Supplementary Information

Table S1. Reduced numbers of $CD4^+CD25^+Foxp3^+$ Treg cells in the spleen of c-Rel-deficient neonatal and adult mice.

Day	Mice (n=6)	Splenocytes per mouse ($x10^{-5}$)	$CD4^+$ (% of splenocytes)	**$CD4^+$ $CD25^+$ (%)[a, b]**	**$CD4^+$ $Foxp3^+$ (%)[a, b]**	**$CD4^+CD25^+$ $Foxp3^+$ (%)[a, b]**	$CD4^+CD25^-$ $Foxp3^-$ (%)[a]
3	WT	60±12	1.4±0.2	**5.1±1.3**	**0.7±0.4**	**0.4±0.1**	94±1.4
	c-Rel$^{-/-}$	40±10	0.4±0.1	**0.6±0.3**	**0.3±0.4**	**0.1±0.1**	98.6±1.7
7	WT	125±15	2.1±0.4	**10±2**	**4.8.±0.8**	**3.2±0.7**	85±1.5
	c-Rel$^{-/-}$	100±12	0.6±0.2	**2.8±1.1**	**1.0±0.2**	**0.6±0.2**	95.7±0.9
21	WT	910±100	10.5±0.6	**5.2±1.3**	**6.2±0.3**	**4.0±1.1**	85.4±2
	c-Rel$^{-/-}$	570±80	5.6±0.3	**1.6±0.5**	**1.3±0.2**	**0.9±0.3**	95±2.7
42	WT	2,100±250	19±2.3	**3.9±1**	**5.5±0.3**	**2.4±0.8**	92.1±2.1
	c-Rel$^{-/-}$	1,700±150	14±2.8	**1.1±0.4**	**2.1±0.3**	**0.5±0.1**	96.3±1.6

[a] % of $CD4^+$ T cells.

[b] The differences between WT and *c-Rel*$^{-/-}$ groups are statistically significant ($p<0.001$) for all days.

