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Raf Signaling but not the ERK Effector SAP-1 Is Required for Regulatory T Cell Development[1]

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Regulatory T cells (T_{reg}) play an important role in immune regulation. Their development in the thymus requires TCR activation and recognition of peptide-MHC, although the downstream signals controlling commitment to the lineage are unclear. To compare the requirements for positive selection and T_{reg} development, we studied knockout and transgenic mice defective in Raf signaling and the ERK effector SRF accessory protein 1 (SAP-1), a member of the ternary complex factor family of Ets domain transcription factors. Although SAP-1 deficient mice display a severe defect in thymocyte positive selection, T_{reg} development was unimpaired as assessed by expression of Foxp3 and the activation markers CD25, GITR, CTLA4, and CD103 in the $CD4^+$ cell population. In contrast, inhibition of Raf signaling by the interfering dominant negative Raf derivative reduced both $Foxp3^+$ and $Foxp3^-$ $CD4^+$ populations. In SAP-1-deficient $CD4^+CD25^+$ T_{reg} cells, TCR crosslinking efficiently induced ERK activation, but transcriptional induction of the immediate early gene Egr-1 was impaired. Nevertheless, neither deletion of SAP-1 nor expression of a dominant negative Raf derivative affected the ability of $CD4^+CD25^+$ T_{reg} cells to suppress $CD4^+CD25^-$ cell proliferation in vitro. Finally the suppressive activity of $CD4^+CD25^+$ T_{reg} cells lacking SAP-1 in an in vivo colitis model was not significantly impaired. The signaling requirements for development of T_{reg} cells in the thymus are thus distinct from those required for "conventional" T cell positive selection, and ERK signaling to SAP-1 is not required for the suppressive activity of T_{reg} cells. *The Journal of Immunology,* **2007, 179: 6836–6844.**

The phenomenon of immune tolerance is a striking feature of the immune system. Avoidance of autoimmunity is achieved both at the level of immune cell development and by direct or indirect suppressive interactions between lymphocyte populations. During thymocyte development, negative selection ensures deletion of cells bearing TCRs with high affinity for self peptide-MHC, whereas positive selection ensures survival of cells bearing functionally rearranged TCRs. The processes of positive selection and negative selection have been extensively studied using transgenic and gene knockout approaches, which have shown that positive selection is completely dependent on signaling through the calcineurin and Ras-ERK pathways (1–3).

Serum response factor (SRF),[3] a transcription factor, and its partnership protein SAP-1 (SRF accessory protein 1) play an important role in transducing Ras-ERK signals to the nucleus (4). SAP-1, a member of the ternary complex factor (TCF) family of Ets-domain transcription factors, is the predominant TCF in the thymus, and is required for positive selection and for TCR-dependent immediate-early gene activation in DP thymocytes (4). Consistent with this, inactivation of Egr-1, a SAP-1 target gene, or its target Id3 also impairs positive selection (5, 6). In contrast, negative selection appears independent of calcineurin and ERK-SAP-1 signaling (2–4), and is more dependent on stress-activated protein kinase/Jun *N*-terminal kinase signaling and its upstream regulators (7, 8). A sharp affinity threshold, which is associated with changes in the relative subcellular localization of active ERK and JNK, separates positive and negative selection outcomes (9).

A second means by which autoimmunity is controlled is through the generation of regulatory T cells (T_{regs}), which play an important role in the maintenance of tolerance as well as regulation of immune responses (reviewed in Refs. 10, 11). $CD4^+Foxp3^+$ T cells represent an important class of T_{regs} in both mouse and human, and the transcription factor Foxp3 appears necessary and sufficient for their generation in the thymus (12–14); reviewed in Ref. 15. $CD4^+Foxp3^+$ T cells comprise a large proportion of thymic $CD4^+CD25^+$ T cells and also express activation markers such as CTLA-4, GITR, and CD103 (12–14). $CD4^+CD25^+$ cells act to suppress a variety of autoimmune phenotypes in adoptive transfer models (16, 17) and have the ability to suppress activation of T cells in vitro in a cell contact-dependent manner (18–20). The development of thymic T_{reg} cells is not well understood: it selects cells expressing TCRs with high affinity for MHC-self peptide, although both the level of peptide expression and the nature of the APC affect the efficiency of T_{reg} commitment (21–27), and is dependent on stromal MHC (28). Curiously, T_{reg} development does not always correlate with substantially increased absolute numbers of T_{reg} cells (27), and indeed recent results suggest that some T_{reg} cells may be generated from a nonproliferative double positive (DP) thymocyte population (29).

Despite the involvement of TCR signaling in commitment to the T_{reg} lineage, the relation of this process to positive and negative

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[3] Abbreviations used in this paper: SRF, serum response factor; SAP-1, SRF accessory protein 1; TCF, ternary complex factor; T_{reg}, regulatory T cells; DP, double positive; PBDu, phorbol dibutyrate; WT, wild type; SP, single positive; DN, dominant negative; p-ERK, phospho-ERK.

selection remains unclear, as does the mechanism by which Foxp3 expression is controlled. In this study, we investigated the role of ERK signaling in T_{reg} cells using SAP-1 knockout animals and transgenic animals expressing a dominant negative Raf (4, 30). Development of T_{reg} cells occurs normally in animals lacking SAP-1, in contrast to positive selection, but remains dependent on Raf signaling. Although SAP-1 is required for T_{reg} cells to exhibit normal levels of Egr-1 induction in response to CD3 crosslinking, this is not associated with impaired T_{reg} function, as assessed by in vitro suppression assays or the ability to suppress colitis in a T cell transfer model. These findings show that signaling to SAP-1 plays different roles in selection of both single positive (SP) T cells and T_{regs}, and is not required for the regulatory function of T_{reg} cells.

Materials and Methods

Mice

SAP-1-deficient mice and mice expressing a dominant negative Raf transgene have previously been described (4, 30). Mice were maintained in specific pathogen-free conditions in the Cancer Research U.K. Biological Resources Unit. Reconstitution experiments were performed as previously described (4). Animal experimentation was approved by the Cancer Research U.K. Research Services Animal Ethics Committee.

Flow cytometry

Cells were prepared from thymi, spleens, lymph nodes, and bone marrow of 6–10 wk old mice. Abs against CD4 (RM4–5), CD25 (7D4), CD90.2 (53-2.1), CD45.1 (A20), CD45.2 (104), CD152 (UC10-4F10–11) and CD103 (M290) for flow cytometry were purchased from BD Pharmingen. Abs against GITR were purchased from R&D Systems, CD8α (5H10) was purchased from Caltag, and Foxp3 staining kit was purchased from eBioscience. For intracellular staining, cells were fixed in 4% paraformaldehyde and permeabilized in 0.3% Saponin, 5% FCS, 10 mM HEPES (pH 7.4) in PBS, and were stained in 0.1% Saponin. Foxp3 staining was performed to the manufacturer's protocol. Cells were analyzed on a FACSCalibur (Becton Dickinson) with CellQuest software. Cells were sorted by either a MoFlo sorter (DakoCytomation) or a FACSAria (Becton Dickinson) to greater than 95% purity.

