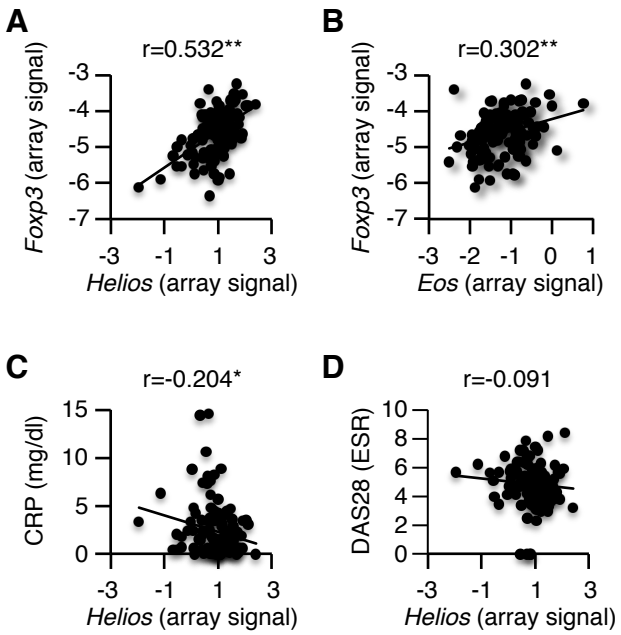

Figure S1. Correlation between Helios gene signals in CD4⁺ T cells and serum CRP in RA patients.

(**A-D**) CD4⁺ T cells were isolated from PBMC of RA patients who were not receiving biologics (n=124), and were subjected to DNA microarray analysis. (**A**) Association between Helios and Foxp3 gene signals. (**B**) Association between Eos and Foxp3 gene signals. (**C, D**) Association between Helios gene signals and CRP (**C**) or DAS28-ESR (**D**). Correlation analysis was performed with Spearman's rank correlation. *p<0.05, **p<0.01.

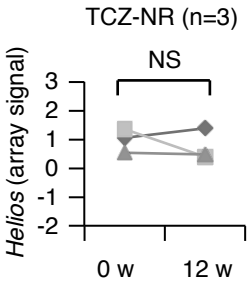

Figure S2. Treatment with Tocilizumab did not increase Helios gene expression in CD4⁺ T cells in non-responders.

CD4⁺ T cells were isolated from PBMC of RA patients who did not response to the treatment with TCZ (TCZ-NR; n=3) at just before (0 w) and 12 weeks (12 w) after the treatment, and were subjected to DNA microarray analysis. Shown are Helios gene signals.

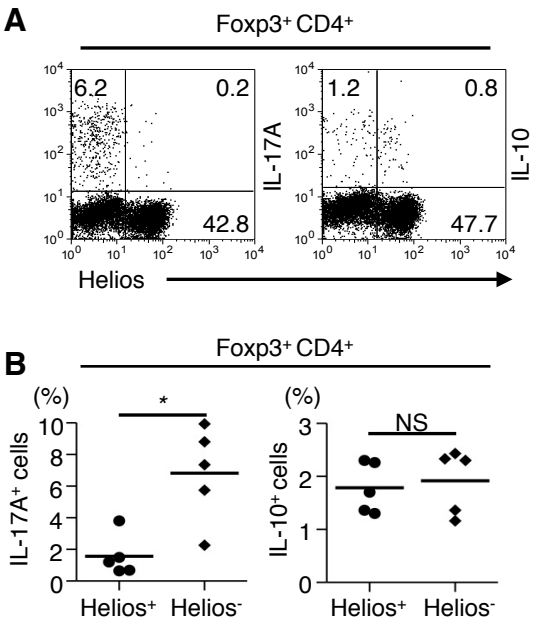

Figure S3. Helios$^+$ Foxp3$^+$ CD4$^+$ T cells produce IL-10 but not IL-17A in humans. CD4$^+$ T cells from PBMCs of HD were stimulated with anti-CD3/anti-CD28 mAb in the presence of TGF-β (10 ng/ml) for 7 days. Cells were then stimulated with PMA/ ionomycin for 4 hours, and the expression of Foxp3, Helios, IL-17A, and IL-10 in CD4$^+$ cells was examined by intracellular staining. Representative dot plots of Helios vs. IL-17A or Helios vs. IL-10 of Foxp3$^+$ CD4$^+$ cells (**A**) and the scatter plot depicting the frequency of indicated cells (**B**) are shown (n = 5 subjects per group). Horizontal lines represent the mean values. *p<0.05, NS=not significant.

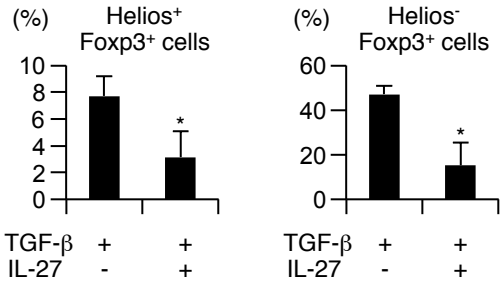

Figure S4. IL-27 inhibits the development of Helios$^+$ and Helios$^-$ iTregs.
Naïve CD4$^+$ T cells from WT mice were stimulated with anti-CD3/anti-CD28 mAb in the presence of TGF-β with or without IL-27 (50 ng/ml) for 3 days and analyzed by flow cytometry. Shown are means ± SD of the frequency of Helios$^+$ Foxp3$^+$ CD4$^+$ T cells (left panel) and Helios$^-$ Foxp3$^+$ CD4$^+$ T cells (right panel) (n=3 mice per group). *p<0.05.