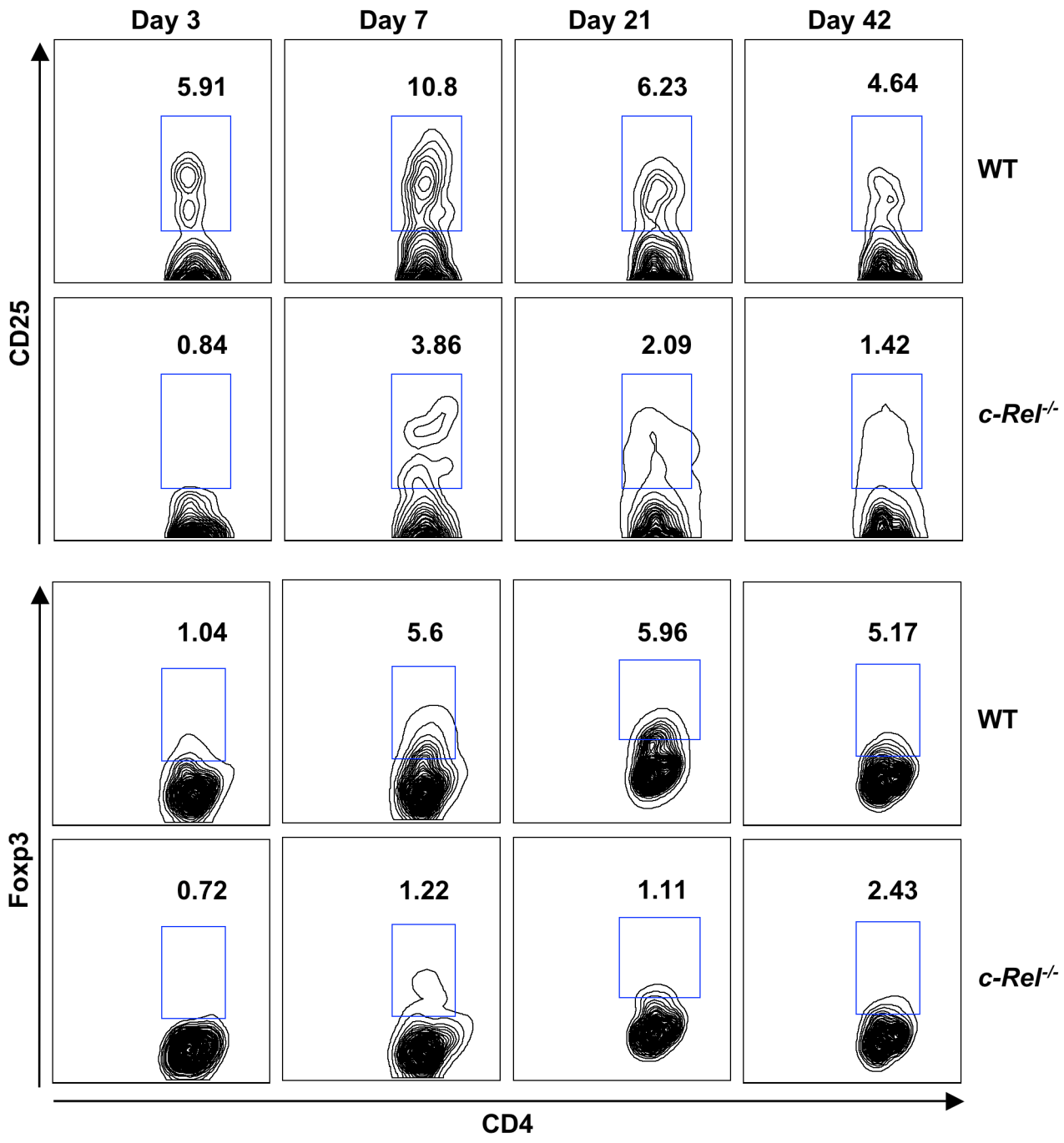

Figure S1. The number of $CD4^+CD25^+Foxp3^+$ regulatory T cells is significantly reduced in the spleen of *c-Rel*$^{-/-}$ mice. Splenocytes from wild type and *c-Rel*$^{-/-}$ C57BL/6 mice (n=6) were collected 3, 7, 21, and 42 days after birth, stained with antibodies to murine CD4, CD8, CD25, and Foxp3, and analyzed by flow cytometry. Results are representative of three independent experiments.