Cell stimulation

T cells were resuspended in RPMI 1640 medium with 10% FCS and 50 μM 2-ME. Cells were stimulated as previously described (4). In brief, for short time courses, thymocytes were stimulated with 10 μg/ml α-CD3 (2C11), and crosslinked by the addition of 75 μg/ml goat anti-hamster. Stimulation was terminated by fixation with 4% paraformaldehyde in PBS or into lysis buffer for RNA/protein preparation. For longer time courses, thymocytes were spun directly onto plates coated with 10 μg/ml α-CD3. In some cases irradiated T cell depleted splenocytes were used as APCs and added at 1:2 ratio of T cell:APC along with 20 ng/ml IL-2 (Chiron). Intracellular staining for phospho-ERK (p-ERK) and Egr-1 was preformed as previously described (4). Immunoblotting was done by standard techniques with detection by monoclonal anti-MAP kinase, activated (Sigma-Aldrich), and anti-MAPK (Sigma-Aldrich).

In vitro suppression assays

$CD4^+CD25^-$ and $CD4^+CD25^+$ T cell populations were sorted as described above to >95%. For thymidine incorporation assays, $CD4^+CD25^-$ T cells (2.5×10^4) were cocultured with irradiated T cell-depleted splenocytes (5×10^4) as APCs, various ratios of $CD4^+CD25^+$ T cells, and 2 μg/ml soluble α-CD3 for 72 h at 37°C. Cultures were pulsed with 1 μCi/well [^{3}H]thymidine for the last 6 h. After 72 h incubation, cells were harvested and analyzed. For CFSE labeled assays, $CD4^+CD25^-$ from SJL (CD45.1) were stained with 5 μM CFSE for 5 min at 37°C and then washed three times in medium. These cells were then cocultured as above with $CD4^+CD25^+$ and irradiated T cell-depleted splenocytes from BL6 mice (CD45.2). After 72 h, cells were analyzed by FACS, with the CD45 marker being used to identify the effector cells.

T cell transfer experiments

Splenocytes were first enriched for $CD4^+$ T cells through magnetic separation according to the manufacturer's instructions. Abs to CD8, Mac-1 and B220 were purchased from BD Pharmingen. Anti-Rat Ig magnetic beads were purchased from Dynal. Enriched $CD4^+$ T cells were then sorted for $CD4^+CD45RB^{high}CD25^-$ and $CD4^+CD45RB^{low}CD25^+$ populations. $Rag2^{-/-}$ mice were injected i.p. with 4×10^5 $CD4^+CD45RB^{high}CD25^-$ T cells alone or were coinjected with 2×10^5 $CD4^+CD45RB^{low}CD25^+$ T cells. Mice were observed daily and weighed weekly; any mice showing clinical signs of severe disease were sacrificed accordingly.

Histology

Tissue sections were stained with H&E as well as Alcian blue (31). Colitis severity was graded semiquantitatively from 0 to 4 as described (16).

Gene expression analysis

RNA was prepared using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Ten to fifty nanograms of RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer's protocol. cDNA was analyzed by real-time PCR using TaqMan probes (Applied Biosystems) or SYBR green incorporation (Invitrogen Life Technologies). Expression levels normalized to GAPDH or HPRT. PCR for Foxp3 and HPRT was performed using published primers and probes (13). PCR for the TCFs and GAPDH was performed using the following primers and probes: SAP-1 primers 5′-ACA ACGCCTGCCAAAAAGC, 5′-GAAAGACTAGGGCTCGTTGC; probe 5′-FAM-ATCGAGCCTGTCGCTGCTGCCT; Elk-1 primers 5′-TCACGG GATGGTGGTGAGT, 5′-GTTCTTGCGCAGTCCCCAT; probe 5′-FAM-CAAGTTGGTGGATGCAGAGGAGGTGG; Net primers 5′-GATGGCG AGTTCAAGCTCCT, 5′-TGGTCTTGTTCTTGCGGAGGC; probe 5′-FAM-AAGGCAGAAGAAGTGGCCAAGCTGTG; GAPDH primers 5′-ACAACTTTGGCATTGTGGAAG, ACAGTCTTCTGGGTGGCAG; GAPDH probe 5′-VIC-CTCATGACCACAGTCCATGCCAT. Egr-1 was measured using SYBR green and the following primers were used: 5′-ATTGATGTCTCCGCTGCAGATC and 5′-TCAGCAGCATCATC TCCTCCA.

EMSA

EMSA was performed as described (32). Cells (0.5×10^6) were activated with 50 ng/ml phorbol dibutyrate (PBDu) for 10 min at 37°C following pretreatment with UO126 (20 μM, 10 min) as necessary. Cells were lysed in 20 mM HEPES (pH 7.9), 10% glycerol, 0.4 M NaCl, 0.4% Triton X-100, 10 mM EGTA, 5 mM EDTA, 1 mM DTT, 0.1 μg of okadaic acid/ml, and protease inhibitors. Binding reactions contained 1 μl of extract in 20 μl 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 1 mM EDTA, 3 mM DTT, 50 ng/ml OVA, 50 ng/ml poly(dI-dC)poly(dIdC), 10% Ficoll 400, protease inhibitors; recombinant SRF DNA binding domain (SRF(133–265)), and 1 ng c-*fos* promoter probe and were incubated for 30 min at room temperature. SAP-1 Ab-induced supershifts were performed by adding anti-SAP-1 (SC-13030X, Santa Cruz Biotechnology) and incubating at room temperature for a further 30 min. All reactions were incubated for a total of 1 hour. Probe was generated by PCR as described previously (33) with primers p10 (5′-CGCACTGCACCCTCGGTGTTGGCTGC-3′) and p11 (5′-ATGG CTCCCCCCAGGGCTACAGGGAAAG-3′). Complexes were resolved in a 5% 37.5:1 acrylamide-bis-acrylamide-0.5 Tris-borate-EDTA gel.

Statistical analysis

Where indicated analysis was performed using unpaired *t* test except for colitis scores, which were analyzed using Mann-Whitney *U* test.

Results

SAP-1-deficient mice show normal numbers of thymic T_{regs}

We previously showed that SAP-1 deficiency results in ~2-fold decrease in the proportion of CD4 SP thymocytes with no appreciable change in thymic cellularity (4). To investigate the role of SAP-1 in T_{reg} cell development, we analyzed $Foxp3^+$ T cells within the CD4 SP population. There was no significant difference between the wild-type (WT) and SAP-1 deficient animals in the absolute numbers of $CD4^+Foxp3^+$ T cells ($0.36 \times 10^6 \pm 0.03$ vs $0.42 \times 10^6 \pm 0.13$), in contrast to the reduction in numbers of $CD4^+Foxp3^-$ SP T cells ($8.54 \times 10^6 \pm 0.67$ vs $5.56 \times 10^6 \pm 0.72$, $p = 0.01$) (Fig. 1*B*). Thus, the proportion of $Foxp3^+$ T cells increased by ~2-fold in SAP-1 deficient animals ($4.0\% \pm 0.1$ vs $8.7\% \pm 0.5$, $p < 0.0001$) (Fig. 1*A*). Furthermore when cells were additionally gated for TCR^{high} expression, no reduction in absolute numbers of

