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FOXP3 renders activated human regulatory T cells resistant to restimulation-induced cell death by suppressing SAP expression

Gil Katz[*,¥], **Kelsey Voss**[*,¥], **Toria F. Yan**[¥], **Yong Chan Kim**[§], **Robert L. Kortum**[¥], **David W. Scott**[§], and **Andrew L. Snow**[¥]

[¥]Department of Pharmacology & Molecular Therapeutics, Uniformed Services University of the Health Sciences, Bethesda, MD

[§]Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD

Abstract

Restimulation-induced cell death (RICD) is an apoptotic program that regulates effector T cell expansion, triggered by repeated stimulation through the T cell receptor (TCR) in the presence of interleukin-2 (IL-2). Although $CD4^+$ regulatory T cells (Tregs) consume IL-2 and experience frequent TCR stimulation, they are highly resistant to RICD. Resistance in Tregs is dependent on the forkhead box P3 (FOXP3) transcription factor, although the mechanism remains unclear. T cells from patients with X-linked lymphoproliferative disease (XLP-1), that lack the adaptor molecule SLAM-associated protein (SAP), are also resistant to RICD. Here we demonstrate that normal Tregs express very low levels of SAP compared to conventional T cells. FOXP3 reduces SAP expression by directly binding to and repressing the *SH2D1A* (SAP) promoter. Indeed, ectopic SAP expression restores RICD sensitivity in human $FOXP3^+$ Tregs. Our findings illuminate the mechanism behind FOXP3-mediated RICD resistance in Tregs, providing new insight into their long-term persistence.

Keywords

Restimulation-induced cell death; FOXP3; apoptosis; regulatory T cells; SAP

1. Introduction

An efficient adaptive immune response relies on the rapid clonal expansion of effector T cells, followed by the timely disposal of these cells to avoid nonspecific damage to host tissues. The adaptive immune system utilizes specific apoptosis programs to regulate T cell expansion and contraction and maintain homeostasis [1]. One of these programs is restimulation-induced cell death (RICD). RICD helps to keep effector T cell expansion in

Corresponding Author: Andrew L. Snow, 4301 Jones Bridge Rd., Room C-2013, Bethesda, MD 20814, Phone: 301-295-3267, andrew.snow@usuhs.edu.

[*]These authors contributed equally to this work

check, triggered by T cell receptor (TCR) restimulation in the presence of interleukin-2 (IL-2) [2-5]. Recent work from our lab has demonstrated that RICD sensitivity can be tuned by alterations in cell metabolism or diacylglycerol kinase (DGK) activity [6, 7]. A better understanding of the underlying mechanisms that influence RICD can therefore lead to novel strategies for controlling the magnitude and potency of a T cell response. However, our knowledge of the molecular signals that govern RICD sensitivity remains inadequate, especially with regard to different T cell populations with variable longevity.

The physiological relevance of RICD is best illustrated in patients with X-linked lymphoproliferative disease (XLP-1), who experience life-threatening, uncontrolled accumulation of $CD8^+$ T cells in response to Epstein-Barr virus (EBV) infection [8]. A marked defect in RICD contributes to unconstrained $CD8^+$ T cell expansion in this setting, resulting in severe immunopathology [9]. XLP-1 patients harbor null mutations in the signaling adaptor SLAM-associated protein (SAP), which is required for RICD [9, 10]. Indeed, work from our lab and others has shown that SAP associates with the signaling lymphocyte activation molecule (SLAM) family receptor NTB-A to potentiate TCR signal strength by displacing SHP-1 and recruiting LCK instead of FYN, which facilitates downstream signaling for apoptosis after TCR re-engagement [11-13]. SAP also represses DGKα to maintain a sufficient pool of diacylglycerol for robust TCR signaling [7, 14], further highlighting SAP as a central player in promoting RICD of effector T cells.