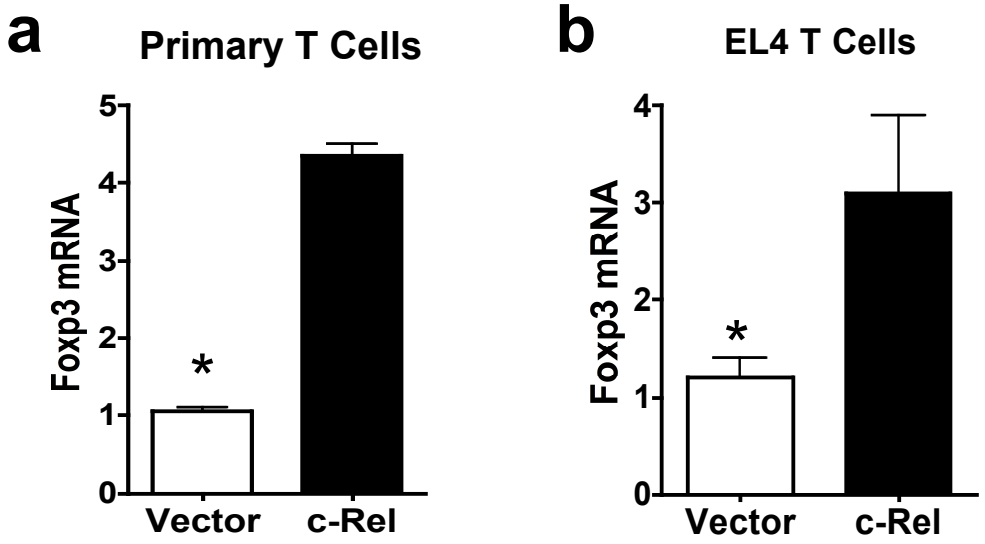

Figure S2. **Over-expression of c-Rel induces Foxp3 expression in both primary CD4$^+$ T cells and EL4 T cells.** Purified primary murine CD4$^+$CD25$^-$ T cells (**a**) and EL4/LAF T cells (**b**) were transfected with a c-Rel-expression pRK5 plasmid or the empty vector using the Nucleofector system and Lipofectamine LTX reagents, respectively. Cells were then stimulated with anti-CD3 (2 μg/ml) plus anti-CD28 (2 μg/ml) (a), or anti-CD3 (2 μg/ml) plus TGF-β (4 ng/ml) (b) for 17 hours. Total RNA was extracted and Foxp3 expression was determined by real-time RT-PCR. The plasmid pEGFP was also used to co-transfect the cells to monitor the transfection efficiency. Data are representative of three experiments. *, $p<0.01$.

