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## TCR signaling fuels T<sub>reg</sub> cell suppressor function

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### Abstract

Acute ablation of T cell antigen receptors (TCRs) in regulatory T cells (T<sub>reg</sub> cells) impairs the suppressive activity of these cells, even though they retain expression of Foxp3 and CD25. TCR signaling imparts a critical role in the suppressive function of T<sub>reg</sub> cells.

The development of Foxp3<sup>+</sup> regulatory T cells (T<sub>reg</sub> cells) in the thymus is dependent on signaling via the T cell antigen receptor (TCR)<sup>1</sup>, but the TCR's role in the function of T<sub>reg</sub> cells in the periphery remains elusive. As acute ablation of T<sub>reg</sub> cells from the normal adult mouse results in the rapid development of a lethal autoimmune syndrome<sup>2</sup>, it is likely that the suppressor function of T<sub>reg</sub> cells is constantly operative *in vivo*, yet evidence for TCR-mediated signaling in this process has been lacking. Levine *et al.*<sup>3</sup> now provide, for the first time, compelling data indicating that continuous expression of the TCR is required for T<sub>reg</sub> cell suppressor function *in vivo*.

The major tool used in this study is deletion of the TCR from peripheral T<sub>reg</sub> cells by administration of tamoxifen to adult mice carrying *Foxp3*<sup>eGFP-Cre-ERT2</sup> (a fusion expressing enhanced green fluorescent protein, Cre recombinase and the estrogen receptor ligand-binding domain protein) that have been crossed to *Trac*<sup>FL/WT</sup> or *Trac*<sup>FL/FL</sup> mice, in which one or both alleles of *Tcra*, specifically the gene segment (*Trac*) encoding the constant region of the TCR  $\alpha$ -chain, were flanked by two *loxP* sites. A minor population (~20%) of TCR-deficient T<sub>reg</sub> cells was generated in the heterozygous cross, whereas in mice descended from the homozygous cross the majority (~70%) of T<sub>reg</sub> cells lacked the TCR. In addition, the authors bred *Trac*<sup>FL/FL</sup> mice to *Foxp3*<sup>YFP-Cre</sup> mice to induce TCR deletion in newly generated T<sub>reg</sub> cells. In all of these models, most of the T<sub>reg</sub> cells that lacked the TCR expressed normal amounts of Foxp3 as well as other T<sub>reg</sub> signature molecules such as CD25, GITR, CD39 and CD73. However, CTLA-4 expression is induced by TCR signaling, as T<sub>reg</sub> cells with TCR ablation induced by *Foxp3*<sup>YFP-Cre</sup> failed to upregulate CTLA-4. Both *Ctla4* mRNA and CTLA-4 protein were also reduced when the *Tcra* gene was deleted by *Foxp3*<sup>eGFP-Cre-ERT2</sup> after tamoxifen treatment, indicating that CTLA-4 expression in T<sub>reg</sub> cells requires continuous TCR signaling. No evidence that loss of the TCR resulted in loss of Foxp3 expression was seen in fate-mapping studies.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

The authors also report that just as in the conventional CD4<sup>+</sup> T cell compartment, T<sub>reg</sub> cells consist of CD44<sup>lo</sup>CD62L<sup>hi</sup> 'naive-like' and CD44<sup>hi</sup>CD62L<sup>lo</sup> 'effector-like' populations. The most striking finding in this study was that the total numbers of TCR-negative T<sub>reg</sub> cells in the two inducible deletion models (*Foxp3*<sup>eGFP-Cre-ERT2</sup> *Trac*<sup>FL/WT</sup> and *Foxp3*<sup>eGFP-Cre-ERT2</sup> *Trac*<sup>FL/FL</sup>, of which only the latter resulted in autoimmunity) were essentially normal, but ablation of the TCR resulted in a marked loss of the T<sub>reg</sub> 'effector-like' cell population. On the other hand, *Foxp3*<sup>YFP-Cre</sup>-mediated deletion of *Tcra* prevented the generation of CD44<sup>hi</sup>CD62L<sup>lo</sup> 'effector-like' T<sub>reg</sub> cells. This finding was also correlated with decreased proliferation and severe reduction in cell numbers of the TCR-negative T<sub>reg</sub> cell population. These results are most consistent with the requirement of a TCR signal for the differentiation as well as maintenance of T<sub>reg</sub> 'effector-like' cells (Fig. 1). While reduction of CD44<sup>hi</sup> T<sub>reg</sub> cells resulted in generalized activation of conventional CD4<sup>+</sup> or CD8<sup>+</sup> T cells in *Foxp3*<sup>eGFP-Cre-ERT2</sup> *Trac*<sup>FL/FL</sup> mice, *Foxp3*<sup>YFP-Cre</sup> *Trac*<sup>FL/FL</sup> mice that completely lacked the CD44<sup>hi</sup> T<sub>reg</sub> cell population developed severe autoimmunity and were moribund by day 13 of life. Thus, the suppressive functions of T<sub>reg</sub> cells are completely dependent on TCR signaling *in vivo*. Although interleukin 2 (IL-2) is required for the 'fitness' of T<sub>reg</sub> cells<sup>4</sup>, IL-2 signaling was normal in TCR-deficient T<sub>reg</sub> cells as measured *ex vivo* through STAT5 phosphorylation. Thus, IL-2 signaling in T<sub>reg</sub> cells does not require TCR activation. Furthermore, administration of IL-2 immune complexes did not reduce the activation of conventional CD4<sup>+</sup> T cells in the *Foxp3*<sup>eGFP-Cre-ERT2</sup> *Trac*<sup>FL/FL</sup> mice.