$CD4^+$ regulatory T cells (Tregs) are a specialized subset of T cells that modulate the immune response and suppress the proliferation of lymphocyte effectors. Given their essential role in maintaining immune homeostasis and preventing autoimmunity [15], Tregs are of special interest regarding their therapeutic potential for tolerance induction in a variety of clinical settings [16, 17]. However, the processes that ultimately maintain Treg cell survival remain unclear [18]. For example, Tregs are extremely resistant to RICD despite frequent stimulation (often via self-antigen), susceptibility to FAS-induced death, and strict dependency on IL-2 derived from effector T cells [19, 20].

The gene encoding SAP, *SH2D1A*, was identified as a FOXP3 target in a genome-wide chromatin immunoprecipitation (chIP) study of human Tregs [21]. We therefore hypothesized that in human Tregs, FOXP3 restricts RICD by directly suppressing SAP expression. Using primary, activated human Tregs and conventional $CD4^+$ T cells (Tcons) derived from healthy human donors, we confirmed that RICD resistance in Tregs is FOXP3 dependent [19]. We now demonstrate that SAP expression is markedly reduced in activated Tregs, and that FOXP3 confers resistance to RICD through repression of the *SH2D1A* promoter. These findings further elucidate the mechanism of RICD resistance in Tregs, providing new insights into Treg homeostasis.

2. Materials and Methods

2.1 Cell isolation and culture conditions

Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats donated by healthy human donors at the National Institutes of Health (NIH) Blood Bank. Access to Blood Bank donors was kindly provided by Dr. Michael Lenardo. $CD4^+$ T cells were

purified from PBMC by immunomagnetic negative selection using the EasySep Human $CD4^+$ T cell enrichment kit (Stem Cell Technologies). Cells were then stained on ice for 30 minutes with the following Abs: anti CD4-FITC (clone RPA-T4), anti-CD25-PE-Cy7 (clone BC96), and anti-CD127-PE (clone A019D5) (Tonbo Biosciences). Tregs and Tcons were sorted on a BD FACSAria cell sorter. The gating strategy is shown in Figure 1, where Tregs were defined as $CD4^+$ $CD25^{hi}$ $CD127^{lo}$ and Tcons were defined as $CD4^+$ $CD25^{lo}$ $CD127^{hi}$ [22].

Sorted cells were activated with anti-CD2/CD3/CD28 antibody-bound biotin beads (Human T cell Activation/Expansion Kit, Miltenyi) in complete RPMI (RPMI 1640 (Life Technologies) + 10% fetal calf serum (FCS) (HyClone) + 1% penicillin/streptomycin (Lonza) + 2 μM ODN [23] for 3 days. Activated T cells were then washed in PBS and subsequently cultured in media as described above with 200 U/mL rIL-2 (PeproTech) and 2 μM ODN at 1×10^6 cells/mL, replacing the media every 3 days. Jurkat T cells were obtained from the American Type Culture Collection (clone E6.1) and cultured in complete RPMI at 37°C and 5% CO_2.

2.2 Flow cytometry and apoptosis assays

RICD assays were performed as described previously [24]. Briefly, 1×10^5 effector T cells were restimulated with 1 μg/ml anti-CD3 mAb (clone OKT3) plus protein A (2 μg/ml) in triplicate wells for 24 hours. Cells were stained with 50nM TO-PRO-3 (Thermo Fisher) to distinguish live and dead cells, and analyzed on a BD Accuri C6 flow cytometer. Death was quantified as percent cell loss, based on quantification of viable cells collected under constant time, where % cell loss = (1 – [number of viable cells (treated) / number of viable cells (untreated)]) × 100.

For surface receptor staining, cells were washed in PBS + 1% FBS + 0.01% sodium azide and incubated with antibodies against CD3, CD25, NTB-A, CD95 (FAS) and CD69 (BD Biosciences) on ice for 30 minutes. Intracellular staining was performed using the FOXP3 intracellular staining kit with anti-FOXP3-APC Ab (eBioscience). All flow cytometry analysis was performed with FlowJo version 10.