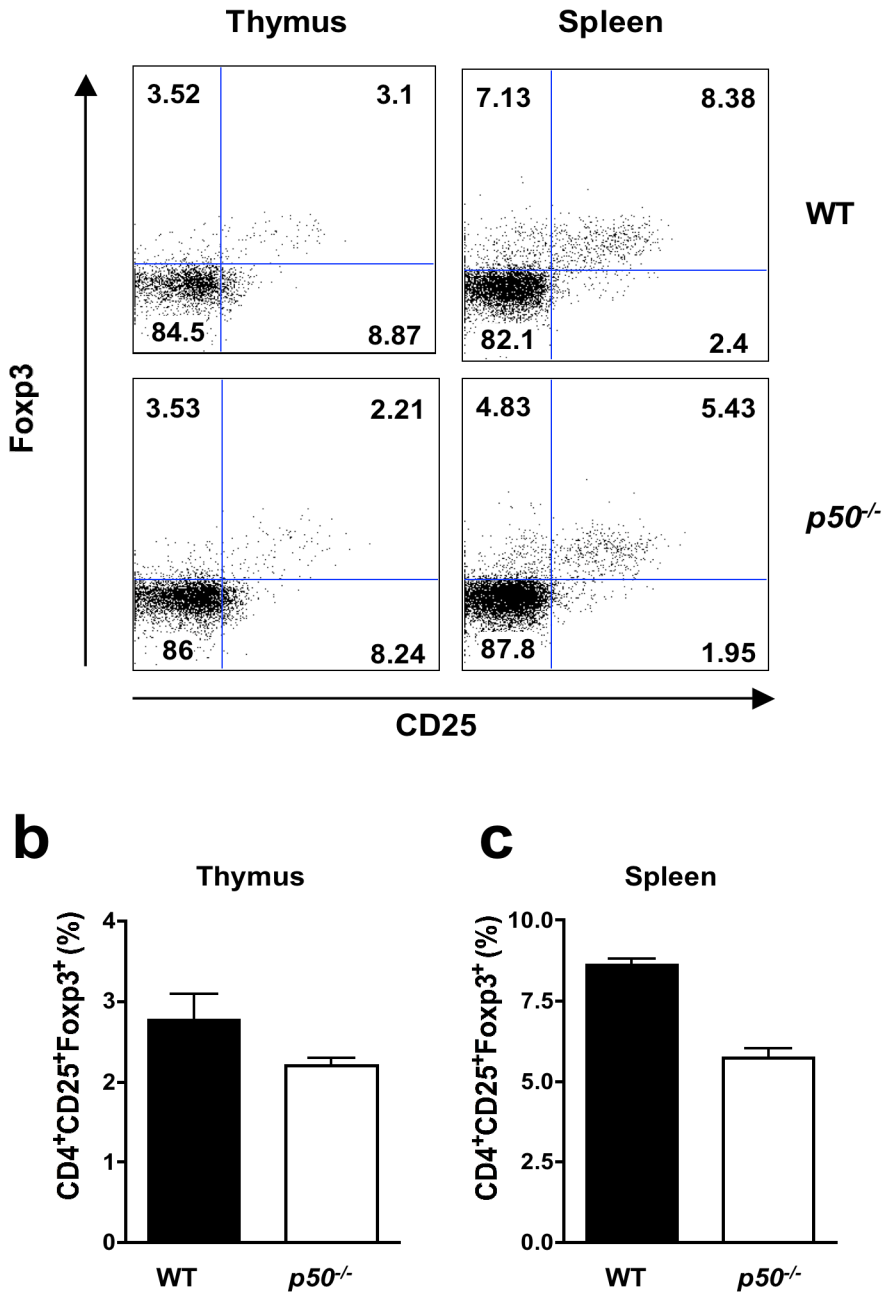

Figure S3. Marginal effect of p50 deficiency on the development of $CD4^+CD25^+Foxp3^+$ T cells. (a) Thymocytes and splenocytes from 6-week-old WT and *p50*$^{-/-}$ mice (n=3) were stained with antibodies to CD4, CD8, CD25, and Foxp3, and analyzed by flow cytometry. **(b)** The percentages of $CD4^+CD25^+Foxp3^+$ thymocytes and splenocytes in WT and *p50*$^{-/-}$ mice are shown. Results are for gated $CD4^+$ T cells, and are pooled from two independent experiments.

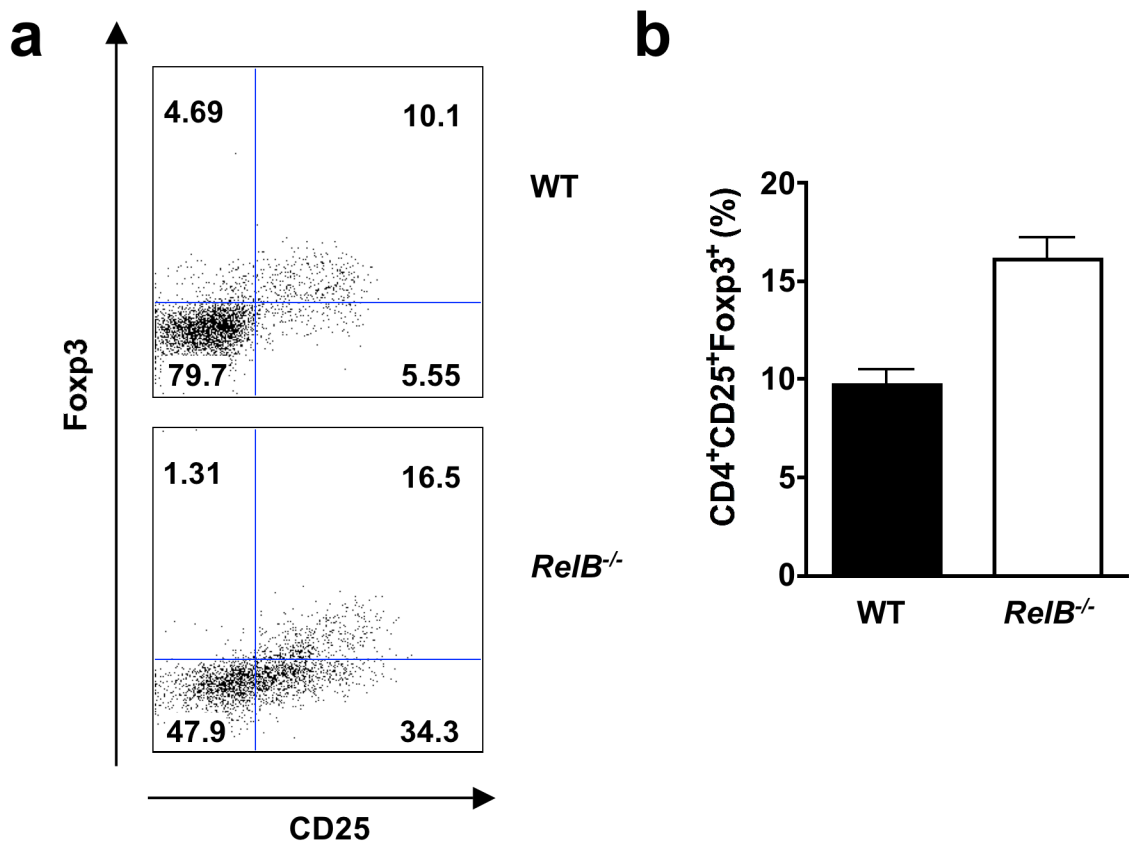

Figure S4. No effect of RelB deficiency on the number of $CD4^+CD25^+Foxp3^+$ T cells. Because *RelB*$^{-/-}$ mice develop neonatal fatal inflammatory diseases (Burkly et al., 1995), we generated bone marrow chimeric mice by intravenously injecting bone marrow cells of three-week-old *RelB*$^{-/-}$ B6 mice into *Rag1*$^{-/-}$ B6 mice (n=3). WT bone marrow cells were used as controls. Four months later, the percentages of $CD4^+CD25^+Foxp3^+$ splenocytes were determined by flow cytometry as described in Figure S2. Results are for gated $CD4^+$ T cells, and are pooled from two independent experiments.

