells (bottom panel), TCF1 levels increase due to absence of Foxp3 and chromatin regions with sox motif become accessible.

chromatin accessibility. TCF1 controls T cell fate through its widespread ability to target silent chromatin, establishing large-scale accessible chromatin landscape in T cells (Johnson et al., 2018). Thus, relatively modest decrease in TCF1 amounts may lead to large-scale loss of chromatin accessibility, thus regulating Treg-cell-specific chromatin accessibility.

To assess how Foxp3 affects the Treg cell epigenome, the authors narrowed down on the effects of genetic variants and chromatin accessibility at Foxp3 binding sites. Analysis of Foxp3 direct binding by chromatin immunoprecipitation sequencing (ChIP-seq) and CUT&RUN (Skene and Henikoff, 2017) showed that genetic variants that disrupted the Forkhead motif impaired Foxp3 binding, while ETS motif also had some effect. However, variations within Forkhead motif did not affect Foxp3-bound chromatin accessibility, while variants in motifs of ETS, IRF, and bZIP strongly affected the chromatin accessibility landscape of Treg cells. Thus, Foxp3 plays a minor role in directly altering the regulatory landscape of Treg cells. To examine how Foxp3 brings about Treg-cell-specific accessibility and gene expression patterns, the authors used ATAC-seq and RNA-seq to compare Treg cells derived from healthy $Foxp3^{GFP\text{-}DTR/WT}$ and $Foxp3^{GFP\text{-}KO/WT}$ F1 females. Foxp3 deficiency affected both chromatin accessibility and gene expression more prominently in activated cells than resting ones and in mature Treg cells compared to early thymic $CD73^{-}$ cells. However, most of these genomic loci were not directly bound by Foxp3, indicating that Foxp3-dependent accessibility and expression changes were mostly mediated by Foxp3-dependent *trans*-regulatory factor.

In search of these *trans*-regulators, transcription factor motif analysis revealed enrichment of HMG (also referred to as Sox) family recognition sites in chromatin regions with reduced accessibility in presence of Foxp3 in resting wild-type (WT) Treg cells as compared to resting Foxp3 deficient cells. Foxp3 bound directly to the *Tcf7* locus, with corresponding decrease in TCF1expression (Figure 1). Mapping TCF1 binding events by CUT&RUN revealed strong allelic bias and higher TCF1 binding in Foxp3-deficient cells. Comparing ATAC-seq peaks, the authors observed TCF1 binding to around 50% of sites that had reduced chromatin accessibility in presence of Foxp3, while only 15% of sites with gained accessibility were occupied by TCF1. Thus, regulation of TCF1 level by Foxp3 enabled Foxp3 to establish Treg-cell-specific repressed chromatin accessibility and gene expression patterns. Together, about half of Foxp3-dependent chromatin repression in Treg cells could be explained by the decreased in TCF1. Importantly, although deleting one copy of *Tcf7* could not prevent autoimmune phenotype of the mice, mapping chromatin accessibility by ATAC-seq showed some degree of loss in chromatin accessibility at same regions previously bound by TCF1 in WT Treg cells.

This observation raised the possibility that other transcription factors may also mediate Foxp3 effects in Treg cells. The authors first considered the transcription factor Lef1 as the possible candidate. Lef1 expression was decreased in Treg cells as compared to Tcon or Foxp3 deficient cells and CUT&RUN experiments revealed that around 90% of TCF1 bound sites also overlapped with Lef1 binding. Treg-cell-specific accessible chromatin regions were also enriched for bZIP and AP1-IRF motifs. CUT&RUN analysis of bZIP and IRF family transcription factors Jun and IRF4 revealed that sites bound by only these factors, and not TCF1, had higher accessibility in Treg cells, suggesting contrasting Foxp3-mediated alteration in chromatin accessibility at TCF1/Lef1 and Jun/IRF4 binding sites. Overall, the work of van der Veeken et al. reveals that although Foxp3 is required for Treg function, the molecular processes through which Foxp3 controls the epigenome of Tregs is indirect. Foxp3 modulates the levels of TCF1 and other transcription factor to alter the chromatin state and establish Treg cell identity.

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Despite these findings, it still remains unanswered how Treg-specific epigenome is established in contrast to many other T cell programs, which also restrict expression levels of proteins such as TCF1. For example, T cell activation also downregulates TCF1, and similar to this study, the HMG motif is the most enriched recognition site in genomic regions that lose accessibility in effector T cells compared with naive T cells (Scott-Browne et al., 2016). The shared and unique aspects of TCF1 mediating chromatin closing in these two contexts remains to be determined. Interestingly, TCF1 can bind to Foxp3 promoter in both human and mice, repressing Foxp3 in CD4$^+$ T conventional cells (Delacher et al., 2020). Treg-cell-specific deletion of *Tcf7* and *Lef1* can also cause spontaneous autoimmunity (Xing et al., 2019). Although these findings suggest that TCF1 and LEF1 activity must be maintained within a certain range to allow proper Treg cell functionality, how the balance between these transcription factors is regulated in T conventional and Treg cells will be a fascinating point of future study. Identifying how other Foxp3 modulated factors enforce Treg identity will aid not only in understanding Treg biology but also in understanding the mechanism of action and regulatory role of other late acting transcription factors on cell identity. Although chromatin accessibility is a key feature of cell fate determination, it remains to be shown if Foxp3 can control some other epigenomic features, such as unknown histone modifications necessary for Treg cell fate. Exciting work lies ahead to fully define the combinatorial action of Foxp3 and other transcription factors in determining Treg cell identity.

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The Dueling Duo: IL10 and TNF Face Off in Microglial Recovery from Endotoxin Challenge

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https://doi.org/10.1016/j.immuni.2020.10.016

The molecular mechanisms that restore microglial quiescence after acute stimulation remain largely unexplored, unlike those that drive microglial activation. In this issue of *Immunity*, Shemer et al. discover that the microglial IL-10 receptor counteracts the pro-inflammatory effects of TNF to allow restoration of microglial quiescence after peripheral endotoxin challenge.

While disease-modulating functions of microglia in septic shock have been an area of research for several decades, how microglia return to quiescent, homeostatic states is not well understood. Microglia exhibit distinct transcriptome signatures depending on the inflammatory model, for example the "damage-associated microglia" or "microglia associated with neurodegeneration" profiles in neurodegenerative pathologies (Keren-Shaul et al., 2017; Krasemann et al., 2017) that contrast with those of acute neuroinflammatory models like experimental autoimmune encephalitis (EAE) or endotoxin challenge (Salter and Stevens, 2017). Persistent and non-resolving microglial inflammatory responses cause