2.3 Western blotting

Cells were lysed in 1% Nonidet P-40 (NP-40) lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, 1 mM NaF) + complete protease inhibitors (Roche) for 30 minutes on ice. Lysates were cleared by centrifugation and boiled in 2× sample buffer (Laemmli buffer + 50 μM 2-βME) and separated on SDS-PAGE gels (Bio-Rad). Using the Trans-Blot Turbo system (Bio-Rad), proteins were transferred to nitrocellulose membranes and subsequently blocked with 2% Tropix I-Block (Applied Biosystems). Blots were probed with the following antibodies: anti-FOXP3 (Novus Biologicals NB600-245), anti-SAP, anti-LCK (Cell Signaling Technology), anti-β-actin (Sigma-Aldrich). After washing in TBS/0.1% Tween20, blots were incubated with horseradish peroxidase-conjugated secondary Abs (Southern Biotech), washed again, and developed using enhanced chemiluminescence (SuperSignal, ThermoFisher).

2.4 Quantitative RT-PCR

Total RNA was isolated from T cells using QIAshredder and RNeasy Mini Plus columns with DNase digestion (Qiagen). cDNA was prepared using the i Script cDNA kit for RT-qPCR (Bio-Rad), and qPCR was performed with Maxima SYBR Green/ROX qPCR Master Mix (ThermoFisher) using a two-step cycling protocol: 95°C for 1 minute, 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds, followed by a final elongation step at 60°C. The primers used were: SAP forward 5′-tctgtatgaaccctgtgttgg-3′, SAP reverse 5′-acaggatgttgtctacttgcc-3′, FOXP3 forward 5′-gatggtacagtctctggagc-3′, FOXP3 reverse 5′-gggaatgtgctgtttccatgg-3′, RPL30 forward 5′-gaatggcatggtcttgaagcc-3′, and RPL30 reverse 5′-ggccaccttcttgtgaatgc-3′.

2.4 SAP promoter cloning and luciferase assays

The pGL3 Firefly luciferase basic vector (Promega) was digested with KpnI and XhoI in order to insert *SH2D1A* promoter inserts of 1000 bp (maximum activity) versus 100bp (minimal activity) as previously published [25]. Segments of the *SH2D1A* promoter were amplified using these primers: SAPprom-F1: 5′ atttgcattaatacagtttagcctcaatcgaag-3, SAPprom-F2: 5′ -atttgcggtaccgttgttggggtgcttctctc-3′, SAPprom-R: 5′-ttactcaccggtggcctggtggactcttgg-3′.

Expression cassettes encoding WT FOXP3 or GFP were PCR amplified and cloned into the pmax vector (Lonza) after cutting with KpnI and XhoI. PCR primers used were: pmaxFOXP3-F 5′-atttgcggtaccgccgccaccatgcccaaccccaggcctg-3′, pmaxFOXP3-R 5′-ttactcctcgagtcaggggccaggtgtaggg-3′, The loss of function mutation in the DNA binding domain of FOXP3 (R397W) was introduced by site-directed mutagenesis using DpnI digestion and the following primers for amplification: R397W-F 5′-tgctttgtgTgggtggagagcgagaagg-3′, R397W-R 5′-gctctccacccAcacaaagcacttgtgcagac-3′. For luciferase assays, Jurkat T cells were triple transfected with 5 μg pmax-GFP or FOXP3 expression vector, 5 μg pSAP-GL3 firefly luciferase vector, 0.5 μg pRL-TK renilla luciferase vector, and the pmax vector and assayed after 24 hours. Luciferase activity was quantified using a Dual Luciferase Assay Kit (Promega) and a Synergy H1 microplate reader (Bio-Tek).