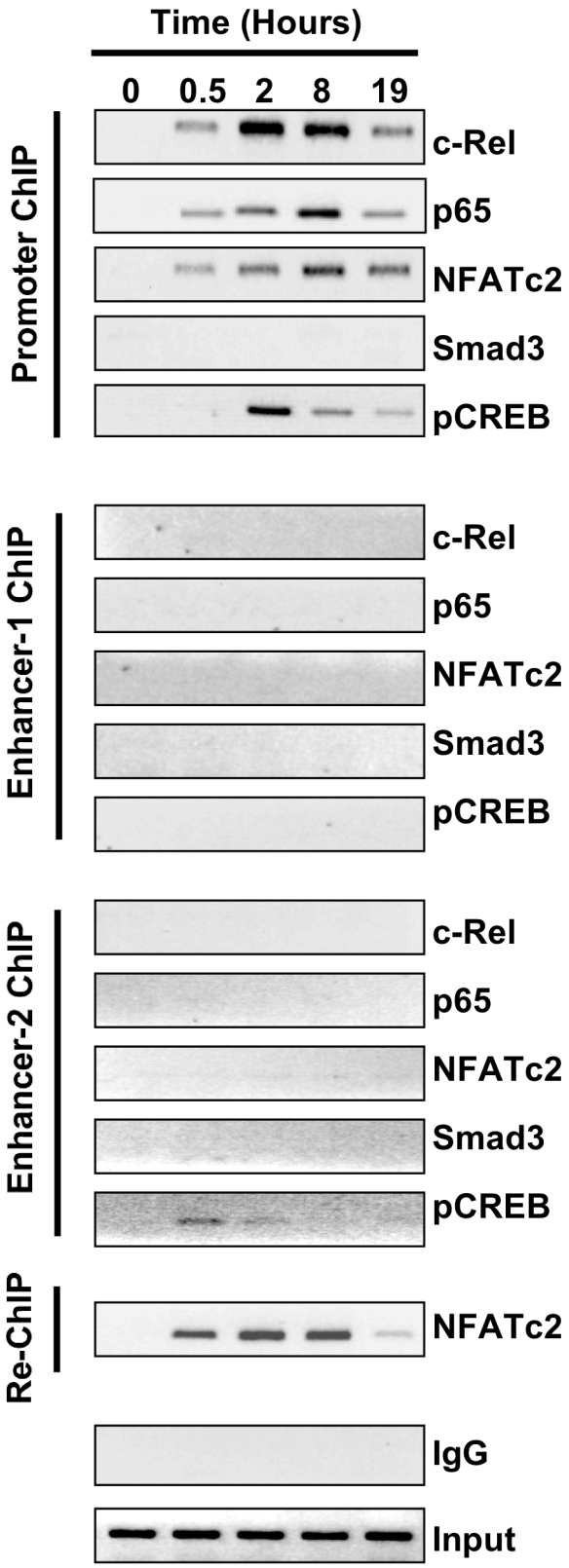

Figure S5. Binding of c-Rel, p65, NFATc2, and pCREB to the *Foxp3* locus as determined by ChIP and Re-ChIP. Purified $CD4^{+}CD25^{-}$ T cells from C57BL/6 mice

were cultured under conditions described in Figure 2b but without TGF-β, and harvested at the indicated times. Cells were then analyzed by ChIP for c-Rel, p65, NFATc2, Smad3, and pCREB bindings to the promoter, enhancer-1, and enhancer-2 of the *Foxp3* gene as described in Experimental Procedures. Re-ChIP was performed for NFATc2 binding to the promoter using DNA precipitated by anti-c-Rel. Control IgG and input DNA shown are for promoter ChIP.