Levine *et al.* also compared gene expression by TCR-sufficient and TCR-deficient T<sub>reg</sub> cells isolated from *Foxp3*<sup>eGFP-Cre-ERT2</sup> *Trac*<sup>FL/FL</sup> mice<sup>3</sup>. Within the 'effector-like' T<sub>reg</sub> cell subset, 155 genes were significantly downregulated in the absence of the TCR, including those encoding transcription factors (NFATc1, c-Rel, IRF4 and Bcl-6) and inhibitory molecules (CD83, CD200, LAG-3, IL-10 and EBI3). Levine *et al.* raise the possibility that IRF4 has a critical role in T<sub>reg</sub> 'effector-like' cell differentiation downstream from TCR signals<sup>3</sup>. The transcription factor IRF4 is also highly upregulated by TCR stimulation in effector T cells. In fact, IRF4 plays a critical role in the differentiation of T<sub>H</sub>2, T<sub>H</sub>17 and follicular helper T cells (T<sub>FH</sub> cells) through its interaction with members of the BATF protein family<sup>5</sup>. In T<sub>reg</sub> cells, IRF4 interacts with Foxp3 and regulates ~20% of 'T<sub>reg</sub> cell-specific' genes, and specific deletion of *Irf4* in T<sub>reg</sub> cells by Foxp3-Cre results in severe autoimmune lymphoproliferative diseases with uncontrolled T<sub>H</sub>2 responses<sup>6</sup>. IRF4 affects the expression of IL-10, ICOS and CCR8, but not CD25, GITR and CLTA-4. However, in the current study inducible deletion of *Irf4* in mature T<sub>reg</sub> cells produced only a very modest effect. Although this small effect size may have resulted from inefficient ablation of IRF4, it is also possible that IRF4 may not be very critical for maintaining the activation status of T<sub>reg</sub> cells. To fully determine the contribution of an IRF4-mediated positive feedforward regulatory loop in the activation of T<sub>reg</sub> cells by the TCR, it is necessary to compare the genes regulated by IRF4 with those regulated by TCR activation. Interestingly, the genes that depend on continuous TCR signaling for their expression are largely regulated by Foxp3. Although Foxp3 by itself is not sufficient to induce and/or maintain the expression of these TCR-inducible genes, Foxp3 is needed to collaborate with TCR signaling to promote gene expression in 'effector-like' T<sub>reg</sub> cells.