2.5 Chromatin immunoprecipitation

To perform chromatic immunoprecipitation (chIP) of Jurkat T cells and/or primary T cells, we used the Simple ChIP Plus enzymatic chromatin IP kit (Cell Signaling Technology) with the addition of anti-FOXP3 antibody Clone 206D (Biolegend) or the Mouse IgG1 isotype κ (BD) control antibody. DNA eluted from IPs was amplified by PCR (Apex 2.0 × Taq RED mastermix) with SAP promoter primers SAP-F 5′-aagttattcctggtggcctc-3′, and SAP-R 5′-ttggttcgatcgagcttgcc-3′. Cycling conditions were 95°C for 180 seconds, 32 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, followed by 72°C for 5 minutes. PCR products were then electrophoresed on a 1% agarose gel (1× TAE) with ethidium bromide for visualization.

2.5 Lentiviral infections and siRNA transfections

pCLS-YFP-2A-FOXP3 and GFP lentiviral vectors were kindly provided by Dr. James Riley [26]. YFP-2A-FOXP3 and GFP expression cassettes were subcloned into the pWPT lentiviral transfer vector using BamHI and SalI. Lentiviruses were produced by co-transfecting the lentiviral plasmid with dR8.2 and VSV-G packaging vectors into 293T cells using calcium-phosphate. Two viral collections were made 48 to 72 h post-transfection, and viral supernatants were pooled and filtered.

For lentiviral transduction of primary T cells, 24 well plates were coated with retronectin overnight at 4°C at 20 μg/ml (Clontech Laboratories). Wells were blocked with 2% BSA in PBS for 30 minutes, and then viral supernatants (or media alone) were added to the plate and centrifuged for 2 hours at 2000 × g at 32°C. Unbound virus was washed from the wells with PBS, and then cells in complete RPMI + 200 U/mL rIL-2 were transferred to the plate and centrifuged at 2000 × g for 15 minutes at 32°C. GFP/YFP expression was measured 24 hours post-infection by flow cytometry to measure transduction efficiency. RICD assays were analyzed on cells counted from gated GFP/YFP^{+} populations.

T cells were electroporated with siRNAs against FOXP3 (FOXP3HSS121456) and SAP (SH2D1AHSS106218) (ThermoFisher) using the Amaxa Nucleofection 4D system and the P3 Primary Cell kit (Lonza). Stealth RNAi negative control medium GC duplex (ThermoFisher) siRNA was used as a non-specific (NS) control, and all assays were conducted 4 days post electroporation for peak knockdown efficiency.

2.6 Statistics

To assess significance in RICD assays, Student t-tests were used in assays comparing two groups, and two-way ANOVA tests were performed for multiple comparisons (Tukey's multiple comparisons test). For all figures, p-values <0.05 are indicated by *, and values <0.01 by **. Prism 7 software (GraphPad) was used for all statistical analyses.

3. Results

3.1 Activated human Tregs display minimal SAP expression and are resistant to RICD

Previous work has shown that human Tregs are resistant to RICD, which is dependent on FOXP3 expression [20]. However, moderate FOXP3-mediated suppression of FASL alone does not completely account for the profound RICD insensitivity in Tregs [19]. To explore this further, we first verified that primary CD25^{hi}CD127^{lo} Tregs sorted from healthy human donors (Figure 1) were resistant to RICD compared to Tcons. As expected, activated Tcons were significantly more sensitive to RICD than Tregs in every donor tested (Figure 2A). However, Tcons and Tregs were equally sensitive to death by direct ligation of the FAS death receptor (Figure 2B). Importantly, apoptosis sensitivity in Tregs could not be explained by alterations in the expression of the T cell receptor (CD3) or FAS, because expression levels were similar in Tcons and Tregs (Figure 2C). The SLAM family receptor NTB-A, which is required for optimal RICD sensitivity [9, 11], also exhibited similar expression in Tregs and Tcons (Figure 2C). As expected, Tregs maintained elevated expression of the high affinity IL-2 receptor CD25 compared to Tcons (Figure 2C).