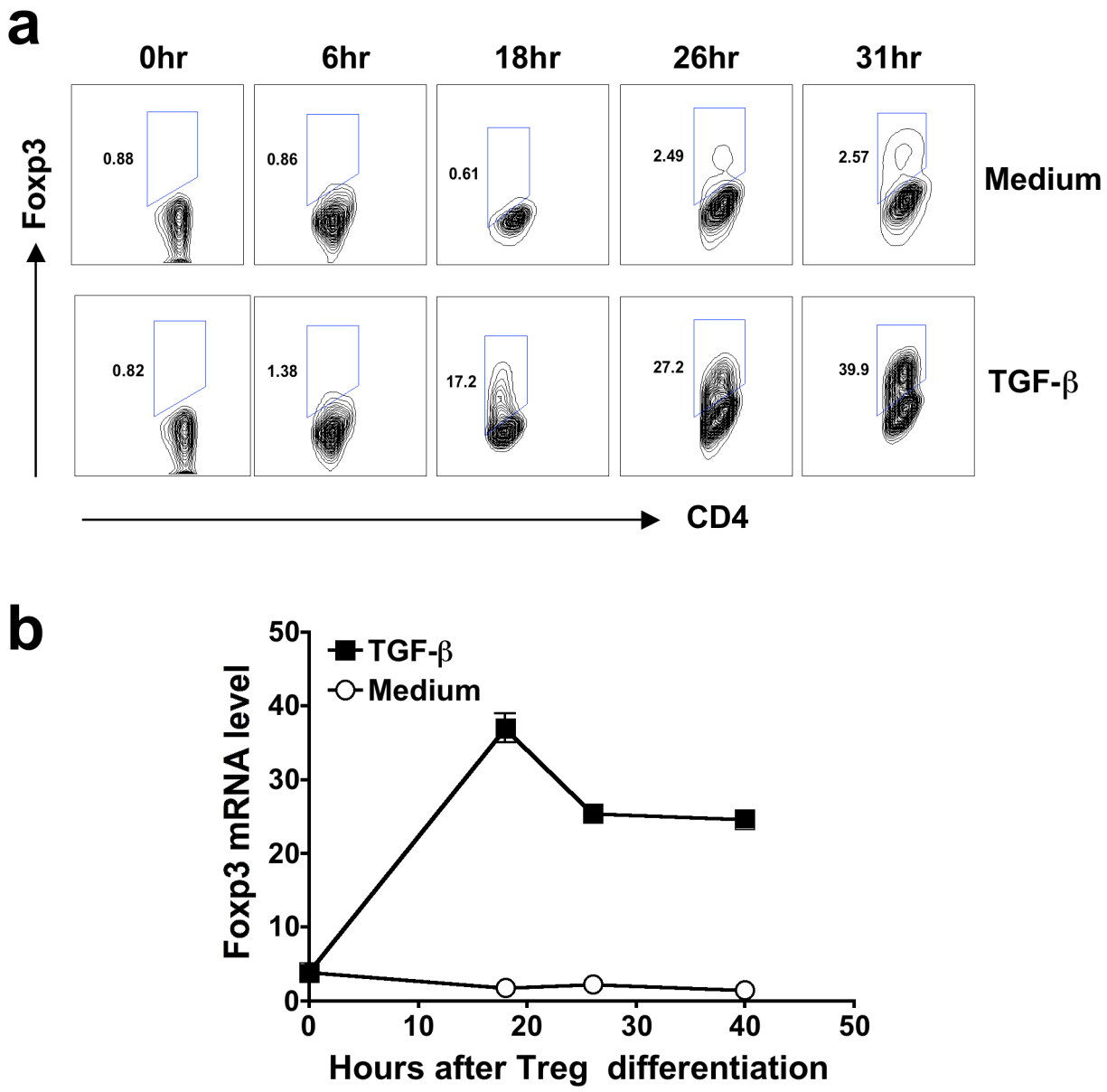

Figure S6. Foxp3 expression during Treg differentiation with or without TGF-β. Purified $CD4^+CD25^-$ splenic T cells were cultured in the presence of plate-bound anti-CD3, soluble anti-CD28, IL-2, anti-IL-4, anti-IFN-γ, with or without TGF-β (4 ng/ml) for the indicated times. (**a**) Cells were stained with antibodies to CD4 and Foxp3, and

analyzed by flow cytometry. (**b**) Total RNA was extracted and Foxp3 mRNA levels were determined by real-time RT-PCR.