Several questions raised by this work merit further investigation. Although it is clear from this study that TCR activation is required for the generation of 'effector-like' T<sub>reg</sub> cells with suppressor function and that CD44<sup>hi</sup> T<sub>reg</sub> cells with TCR ablation have changed their gene expression pattern, the requirements for TCR signaling for the maintenance of the CD44<sup>hi</sup>CD62L<sup>lo</sup> T<sub>reg</sub> cell population *in vivo* remain to be further elucidated. Approximately 50% of this subpopulation cycles every 2–3 days *in vivo*, as measured by Ki67 staining. Yet it is unknown whether TCR-derived signals play any role in this process, which must also include the death of a major proportion of the cycling cells, as absolute numbers of T<sub>reg</sub> cells remain constant for the life of the animal. In this regard, the cycling of T<sub>reg</sub> cells *in vivo* closely resembles the cycling of conventional memory phenotype CD44<sup>hi</sup>CD4<sup>+</sup> T cells<sup>7</sup>, and the factors that regulate the proliferation of CD44<sup>hi</sup>CD4<sup>+</sup> T cells also remain to be fully characterized. Normal adult thymus does contain a population of CD44<sup>hi</sup> T<sub>reg</sub> cells that cycle at a frequency slightly less than that of CD44<sup>hi</sup> T<sub>reg</sub> cells in the periphery (M. Holt and E.M.S., unpublished observations). It is thus possible that there are two waves of TCR-induced activation of T<sub>reg</sub> cells, with the first wave, in the thymus, resulting in partial T cell activation and the second wave, in the periphery, inducing suppressive activity. It will be important to determine whether T<sub>reg</sub> cells activated in the thymus already express suppression-related genes or whether these genes need to be induced in the periphery by TCR stimulation.

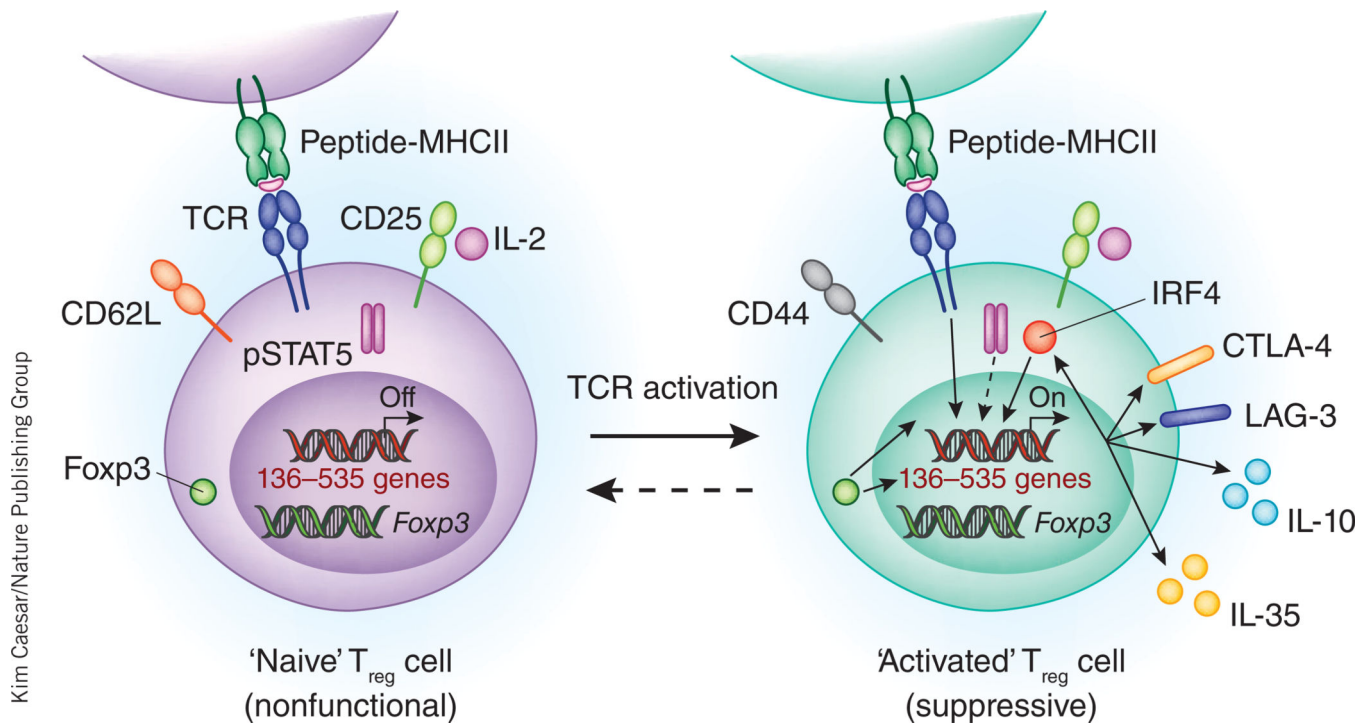
Studies performed *in vitro* have suggested that the suppressor function of T<sub>reg</sub> cells requires TCR activation but that, once activated, suppressor function is nonspecific<sup>8</sup>. Does a similar situation exist *in vivo*? It also remains unclear whether upon TCR activation, CD44<sup>lo</sup>CD62L<sup>hi</sup> 'naive-like' T<sub>reg</sub> cells can turn into CD44<sup>hi</sup>CD62L<sup>lo</sup> 'effector-like' T<sub>reg</sub> cells. In the future, it will be important to understand the dynamics between these two states. What is the rate of such transition at steady state? Are the TCR repertoires of these two populations the same, or is the CD44<sup>hi</sup> CD62L<sup>lo</sup> 'effector-like' population more self-reactive? Can CD44<sup>hi</sup>CD62L<sup>low</sup> 'effector-like' T<sub>reg</sub> cells revert to CD44<sup>lo</sup>CD62L<sup>hi</sup> 'naive-like' T<sub>reg</sub> cells or are they terminally differentiated?

