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Retinoic Acid Can Directly Promote TGF-β-Mediated Foxp3+ Treg Cell Conversion of Naive T Cells

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The article by Hill et al. (2008), published in the November 14, 2008 issue of *Immunity*, describes a mechanism by which retinoic acid (RA) enhances TGF-β-induced Foxp3 expression. The authors propose that RA does not act directly on naive T cells during activation in culture but rather indirectly via negative regulation of an accompanying population of effector or memory $CD4^+$ $CD44^{hi}$ cells. They reasoned that the increased generation of $Foxp3^+$ cells in response to RA in culture, as described previously (Coombes et al., 2007; Elias et al., 2008; Mucida et al., 2007; Sun et al., 2007; Xiao et al., 2008), represented the lifting by RA of inhibition imparted by accompanying $CD4^+CD44^{hi}$ T cells, rather than by direct or indirect effects of RA on the Foxp3 expression of the primed naive T cells themselves.

In order to assess the effects of RA on naive T cells in the absence of accompanying $CD4^+CD44^{hi}$ T cells, we sorted ($CD4^+CD25^-CD44^{low}$ $CD62L^+$) GFP^- T cells (more than 99.7% purity) from Foxp3-eGFP reporter mice (Figure S1A available online) by flow cytometry. After 4 days of stimulation with anti-CD3 and anti-CD28, we stained CD4 cells with 7AAD to exclude dead cells; additionally, forward and side scatter (area versus width) was used to exclude doublets, and we evaluated Foxp3 expression via GFP staining. Addition of RA enhanced Foxp3 induction more than 50% by use of 1 or 10 ng/ml doses of TGF-β (Figure S1B). Because the sorting efficiency is not 100%, it is possible that extremely low numbers of "accompanying" memory or effector cells could still influence these results. To exclude this possibility, we used FACS-sorted $CD4^+CD25^-CD44^{lo}$ $CD62L^+$ T cells, isolated from B7-1 and B7-2 double-deficient mice ($Cd80^{-/-}$ $Cd86^{-/-}$), which even before sorting already contain less than 5% of memory or effector $CD44^{hi}$ cells (data not shown). RA also greatly enhanced Foxp3 induced by TGF-β in $CD4^+CD25^-CD44^{lo}$ $CD62L^+$ naive T cells isolated from $Cd80^{-/-}$ $Cd86^{-/-}$ mice (Figure S1C). Moreover, we showed previously that RA is able to counterbalance the inhibitory effects of costimulation on TGF-β-mediated Foxp3 induction, with either $CD4^+CD25^-$ or $CD4^+Foxp3^-$ T cells (Benson et al., 2007). To confirm these results, we used OTII TCR transgenic $CD4^+CD25^-CD44^{lo}$ $CD62L^+$ cells sorted by flow cytometry and tested the effects of RA by using increasing doses of anti-CD28 stimulation. We found that RA markedly enhanced TGF-β-mediated Foxp3 induction on pure naive $CD4^+$ T cells that were stimulated with anti-CD3 and various doses of anti-CD28 (Figure S1D). The enhanced Foxp3 expression mediated by RA is more pronounced on naive monoclonal

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SUPPLEMENTAL DATA

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OTII TCR transgenic T cells as compared to polyclonal T cells, consistent with a lesser frequency of “contaminating” memory T cells.

Finally, because we showed previously that RA-mediated enhanced expression of Foxp3 is greatly reduced in the absence of IL-2 (Mucida et al., 2007), we investigated the effects of RA on naive T cells with various doses of exogenous IL-2. Although IL-2-deficient mice develop inflammatory disorders, *Il2*$^{-/-}$*Cd80*$^{-/-}$*Cd86*$^{-/-}$ mice are healthy and, more importantly, they do not contain T regulatory cells. At steady state, ~99% of all CD4^{+} T cells isolated from *Il2*$^{-/-}$*Cd80*$^{-/-}$*Cd86*$^{-/-}$ mice are naive (data not shown). The CD4^{+} T cells were further sorted by flow cytometry so that highly purified naive CD4^{+}CD25^{-}CD44^{lo} CD62L^{+} cells (more than 99.9% purity) were obtained. The sorted naive CD4^{+} *Il2*$^{-/-}$*Cd80*$^{-/-}$*Cd86*$^{-/-}$ T cells were tested for TGF-β-induced Foxp3 expression in the presence of increasing doses of IL-2 and anti-CD3 and anti-CD28 coated beads, with or without RA. The data showed that 1 nM RA distinctly enhanced TGF-β (1 ng/ml)-mediated Foxp3 induction in pure naive CD4^{+} T cells at all doses of IL-2 examined (Figures S1E and S1F). Strikingly, although the expression of Foxp3 was much reduced, RA enhanced TGF-β-mediated Foxp3 induction not only in the absence of memory or effector T cells but also in the absence of IL-2.

These data demonstrate that RA mediates enhanced TGF-β-induced Foxp3 expression upon activation of pure naive T cells in the absence of accompanying CD4^{+}CD44^{hi} T cells. In addition, we confirmed, as Hill et al. (2008) proposed, that RA also efficiently counteracts inhibitory effects of CD44^{hi} T cells on Foxp3 induction (*d*ata not shown), which indicates that RA is able to enhance Foxp3 expression both, via effects directly on the primed naive T cells as well as indirectly via inhibitory effects on accompanying CD4^{+}CD44^{hi} T cells.

There is no doubt that the new findings by Hill et al. (2008) add an important new pathway by which RA can enhance Foxp3 induction, which had been suggested previously (Elias et al., 2008; Mucida et al., 2007; Xiao et al., 2008).Nevertheless, published data, together with the data presented here, disagree with the central statement proposed by Hill et al. (2008) that the enhanced expression of TGF-β driven Foxp3 mediated by RA is an indirect effect that requires suppression of accompanying CD4^{+}CD44^{hi} T cells rather than via direct or indirect effects on the primed T cells themselves. Under physiological conditions, naive T cells may be exposed to cytokines and effector or memory cells, and hence it is likely that during priming of naive T cells, both mechanisms of RA-mediated enhanced TGF-β driven Foxp3 expression in the primed T cells will synergize in vivo. Therefore, elucidating and understanding both processes by which RA affects naive and already differentiated T cells is important and may lead to the identification of possible targets for therapeutic interventions to treat various inflammatory and autoimmune diseases.

Supplementary Material

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