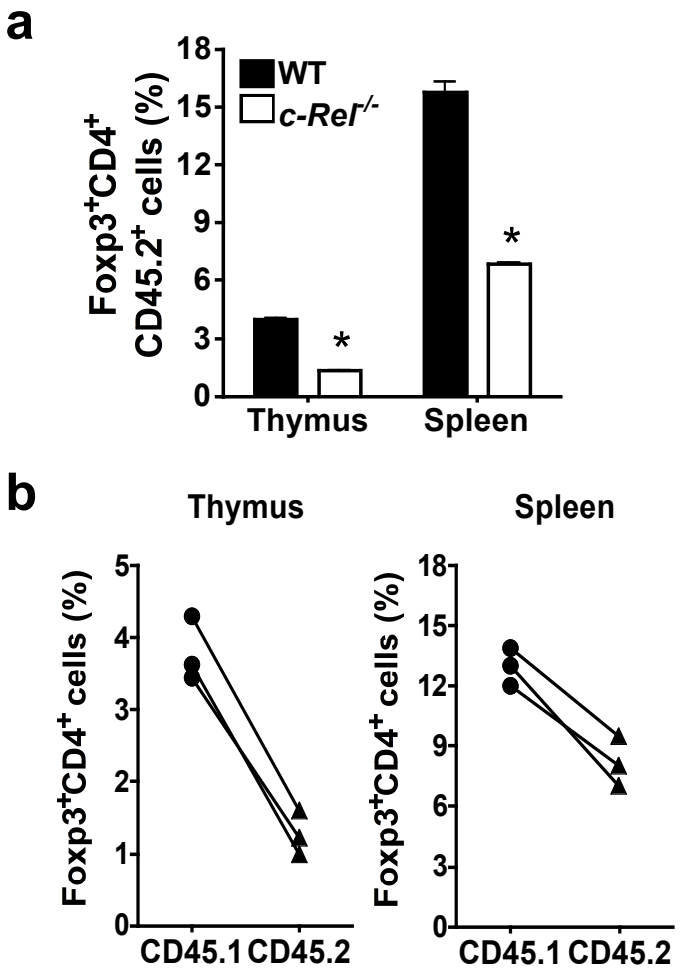

Figure S7. c-Rel regulates Treg development in a cell-autonomous manner. (a) Mixed bone marrow chimeric mice were generated by injecting T cell-depleted bone marrow cells from CD45.2$^+$ *c-Rel*$^{-/-}$ or WT C57BL/6 mice, mixed with CD45.1$^+$ WT bone marrow cells at a ratio of 1:4 (CD45.2$^+$:CD45.1$^+$), into irradiated CD45.2$^+$ WT C57BL/6 recipients (n=4). Seven weeks later, the frequencies of Foxp3$^+$CD4$^+$CD45.2$^+$ cells were determined by flow cytometry. *, $p<0.01$. **(b)** Mixed bone marrow chimeric mice were generated by injecting T cell-depleted CD45.2$^+$ *c-Rel*$^{-/-}$ bone marrow cells, mixed with CD45.1$^+$ WT bone marrow cells at a ratio of 1:1 (CD45.2$^+$:CD45.1$^+$), into irradiated CD45.2$^+$ C57BL/6 recipients (n=3). Seven weeks later, the frequencies of Foxp3$^+$CD4$^+$ cells were determined by flow cytometry. Connected symbols indicate values from the same mouse.