An additional issue raised by these studies is how the results might influence the design of therapies to maximize the abundance of T<sub>reg</sub> cells or, more importantly, their suppressive function *in vivo*. For example, would blocking the TCR specifically on T<sub>reg</sub> cells (if possible) impair their function and represent an adjunct to tumor therapy? At present, low-dose IL-2 therapy has been advocated as a potentially safe, effective approach to augmenting T<sub>reg</sub> cell function in a variety of experimental studies and human clinical trials<sup>9</sup>. Yet the target of low-dose IL-2 would be the nonsuppressive CD44<sup>lo</sup>CD62L<sup>hi</sup> population that expresses large amounts of CD25 (ref. 10). Do the beneficial effects of this therapy depend on simultaneous TCR activation and differentiation of the CD44<sup>lo</sup>CD62L<sup>hi</sup> T<sub>reg</sub> cells to 'suppressor effector-like' CD44<sup>hi</sup>CD62L<sup>lo</sup> T<sub>reg</sub> cells? The failure of IL-2 immune complexes to reverse the activation of conventional CD4<sup>+</sup> T cells in the present study suggests that this is unlikely to be the mechanism. Conversely, how can we directly augment the numbers and function of the CD44<sup>hi</sup>CD62L<sup>lo</sup> 'effector-like' T<sub>reg</sub> cells? Smigielski *et al.* suggest that their maintenance depends of ICOS-ICOSL signaling<sup>10</sup>, but this pathway does not appear to mediate their proliferation, as inhibition of ICOS signaling did not reduce the

cycling of the CD44<sup>hi</sup>CD62L<sup>lo</sup> T<sub>reg</sub> cells. Lastly, one potential alternative approach for the expansion of 'effector-like' T<sub>reg</sub> cells may be the delivery of costimulation in the absence of an overt TCR signal—for example, the delivery of CD28-mediated costimulation with a CD28 superagonist monoclonal antibody<sup>11</sup>. It remains to be determined whether this type of stimulation would selectively expand CD44<sup>hi</sup>CD62L<sup>lo</sup> T<sub>reg</sub> cells.

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**Figure 1.**

T<sub>reg</sub> cells require continuous TCR stimulation to maintain their activated phenotype and suppressive activities *in vivo*. Upon TCR activation presumably by self-peptide, CD44<sup>lo</sup>CD62L<sup>hi</sup> 'naive' T<sub>reg</sub> cells may become CD44<sup>hi</sup>CD62L<sup>lo</sup> 'activated' T<sub>reg</sub> cells. Up to 535 genes are induced during this process. Among them, at least 136 genes require continuous TCR stimulation for their expression. The 136 genes encode many important molecules, including LAG-3 and IL-10, which are involved in exerting the suppressive functions of T<sub>reg</sub> cells. Consequently, inducible ablation of the TCR in T<sub>reg</sub> cells results in loss of suppressor function and autoimmunity. Induction of these 136 genes by TCR activation requires Foxp3 and is partially reinforced by IRF4-mediated positive feedforward regulation. Although continuous TCR signaling in T<sub>reg</sub> cells is essential for their suppressive function, it is not required for Foxp3 expression, IL-2 signaling or the expression of many T<sub>reg</sub> 'signature' molecules such as CD25 and GITR. Whether activated T<sub>reg</sub> cells will return to the naive state upon cessation of TCR signaling is unknown.