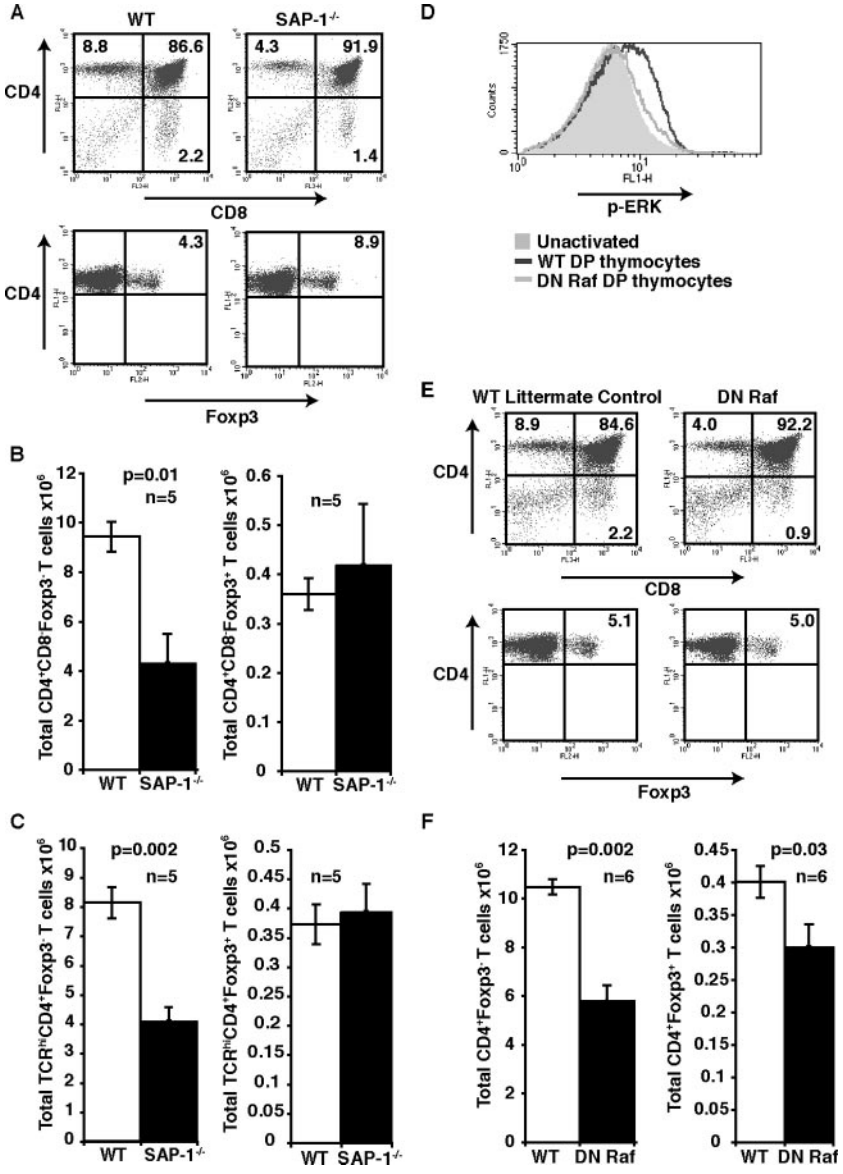

FIGURE 1. $CD4^+Foxp3^+$ T cells develop in SAP-1 deficient and DN Raf animals. *A*, Thymocytes from WT and SAP-$1^{-/-}$ mice were stained for CD4, CD8, and Foxp3. Analysis in lower panels was performed on $CD4^+$ SP T cells. Percentage of cells residing within quadrants is shown on the dot plots. *B*, Absolute numbers of $CD4^+CD8^-Foxp3^-$ T cells (*left*) and $CD4^+CD8^-Foxp3^+$ T cells (*right*) were quantified for both WT and SAP-$1^{-/-}$ mice. □, WT; ■, SAP-$1^{-/-}$. *C*, Cells were gated on TCR^{high} and then analyzed for expression of CD4 and Foxp3. Absolute numbers of $TCR^{high}CD4^+$T cells (*left*) and $TCR^{high}CD4^+Foxp3^+$ T cells (*right*) were quantified as in *B*. □, WT; ■, SAP-$1^{-/-}$. *D*, DP thmocytes were activated by α-CD3 crosslinking for 2 min and then stained for p-ERK. Gray shaded area, unactivated; dark gray line activated WT; light gray line, activated DN Raf. *E*, Thymocytes from WT and DN Raf mice were stained for CD4, CD8 and Foxp3 and analyzed as in *A*. Percentage of cells residing within quadrants is shown on the dot plots. *F*, Absolute numbers of $CD4^+CD8^-Foxp3^-$ T cells (*left*) and $CD4^+CD8^-Foxp3^+$ T cells (*right*) were quantified for both WT and DN Raf mice. □, WT; ■, DN Raf.

$CD4^+Foxp3^+TCR^{high}$ cells was observed in SAP-1-deficient animals, although a substantial reduction in the absolute numbers of $CD4^+Foxp3^-TCR^{high}$ cells was detected ($8.1 \times 10^6 \pm 0.5$ vs $4.0 \times 10^6 \pm 0.5$, $p = 0.002$) (Fig. 1*C*). The positive selection defect in SAP-1 deficient animals results in lower numbers of $CD4^+$ and $CD8^+$ T cells in secondary lymphoid organs (4). As in the thymus, the proportion of $CD4^+Foxp3^+$ T cells in spleen and lymph node increased almost 2-fold (Spleen: WT 12.7% ± 0.8, SAP-$1^{-/-}$ 24.1% ± 1.6, ($p < 0.0001$); LN: WT 13.8% ± 0.5, SAP-$1^{-/-}$ 23.1% ± 1.3, $p < 0.0001$). Thus, in contrast to the development of conventional CD4 SP T cells, the development of $CD4^+Foxp3^+$ thymocytes appears to be unaffected by the loss of SAP-1.

Dominant negative (DN) Raf mice display defective development of $CD4^+Foxp3^+$ thymocytes

To gain further insight into the role of SAP-1 linked signal pathways in T_{reg} development, we analyzed thymocytes from transgenic mice expressing a dominant interfering Raf derivative (DN Raf). This protein interferes with Ras-ERK signaling, and consistent with this, these animals were previously shown to exhibit a severe positive selection defect (30); in addition, DN Raf expression is likely to inhibit *Mst*II- and Ask1-induced apoptosis, because these kinases interact with its regulatory domain (34, 35). We used FACS analysis for activation-loop-phosphorylated ERK to demonstrate that the DN Raf transgene indeed substantially inhibits ERK activation upon TCR crosslinking in DP thymocytes (Fig. 1*D*). DN Raf thymocytes exhibited a slight increase in thymic cellularity (WT $133.8 \pm 5.5 \times 10^6$, DN Raf $161.4 \pm 8.4 \times 10^6$; $p < 0.05$) together with ~2-fold reduced proportion of $CD4^+FoxP3^-$ SP cells, comparable to that observed in animals lacking SAP-1 (Fig. 1*E*). In contrast to animals lacking SAP-1, however, the proportion of $CD4^+Foxp3^+$ T cells in DN Raf animals was not significantly different from WT littermates (4.5 ± 0.3% vs 5.6 ± 0.5%; $n = 6$). Thus, DN Raf expression reduces the absolute numbers of DN Raf $CD4^+Foxp3^+$ thymocytes compared with WT littermates ($0.40 \pm 0.02 \times 10^6$ vs $0.30 \pm 0.04 \times 10^6$, $p = 0.03$; $n = 6$) (Fig. 1F). Taken together with data in the previous section, these results suggest that although T_{reg} development involves Raf signaling it does not require the nuclear ERK effector SAP-1.