T cells deficient in SAP expression, such as in XLP-1 patients, are highly resistant to RICD [11]. We therefore asked whether human Tregs express less SAP compared to Tcons. Indeed, genome-wide ChIP analysis of human Tregs uncovered *SH2D1A* as a FOXP3 target gene, although this particular finding was not subjected to further validation [21]. We therefore hypothesized that FOXP3 confers RICD resistance in activated Tregs by repressing the *SH2D1A* promoter and reducing SAP expression. We observed that SAP mRNA levels were markedly reduced in activated Tregs compared to Tcons, exhibiting an inverse relationship with FOXP3 expression (Figure 3A). As expected, this correlated with decreased SAP protein in Treg cell lysates (Figure 3B). Overall, our survey of cells from multiple human donors indicated that activated Tregs contain very little SAP expression compared to Tcons, and are extremely resistant to RICD, similar to XLP-1 patient-derived SAP-deficient T cells.

3.2 Expression of FOXP3 suppresses SH2D1A promoter activity

We next tested whether ectopic FOXP3 expression can repress *SH2D1A* gene promoter activity. In silico analysis of a ~1000 bp sequence of the proximal *SH2D1A* promoter revealed at least three putative FOXP3 binding sites [21, 25] (Figure 4A). In order to measure *SH2D1A* promoter activity +/- FOXP3 expression, we employed a luciferase reporter system in Jurkat T cells. Both wild-type (WT) and a loss-of-function mutant of FOXP3 (R397W, defective in DNA binding [27]) were cloned into the pmax expression vector; GFP served as a negative control. The ~1000 bp fragment of the proximal *SH2D1A* promoter was cloned into the pGL3 firefly luciferase vector to create a reporter plasmid of *SH2D1A* promoter activity (~1000bp), with a smaller ~100 bp promoter fragment with minimal activity serving as a negative control. As predicted, the ~1000 bp promoter sequence displayed ~3-fold higher activity than the minimal ~100 bp sequence (Figure 4B). With concomitant transfection of GFP or FOXP3 expression plasmids, we found that robust transient expression of WT FOXP3 substantially reduced luciferase expression driven by the 1000bp *SH2D1A* promoter (Figure 4B). In contrast, FOXP3 R397W failed to repress SAP promoter activity despite comparable overexpression (Figure 4B).

3.3 FOXP3 directly binds to the SH2D1A promoter

The aforementioned results strongly suggested that FOXP3 can directly suppress *SH2D1A* promoter activity through its DNA binding function. To assay for direct FOXP3 binding to the *SH2D1A* gene, we first derived Jurkat T cells stably expressing FOXP3, as opposed to the transient overexpression system described above. Lentiviruses encoding YFP-2A-FOXP3 (allowing for bicistronic expression) or GFP [26] were used to transduce Jurkat T cells. Robust expression of GFP/YFP was maintained over several weeks in culture, indicating stable integration (Figure 5A). FOXP3 protein expression was also readily detected by Western blot (Figure 5B), although at reduced levels compared to transient Jurkat transfections (Figure 4C). As expected, endogenous SAP expression was reduced in Jurkat T cells that stably expressed FOXP3 compared to the GFP control (Figure 5B).

To provide direct evidence of FOXP3 binding to the *SH2D1A* promoter, we lysed Jurkat T cells stably transduced with FOXP3 or GFP and performed chromatin immunoprecipitations (chIP) using an anti-FOXP3 or isotype control antibody. Indeed, we detected an enrichment

of *SH2D1A* promoter DNA in FOXP3 IPs relative to an IgG isotype control IP (Figure 5C). Importantly, Jurkat cells expressing GFP alone did not show the same amplification of the *SH2D1A* promoter region after FOXP3 IP. Immunoprecipitation with an anti-histone H3 antibody reliably detected both *SH2D1A* and the housekeeping gene *RPL30*, which confirmed that our ChIP procedure was efficient and selective based on the IP Ab used.