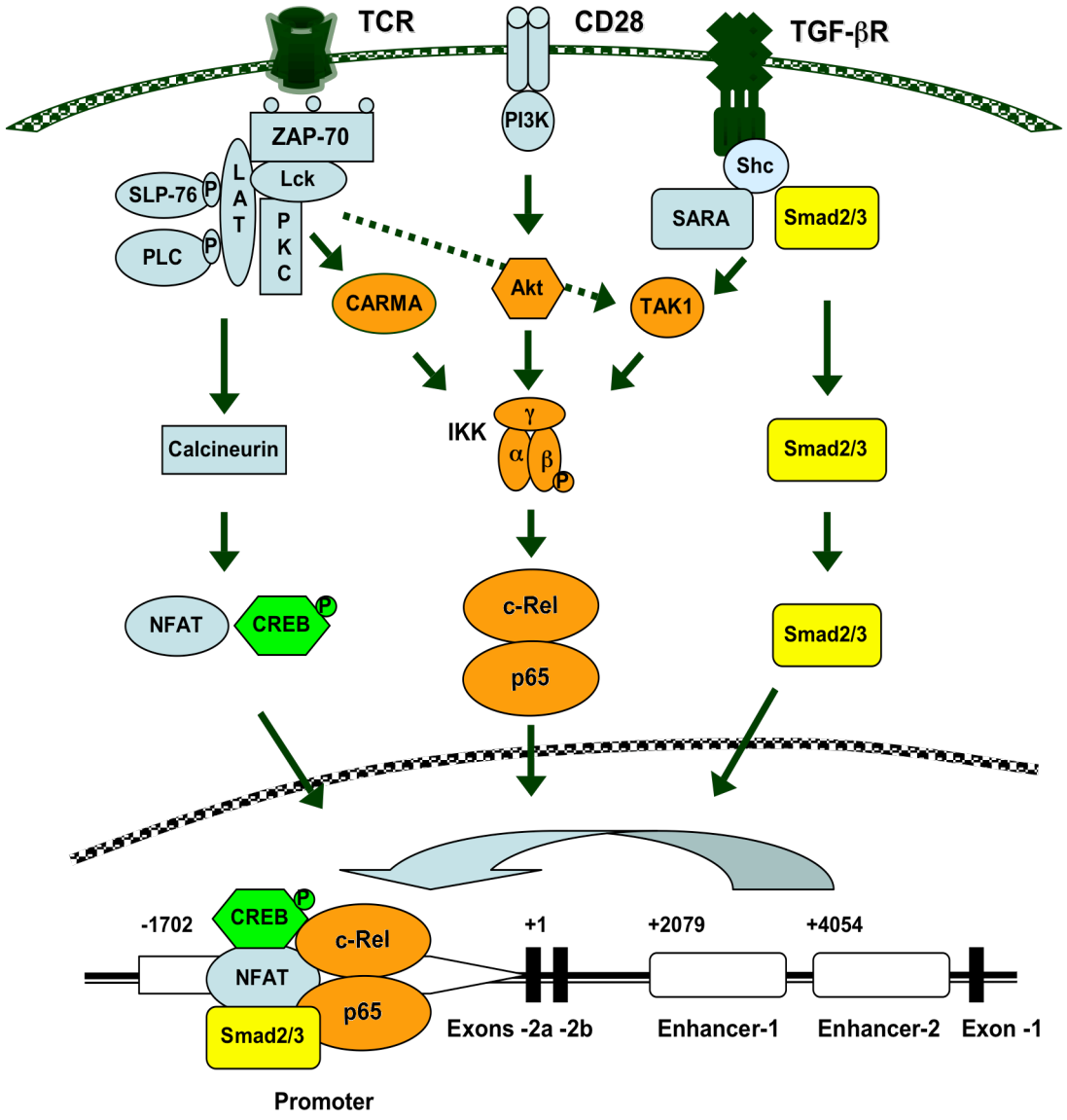

Figure S8. The *Foxp3*-specific c-Rel enhanceosome model. Under Treg-inducing conditions, TCR, CD28, and TGF-β receptors (TGF-βR) can all activate Rel/NF-κB through the IKK complex. Additionally, TCR also activates NFAT and CREB, and TGF-β receptors activate Smad. The c-Rel enhanceosome formed at the *Foxp3* locus is shown (see text for details). Lck, leukocyte-specific protein tyrosine kinase; PKC, Protein Kinase C; PLC, phospholipase C; SARA, Smad anchor for receptor activation; P, phosphorylated.