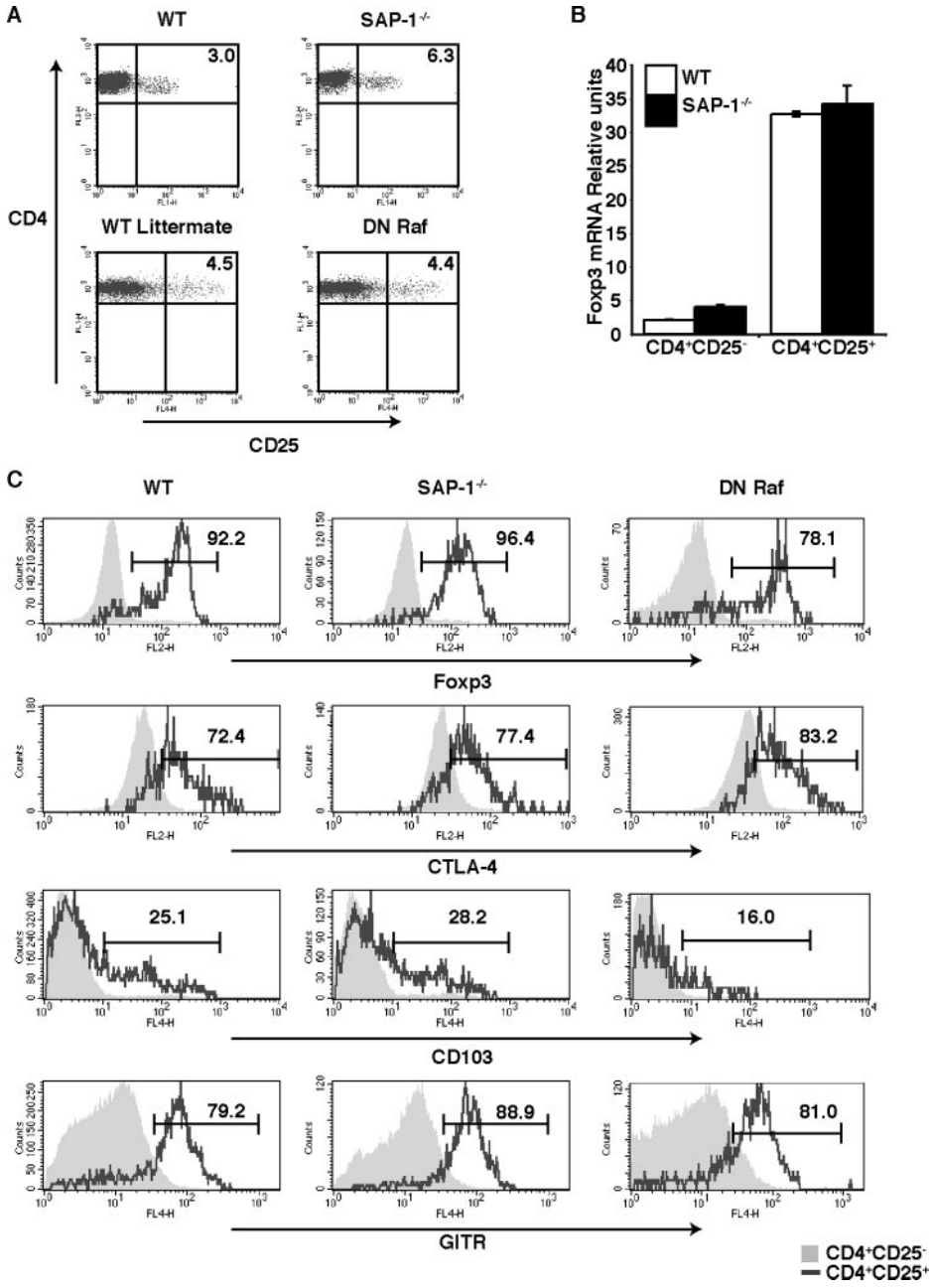

FIGURE 2. SAP-$1^{-/-}$ and DN Raf $CD4^+CD25^+$ express high levels of regulatory T cell markers. *A*, Thymocytes from WT, SAP-$1^{-/-}$, and DN Raf mice were stained for CD4, CD8, and CD25. Percentage of cells residing within quadrants is shown on the dot plots. *B*, Relative levels of Foxp3 mRNA expression normalized to GAPDH expression in $CD4^+CD25^-$ and $CD25^+$ thymocytes. □, WT; ■, SAP-$1^{-/-}$. *C*, $CD4^+CD25^+$ thymocytes from WT, SAP-$1^{-/-}$, and DN Raf animals express high levels of T_{reg} markers. Thymocytes stained for Foxp3 (*top row*) CTLA-4 (*second row*), CD103 (*third row*), and GITR (*bottom row*). Gray shaded area, $CD4^+CD25^-$; gray line $CD4^+CD25^+$.

Normal $CD4^+CD25^+$ T cell markers in SAP-$1^{-/-}$ and DN Raf animals

Previous studies have shown that $CD4^+Foxp3^+$ cells account for the majority of thymocytes expressing the activation marker CD25 (12–14). The effects of SAP-1 inactivation or DN Raf expression on the proportion of $CD4^+CD25^+$ thymocytes within the $CD4^+$ population mirrored those seen with the $CD4^+Foxp3^+$ population, increasing by ~2-fold in animals lacking SAP-1 animals (3.8% ± 0.3 vs 7.2% ± 0.8; $p = 0.0002$) and remaining the same in DN Raf animals (4.2% ± 0.2 vs 4.7% ± 0.4) (Fig. 2*A*). Examination of thymocyte RNA by quantitative RT-PCR showed that Foxp3 mRNA was barely detectable in $CD4^+CD25^-$ cells but was present at high levels in $CD4^+CD25^+$ thymocytes; inactivation of SAP-1 had no effect on this differential mRNA expression (Fig. 2*B*). Approximately 90% of $CD4^+CD25^+$ thymocytes expressed Foxp3 protein, as assessed by intracellular staining, consistent with previous reports that the majority of T_{reg} cells are found within the $CD4^+CD25^+$ population. Again, deletion of SAP-1 or expression of DN Raf had no effect on Foxp3 protein expression (Fig. 2*C*, *top*). Similar results were observed upon analysis of peripheral lymphoid organs (Spleen: WT 95.1% ± 0.8 Foxp3-positive $CD4^+$ cells, SAP-$1^{-/-}$ 95.1% ± 1.9; DN Raf 86.1% ± 3.3; LN: WT 89.8% ± 3, SAP-$1^{-/-}$ 89.5% ± 2.7, DN Raf 88.6% ± 0.6). Furthermore, bone marrow reconstitution experiments indicated that the apparently normal development of SAP-1 deficient $CD4^+CD25^+$ T cells was cell autonomous (data not shown).