To confirm these findings, lysates derived from activated, primary human Tregs and Tcons were also subjected to chIP with FOXP3 and IgG Abs and analyzed for *SH2D1A* promoter binding. As expected, activated Tregs showed enrichment of *SH2D1A* promoter DNA in FOXP3 IPs, whereas Tcons did not (Figure 5D). Taken together, these results demonstrate that FOXP3 can directly bind to the *SH2D1A* promoter, where it is readily detected in activated human Tregs.

3.4 FOXP3-mediated RICD resistance in Tregs is dependent on repression of SAP expression

To further explore the mechanism of FOXP3-dependent RICD resistance in human Tregs, we next investigated whether FOXP3-mediated repression of SAP is required for RICD resistance. Freshly isolated Tregs were transfected with non-specific (NS), FOXP3-specific, or FOXP3- plus SAP-specific siRNA and stimulated *in vitro*. Knockdown of FOXP3 expression sensitized Tregs to RICD, confirming previous reports [19]. However, concomitant knockdown of SAP completely blocked the increase in RICD afforded by FOXP3 knockdown (Figure 6A). Reduced target protein expression was confirmed for each siRNA transfection by Western blot analysis, and also revealed a modest but significant increase in SAP expression when FOXP3 was silenced in Tregs (Figure 6B-C).

If silencing FOXP3 renders Tregs susceptible to RICD by boosting SAP expression, we reasoned that increasing SAP expression alone should also restore RICD sensitivity. To this end, we infected primary activated Tregs with lentiviral vectors expressing YFP-2A-SAP or GFP. At 24 hours post-infection, we restimulated cells with OKT3 and assayed death in the GFP/YFP$^+$ gated population of transduced cells. Ectopic SAP expression dramatically restored RICD sensitivity in YFP$^+$ transduced Tregs, relative to cells transduced with GFP alone (Figure 6D). These results definitively show that RICD resistance in human Tregs is specifically dependent on FOXP3-mediated repression of SAP expression. Furthermore, ectopic expression of SAP alone (using a FOXP3-irrepressible promoter) is sufficient to sensitize activated Tregs to RICD.

4. Discussion

A sufficient CD4$^+$ Treg population must be preserved in order to stave off autoimmunity. A better understanding of apoptosis sensitivity in Tregs is therefore vital to comprehend mechanisms of immune homeostasis and tolerance. Tregs are CD25^{hi}, consume high amounts of IL-2, and experience frequent stimulation from self-antigens [19, 20]. Whereas IL-2 consumption and TCR restimulation would typically induce RICD in conventional effector T cells, Tregs have adopted unique strategies to evade apoptosis and persist under these conditions [30]. In fact, Treg survival and function is dependent on IL-2 consumption for induction of *FOXP3* mRNA expression and upregulation of the pro-survival protein

MCL-1 to combat pro-apoptotic BIM expression [31]. Surprisingly, Tregs are able to persist despite experiencing even stronger TCR signals in the periphery [32]. We demonstrate here that FOXP3-dependent suppression of SAP renders Tregs resistant to RICD. Although protecting Tregs under these conditions is important for maintaining tolerance, these adaptations to evade RICD could also explain why Tregs are able to persist in the antigen-rich tumor microenvironment, which is likely detrimental to the anti-tumor response [33-35].

Work from our group and others has illuminated a central role for SAP/SLAM receptor signaling in governing RICD sensitivity in T cells [10, 11, 13]. Our results further underscore the importance of SAP-driven signaling in this process, demonstrating that Tregs have evolved to disable this signaling pathway via FOXP3-dependent SAP repression to resist RICD. We posit that other T cell subsets might manipulate SAP-SLAM receptor signaling to elude RICD (e.g. Th17 cells [36]) or adjust RICD sensitivity at distinct phases of the effector T cell response. For example, effector cells that progress into long-lived memory T cell pools could be shaped in part by SAP-SLAM receptor signaling alterations that promote their persistence. After all, dynamic changes in this pathway shape RICD sensitivity at different phases of the conventional effector T cell response; SAP expression is initially kept at low levels after T cell activation, then gradually increases as effector T cells fully differentiate and become more sensitive to RICD [37, 38]. Moreover, transient induction of FOXP3 expression after activation of human Tcons may help preserve these cells from premature RICD during the early clonal expansion phase [39]. However, further work is required to determine whether FOXP3 can repress SAP expression in this context.