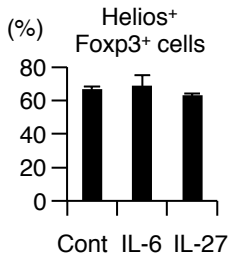

Figure S5. The expression of Helios was not decreased by IL-6 or IL-27 in thymus-derived naturally occurring Tregs.

$CD4^+$ $hCD2^+$ cells were isolated from LN of $Foxp3^{hCD2}$ mice and stimulated with anti-CD3 mAb in neutral conditions, IL-6 conditions, or IL-27 conditions for 3 days. The expression of Foxp3 and Helios in $CD4^+$ $hCD2^+$ cells was analyzed by intracellular staining. Shown are means ± SD of the frequency of $Helios^+$ $Foxp3^+$ $CD4^+$ T cells (n=3 mice per group).

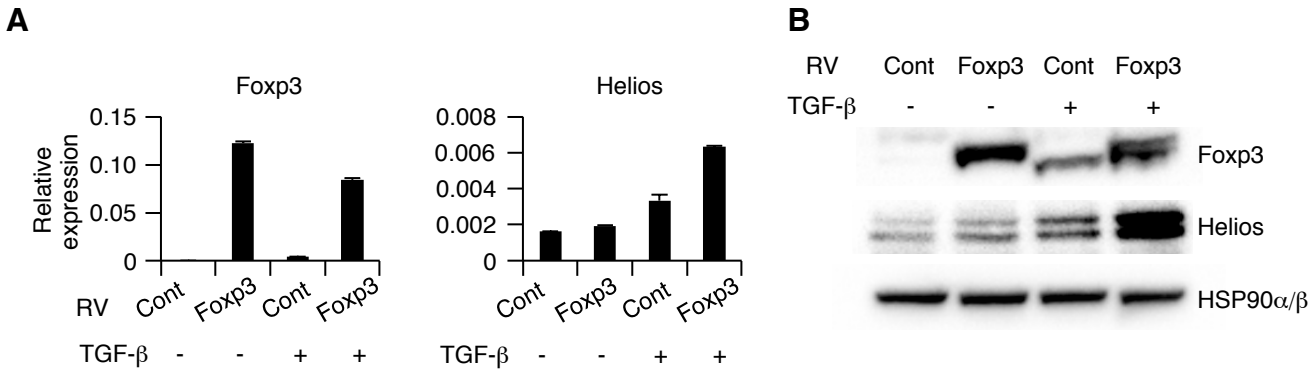

Figure S6. Foxp3 expression and TGF-β signaling cooperatively induce Helios expression. (**A**, **B**) Naïve CD4$^+$ T cells from WT mice were stimulated with anti-CD3/anti-CD28 mAb in neutral conditions or Treg conditions for 24 hours, infected with retroviruses (RV) of PIN-Flag-Foxp3 or PIN (as a control), and then cultured in the presence or absence of TGF-β (0.5 ng/ml) for 3 days. The cells infected with retroviruses (NGFR$^+$ cells) were isolated by magnetic cell sorting. (**A**) Total RNAs were prepared from these cells, and mRNA levels of Foxp3 and Helios were analyzed by qPCR. Shown are means ± SD of the relative expression of Foxp3 and Helios (n=3 mice per group). (**B**) Whole cell extracts were prepared and subjected to immunoblotting with antibodies against Foxp3, Helios, and HSPα/β (as a control).

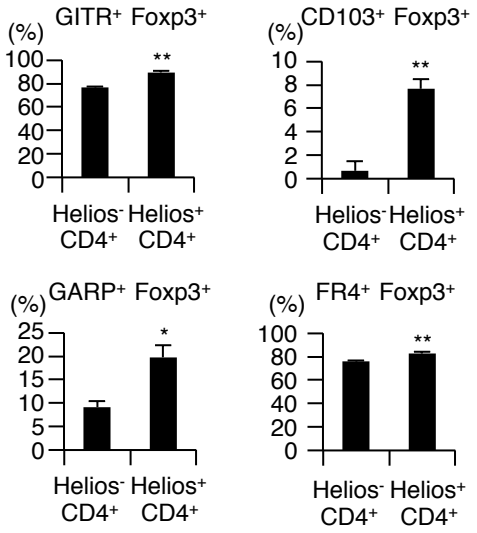

Figure S7. The expression of Treg-related molecules in murine Helios$^+$ or Helios$^-$ iTregs. Naïve CD4$^+$ T cells from WT mice were stimulated with anti-CD3/anti-CD28 mAb in Treg conditions for 3 days, and the expression levels of Treg-related molecules in Helios$^+$ iTregs were compared to those in Helios$^-$ iTregs by flow cytometry. Shown are means ± SD of the frequency of indicated cells (n=3 mice per group). *p<0.05. **p<0.01.