Thymic $CD4^+CD25^+$ cells also express a number of surface markers associated with the T_{reg} phenotype, including CTLA-4, GITR and CD103. These surface molecules are all more highly expressed on T_{regs} than on naive $CD4^+$ T cells, although they can be induced upon activation of naive T cells. Both thymic and peripheral SAP-$1^{-/-}$ and DN Raf $CD4^+CD25^+$ T cells expressed all of these markers at a similar level to WT

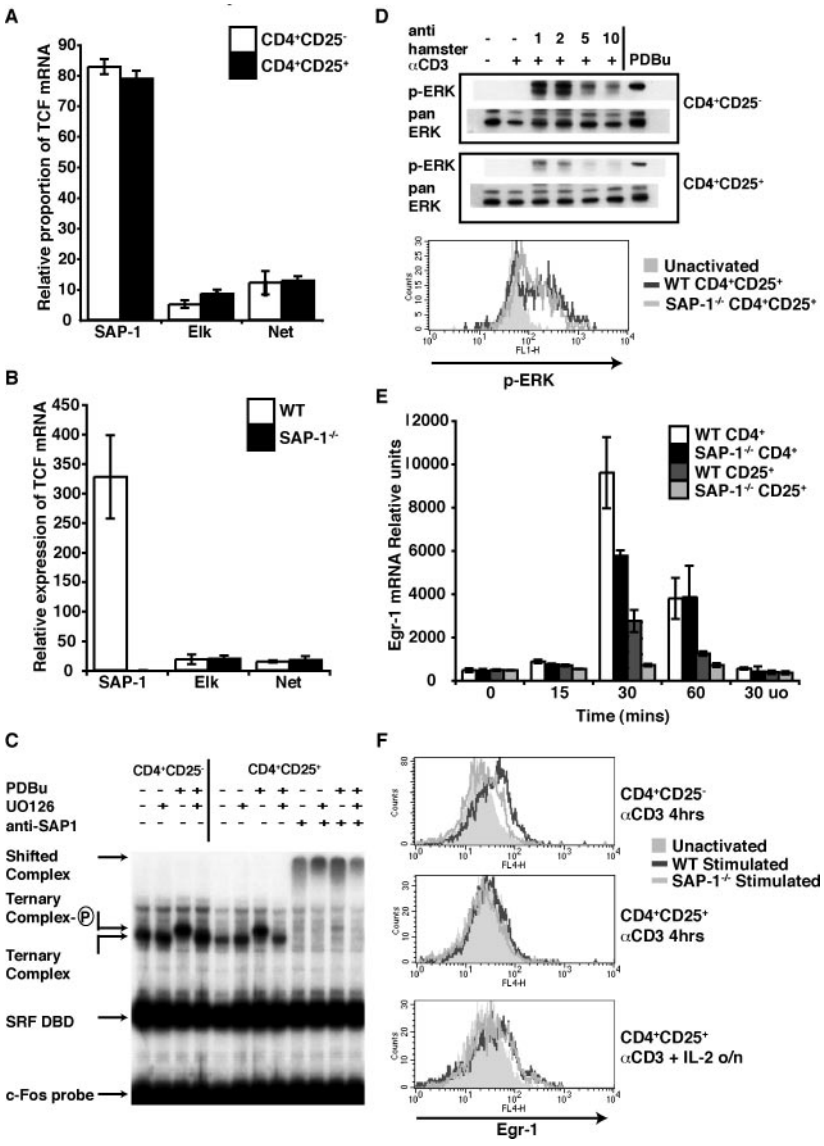

FIGURE 3. TCFs are expressed in T_{regs} but responses to TCR stimulus are reduced. *A*, Relative contribution of the three TCF mRNAs in thymic T_{regs} compared with conventional $CD4^+$ thymocytes. Expression measured by real-time RT-PCR and normalized to GAPDH. □, $CD4^+CD25^-$; ■, T_{regs}. *B*, Relative expression of TCF expression in WT and SAP-$1^{-/-}$ T_{regs} as measured by real-time RT-PCR shows no compensatory increase in Elk-1 or Net mRNA upon loss of SAP-1 expression. □, WT; ■, SAP-$1^{-/-}$. *C*, Gel mobility shift assay performed with c-Fos serum response element probe on peripheral $CD4^+CD25^-$ or $CD4^+CD25^+$ T cell extract with or without PBDu stimulation, in the presence of recombinant SRF DNA binding domain. MEK inhibitor UO126 or anti-SAP-1 Ab were added to the reactions where indicated. Identities of the complexes are indicated. *D*, p-ERK levels measured by Western blot (*top*) and intracellular staining (*bottom*) upon TCR activation by α-CD3 crosslinking of peripheral and thymic T_{regs}. Intracellular staining: gray shaded area, unactivated; gray lines, α-CD3 crosslinking for 2 min; dark gray, WT $CD4^+CD25^+$ thymocytes; light gray SAP-$1^{-/-}$ $CD4^+CD25^+$ thymocytes. *E*, Egr-1 mRNA induction upon TCR activation measured by real-time RT-PCR and normalized to hypoxanthine phosphoribosyltransferase. *F*, Egr-1 protein levels measured by intracellular staining. *Top*, α-CD3 activation of $CD4^+CD25^-$ thymocytes (4 h); *middle*, α-CD3 activation of $CD4^+CD25^+$ thymocytes (4 h); *bottom*, overnight stimulation of $CD4^+CD25^+$ thymocytes with α-CD3, APCs, and IL-2.

$CD4^+CD25^+$ T cells (Fig. 2*C*; data not shown) and at a higher level than their $CD25^-$ counterparts. Taken together, these data indicate that the T_{reg} cells from animals deficient for SAP-1 or expressing the DN Raf transgene appear normal, and we therefore used $CD4^+CD25^+$ cells for further functional studies.

SAP-1 is the predominant TCF in T_{regs}

Previous studies have shown that the suppressive effects of T_{reg} cells in in vitro coculture assays are dependent on activation of the T_{reg} TCR (18–20). As a prelude to functional studies, we therefore examined TCR-mediated activation of immediate-early gene expression in $CD4^+CD25^+$ cells. SAP-1 is the major TCF family member expressed in thymus; accounting for ~70% of TCF mRNA in total $CD4^+$ thymocytes, with the remainder comprising 20% Net and 10% Elk mRNA, as determined by RNase protection analysis (4). RT-PCR analysis indicated that the relative expression levels of the three TCF family members are similar in $CD4^+CD25^+$ and $CD4^+CD25^-$ thymocytes, with SAP-1 accounting for 80% of the TCF transcripts (Fig. 3*A*). Deletion of SAP-1 did not result in compensatory up-regulation of other TCF family members in $CD4^+CD25^+$ thymocytes (Fig. 3*B*).

To assess the TCF activity in T_{reg} cells at the biochemical level, peripheral T cell extracts were analyzed by gel mobility shift assay, in which formation of ternary complexes between TCFs and the DNA-binding domain of their transcription factor partner SRF can be measured directly (36). In both $CD4^+CD25^-$ and $CD4^+CD25^+$ populations, a well-defined ternary complex was formed on the SRF DNA-binding domain, which Ab supershift analysis confirmed to contain predominantly SAP-1 (Fig. 3*C*). Extracts from PDBu-stimulated cells generated a ternary complex of reduced mobility, characteristic of phosphorylation of the SAP-1 C terminus (4). No reduction in mobility was observed with extracts of cells stimulated with PDBu in the presence of U0126, indicating that it occurs through activation of MEK-ERK signaling (Fig. 3*C*). Similar results were obtained when complex formation on endogenous SRF was analyzed (data not shown). Thus, as in total thymocyte extracts (4), the majority of TCF activity in $CD4^+CD25^+$ extracts is accounted for by SAP-1, and activation of ERK in these cells induces its C-terminal phosphorylation.

TCR activation in $CD4^+CD25^+$ T cells induces immediate-early gene expression

We next tested whether TCR activation induces ERK activation in $CD4^+CD25^+$ T cells. TCR signaling was triggered by Ab crosslinking of surface-bound α-CD3 and levels of activation-loop-phosphorylated ERK were then measured by immunoblot. Rapid

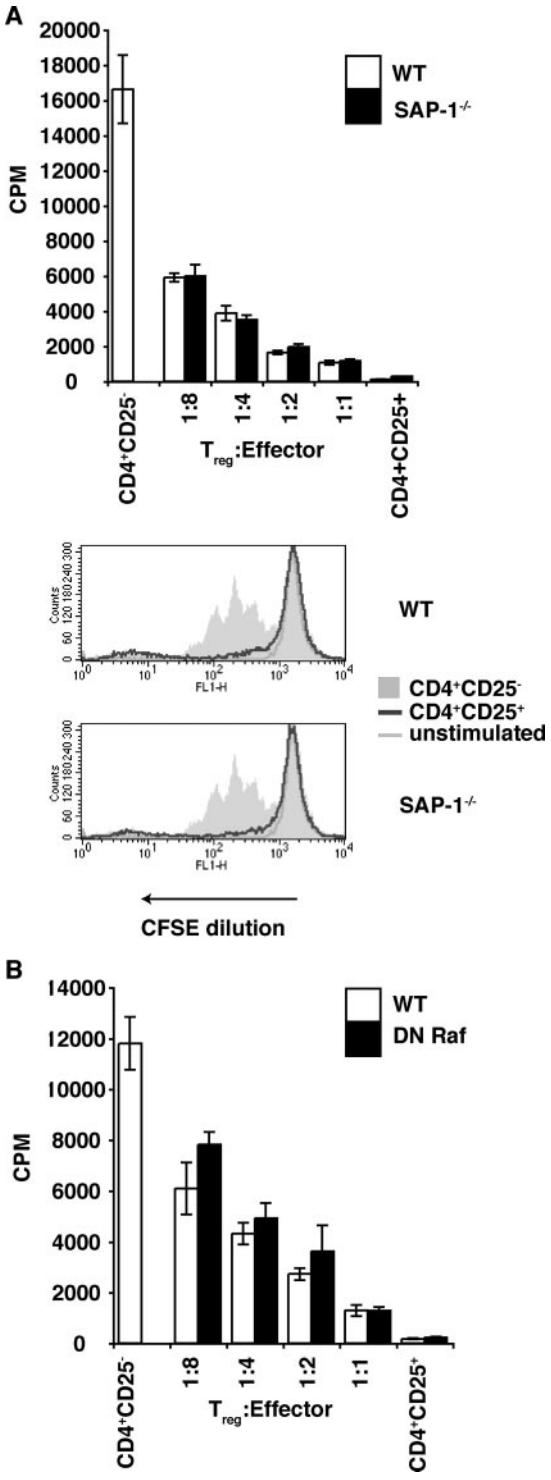

FIGURE 4. In vitro suppression assays. *A*, Thymidine incorporation assays were used to assess in vitro suppressive function at a range of effector:T_{reg} ratios. Thymic $CD4^+CD25^-$ effectors and T_{regs} were used. □, WT; ■, SAP-$1^{-/-}$. CFSE suppression assays performed at a 1:1 ratio of $T_{effector}$:T_{reg}. Gray shaded area, activated effectors at 72 h; dark gray line, 1:1 ratio of $T_{effectors}$:T_{regs}; light gray line, unstimulated effectors. *Top*, WT thymocytes; *bottom*, SAP-$1^{-/-}$ thymocytes. *B*, Suppression assay, assessed by thymidine incoportaion after 72 h. □, WT; ■, DN Raf T cells. $CD4^+CD25^-$ effectors and T_{regs} were taken from lymph nodes.

and transient activation of ERK occurred in both $CD4^+CD25^-$ and $CD4^+CD25^+$ T cell populations, although the degree of activation appeared less in $CD4^+CD25^+$ cells than in $CD4^+CD25^-$ cells (Fig. 3*D*, *top*). As previously shown for DP thymocytes, the level of ERK phosphorylation in $CD4^+CD25^+$ cells was unaffected by the deletion of SAP-1, as assessed by intracellular staining for activated ERK (Fig. 3*D*, *bottom*).

We next examined expression of the TCF target gene Egr-1. $CD4^+CD25^-$ and $CD4^+CD25^+$ populations were activated and levels of Egr-1 mRNA were assessed by quantitative RT-PCR. Maximal induction of Egr-1 transcripts in $CD4^+CD25^-$ T cells was observed 30 min following stimulation. In $CD4^+CD25^+$ cells, stimulation induced Egr-1 with similar kinetics, but to a lower maximal level consistent with the lower level of ERK activation in these cells; activation was substantially reduced in cells lacking SAP-1 (Fig. 3*E*). In both $CD4^+CD25^-$ and $CD4^+CD25^+$ cells induction was blocked by the MEK inhibitor UO126, indicating that it was ERK dependent (Fig. 3*E*). In WT $CD4^+CD25^-$ cells significant up-regulation of Egr-1 protein occurred by 4 h following stimulation, as assessed by intracellular staining, and this was significantly reduced upon deletion of SAP-1 (Fig. 3*F*, *top*). In contrast no up-regulation of Egr-1 protein was detectable in $CD4^+CD25^+$ cells at this time (Fig. 3*F*, *middle*). Previous reports have demonstrated that $CD4^+CD25^+$ cells become proliferative in vitro following TCR activation in the presence of exogenous IL-2 (37). Indeed, a small increase in Egr-1 protein level was observed in $CD4^+CD25^+$ following activation and overnight culture with added IL-2 and APCs, although this was not affected by deletion of SAP-1 (Fig. 3*F*, *bottom*).

SAP-1 deficient and DN Raf T_{regs} are suppressive in vitro

$CD4^+CD25^+$ cells can inhibit proliferation of $CD4^+CD25^-$ effector cells in a suppressive interaction that requires cell contact and activation of their TCR (18–20). Thymic $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were mixed at a range of ratios in the presence of αCD3 and T cell depleted irradiated splenocytes and incubated for 72 h. Proliferation of the effector cells was assessed by either thymidine incorporation or by CFSE dilution. No significant proliferation of either WT or SAP-1 deficient $CD4^+CD25^+$ T cells alone was detectable in this assay, while $CD4^+CD25^-$ cells proliferated efficiently (Fig. 4*A*). As the $CD4^+CD25^+$/$CD4^+CD25^-$ ratio was increased, proliferation of the $CD4^+CD25^-$ cells was inhibited, being effectively blocked at 1:1 ratio. No significant difference could be detected in the abilities of WT and SAP-1 deficient $CD4^+CD25^+$ cells to suppress the proliferation of WT $CD4^+CD25^-$ T cells, whether assessed by thymidine incorporation or CFSE dilution (Fig. 4*A*). Similar results were obtained when SAP-1 deficient $CD4^+CD25^-$ T cells were used as effector cells, and with $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations purified from lymph nodes (data not shown). $CD4^+CD25^+$ T cells purified from lymph nodes of DN Raf mice behaved similarly in this assay, suppressing proliferation of both WT and DN Raf $CD4^+CD25^-$ T cells virtually completely at 1:1 ratio (Fig. 4*B* and data not shown).

SAP-1 deficient $CD4^+CD25^+$ cells are functional in vivo

Finally, we investigated whether the SAP-$1^{-/-}$ T_{regs} were capable of suppression in vivo using the adoptive transfer colitis model (38–40). In this system transfer of naive $CD4^+RB^{high}CD25^-$ T cells into immunodeficient hosts such as Scid or $Rag^{-/-}$ mice induces colitis, characterized by weight loss and inflammation of the colon. Induction of colitis in this model is dependent on gut intestinal flora, and can be driven by IL-23 (41). Development of colitis can be prevented by coinjection of $CD4^+CD25^+$ T_{regs}, and apparently reversed by $CD4^+CD25^+$ cell transfer 4 wk subsequent to the initial transfer (38). In contrast to the contact-dependent

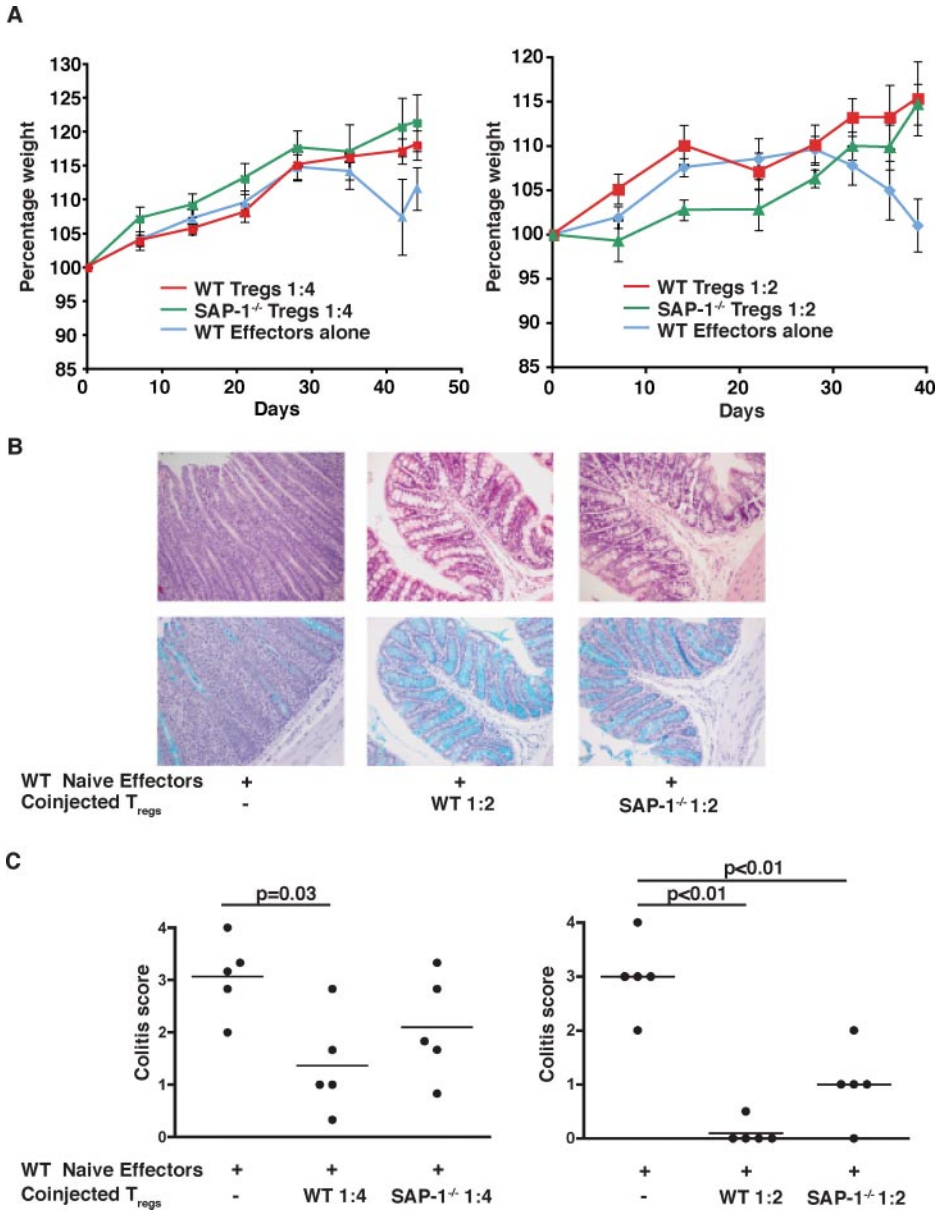

FIGURE 5. SAP-$1^{-/-}$ T_{regs} are function in vivo. T cell transfer experiments were used to assess the in vivo function of T_{regs} through prevention of colitis induction in recipient mice. $Rag2^{-/-}$ mice received either WT naive effectors alone or were coinjected with WT or SAP-$1^{-/-}$ T_{regs} and assessed for presence of disease. *Left*, T_{reg}:$T_{effector}$ 1:4; *right* T_{reg}:$T_{effector}$ 1:2. *A*, Data represent the percentage of initial body weight. Blue line, WT naive effectors alone; red line, WT T_{regs} cotransferred with WT effectors; green line, SAP-$1^{-/-}$ T_{regs} cotransferred with WT effectors. Weight loss was significantly prevented by cotransfer of $CD4^+CD25^+$ T cells ($p = 0.02$ for both WT and SAP-$1^{-/-}$) at 1:2 T_{reg}:$T_{effector}$ ratio (*right*). *B*, Histological sections of distal colon. *Left*, naive WT effectors only; *middle*, cotransfer of WT T_{regs} with effectors (1:2); *right*, cotransfer of SAP-$1^{-/-}$ T_{regs} with effectors (1:2). *Upper*, H & E staining; *lower*, Alcian blue staining for goblet cells. *C*, Colitis scores: *left*, T_{reg}:$T_{effector}$ 1:4; *right*, T_{reg}:$T_{effector}$ 1:2.

mechanism of suppression in vitro, suppression of colitis in this model by T_{regs} is dependent on TGFβ and on IL-10 (42, 43).

$Rag2^{-/-}$ mice were injected with naive WT $CD4^+RB^{high}CD25^-$ T cells either alone or in combination with WT or SAP-1 deficient $CD4^+CD25^+$ T cells. Animals injected with effector cells alone, but not those coinjected with $CD4^+CD25^+$ T cells, began losing weight by 4 wk (Fig. 5*A*). Weight loss observed at 40 days post transfer was significantly prevented by the cotransfer of T_{regs} at a ratio of 1:2 (WT $CD4^+CD25^+$ $p = 0.02$, SAP-$1^{-/-}$ $CD4^+CD25^+$ $p = 0.02$). Because wasting does not necessarily correlate with severity of colitis, we performed histological analysis assessing pathology according to the level of T cell infiltrates, loss of colon architecture, and depletion of goblet cells. In mice injected with naive $CD4^+CD45RB^{high}CD25^-$ T cells alone, histological analysis showed clear changes in colon architecture involving elongation of crypt length; substantial lymphocyte infiltration was also apparent (Fig. 5*B*, *left*). In contrast, animals receiving a coinjection of WT $CD4^+CD25^+$ T cells showed no such changes (Fig. 5*B*, *center*). Similarly, staining with Alcian blue revealed loss of goblet cells in those animals injected with WT naive effectors alone but not those in which WT $CD4^+CD25^+$ T cells were cotransferred (Fig. 5*C*, *center*). Pathology was quantified using a standard colitic scoring scheme (0 to 4 with increasing severity). All of the animals injected with $CD4^+CD45RB^{high}CD25^-$ T cells alone developed marked colitis (score of 2 or over) whereas transfer of WT $CD4^+CD25^+$ T cells at 1:2 ratio to effectors prevented development of disease. Similar results were observed with SAP-1 deficient $CD4^+CD25^+$ cells (Fig. 5*C*). A two-fold lower dose of $CD4^+CD25^+$ cells (1:4 T_{reg}:$T_{effector}$ ratio), from either WT or SAP-$1^{-/-}$ mice also reduced the incidence of severe disease (Fig. 5*C*). These results indicate that $CD4^+CD25^+$ T cells lacking SAP-1 retain suppressor activity both in vitro and in vivo.

Discussion

In this work, we used two animal models to study the relationship between thymocyte positive selection and the development of CD4 regulatory T cells. Animals lacking the nuclear ERK effector SAP-1, a member of the TCF family of Ets domain proteins, exhibit a 50% reduction in positive selection (4). Despite this, in SAP-1-deficient animals, we found no defect in development of regulatory T cells, which appeared normal according to expression of Foxp3 and of other markers associated with T_{reg} cells including

CD25, GITR, CTLA-4, and CD103. In contrast, we found that T cell-restricted expression of a dominant interfering DN Raf transgene (30) inhibited both positive selection and $CD4^+Foxp3^+$ cell development to a comparable extent, although it did not affect T_{reg} suppressive function in vitro.

DN Raf, which comprises the N-terminal regulatory domain of Raf, exerts its effects by titrating Ras, its upstream regulator, and we confirmed that its expression inhibits ERK signaling. However, DN Raf also binds two proapoptotic MAPKKKs, *Mst*II and Ask1 (34, 35). Two considerations lead us to propose that its effects on T_{reg} development are due to its effects on ERK signaling, however. First, studies by the Hedrick group have shown that thymocyte-specific inactivation of both ERK1 and ERK2 reduced $CD69^{high}TCR^{high}$ thymocyte numbers by >99% (2). Because T_{reg} cells represent around 4% of the mature $CD4^+$ thymocyte population, it is therefore likely that T_{reg} generation is also impaired in these animals. Second, the Raf N-terminus acts to inhibit the proapoptotic function of Ask1 and *Mst*II (34, 35), and so its expression might if anything be expected to increase rather than decrease cell numbers. We thus favor the interpretation that T_{reg} development requires Ras signaling to ERK but is independent of the nuclear ERK effector SAP-1, or SAP-1 target gene expression.

The two other TCF family members, Elk-1 and Net, are also targets for ERK signaling, and are also expressed in thymus, albeit at lower level than SAP-1. Animals lacking Elk-1 or Net show no obvious signs of autoimmunity (44, 45), and possess normal numbers of $CD4^+Foxp3^+$ thymocytes; moreover, simultaneous inactivation of Elk-1 and SAP-1, or Net and SAP-1, does not affect T_{reg} numbers, even though the positive selection defect is even more pronounced in the Elk-$1^{-/-}$ SAP-$1^{-/-}$ animals (J. Willoughby, R. Nicolas, P. Costello, A. Nordheim, B. Wasylyk, and R. Treisman, unpublished observations). It is therefore unlikely that the other TCF family members play specific roles in T_{reg} development, although we cannot rule out the possibility that T_{reg} development requires a low threshold level of TCF activity. In any case, such properties still indicate different signaling requirements for T_{reg} development and positive selection.

A simple interpretation of our findings is that the signaling pathways downstream of the TCR that lead to expression of Foxp3 and commitment to the T_{reg} lineage are different from those involved in positive selection, even though thymic development of $CD4^+CD25^+$ T_{reg} cells is dependent on TCR-MHC:self-peptide interactions (21–24, 27). It remains possible, however, that as the strength of signal increases, the dependence of positive selection on ERK-SAP-1 decreases, such that high affinity TCRs, such as those inducing T_{reg} selection, no longer require ERK-SAP-1 signaling to escape death by neglect. According to this view, inactivation of ERK-SAP-1 signaling would differentially affect selection according to the avidity of the TCR-peptide interaction, and it might be interesting to test this idea using transgenic TCR models. Our findings that DN Raf inhibits T_{reg} development raises the possibility that ERK signaling controls activation of Foxp3 expression, but the nature of the link, if any, remains to be determined.

Naturally occurring T_{regs} have distinct responses to TCR signaling when compared with conventional $CD4^+$ T cells. Initially described as being anergic, recent data have shown that T_{regs} can proliferate in vitro if exogenous IL-2 is added to the culture (37) and in vivo data has shown that these cells can expand and proliferate. TCR signaling in T_{regs} thus results in a distinct outcome from that seen in $CD4^+$ T cells. We confirmed that TCR activation in T_{reg} cells results in ERK activation and transcription of the immediate-early gene Egr-1, although to a lesser extent than in $CD4^+CD25^-$ cells. Indeed, at the protein level little Egr-1 induction was observed in the absence of secondary stimulation by IL-2. As in $CD4^+CD25^-$ cells, SAP-1 is the predominant TCF in T_{reg} cells, and its inactivation results in reduced Egr-1 transcription in response to TCR activation. It is tempting to speculate that the reduced efficiency of IE gene induction in T_{reg} cells underlies their nonproliferation, because reduction of the TCF gene dose in T cells impairs proliferation in response to TCR activation (P. Costello and R. Treisman, unpublished observations).

Despite the reduced activation of immediate-early gene transcription seen in SAP-1 deficient $CD4^+CD25^+$ cells, we found that these cells remained fully competent to suppress proliferation of $CD4^+CD25^+$ cells in vitro, as did DN Raf $CD4^+CD25^+$ cells. $CD4^+CD25^+$ cells lacking SAP-1 also retained the ability to inhibit colitis in the T cell transfer model. Together, the data suggest that although the Raf/ERK signaling pathway plays a role in the development of T_{reg} thymocytes, it does not appear to be required for their suppressive functions, even though these involve TCR activation.

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Disclosures

The authors have no financial conflict of interest.

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