Our findings confirm previous work showing FOXP3 is necessary for maintaining RICD resistance in Tregs [19, 20]. Interestingly, ectopic SAP expression was sufficient to sensitize Tregs to RICD, suggesting SAP can override any direct FOXP3-mediated repression of FASL itself to promote apoptosis. On the other hand, ectopic FOXP3 expression alone was not sufficient to significantly reduce SAP expression in Tcons and confer resistance to RICD (data not shown). Indeed, only considerable overexpression of FOXP3 repressed *SH2D1A* promoter activity in our reporter assays, and reduced endogenous SAP expression by ~30% in stably-transduced Jurkat T cells. We speculate that additional binding partners may be required to stabilize and/or cooperate with FOXP3 to suppress SAP expression, similar to other FOXP3 target genes [40, 41]. This idea is also consistent with studies that indicate FOXP3 expression alone is not enough to convert Tcons to Tregs with suppressive function, unless expression levels are artificially high [42, 43]. There could also be competition among other transcription factors in Tcons that is not relevant in Tregs. High levels of retrovirally-introduced FOXP3 in Tcons are necessary to efficiently out-compete endogenous AP-1 for NFAT binding and repression of several activation-induced genes [42]. Similarly, a Treg-specific co-factor may cooperate or synergize with FOXP3 to repress SAP expression by interfering with Ets-1/Ets-2 binding or transactivation at the *SH2D1A* promoter [25]. Finally, additional exogenous factors may also aid in FOXP3-dependent SAP suppression. For example, Bhaskaran and colleagues showed that TGF-β1 promotes RICD resistance in $FOXP3^+$ Tregs, perhaps via increased expression of cFLIP [44].

In conclusion, new mechanistic knowledge regarding apoptosis resistance in Tregs could be utilized to manipulate immune responses by changing Treg apoptosis sensitivity. Tuning T

cell apoptosis sensitivity to shape immune responses has great potential in attenuating autoimmunity and/or enhancing eradication of pathogens and tumors [4]. However, this approach also requires a fuller understanding of how T cell subsets could be differentially impacted. In addition, adoptive Treg cell therapies could be improved by maintaining and/or enhancing RICD resistance. Adoptive transfer of infused Tregs has been used in human clinical trials to help prevent graft versus host disease (GVHD) in transplant recipients [45]. Although GVHD rates declined, no infused Tregs were detected in the blood after 14 days, which likely suggests many of the infused Tregs died by apoptosis. Strategies that specifically stabilize FOXP3-dependent repression of the *SH2D1A* promoter could improve clinical usefulness of Tregs by promoting their longevity *in vivo*. Stabilizing RICD resistance could also preserve infused FOXP3-transduced "regulatory" T cells from patients with immune-dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, which lack natural Tregs due to debilitating *FOXP3* mutations [46]. In conclusion, our findings provide new insight into the puzzling TCR-mediated apoptosis resistance demonstrated by $CD4^+$ Tregs, highlighting the importance of SAP suppression in this phenotype.

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Highlights (Katz & Voss et al.)

- FOXP3^{+} regulatory T cells (Tregs) are resistant to restimulation-induced cell death.
- Human Tregs express much less SAP compared to conventional T cells.
- FOXP3 binds to and suppresses the SH2D1A (SAP) promoter in human Tregs.
- RICD resistance in regulatory T cells is dependent on FOXP3-dependent repression of SAP.
- Ectopic SAP expression is sufficient to restore RICD sensitivity in regulatory T cells.

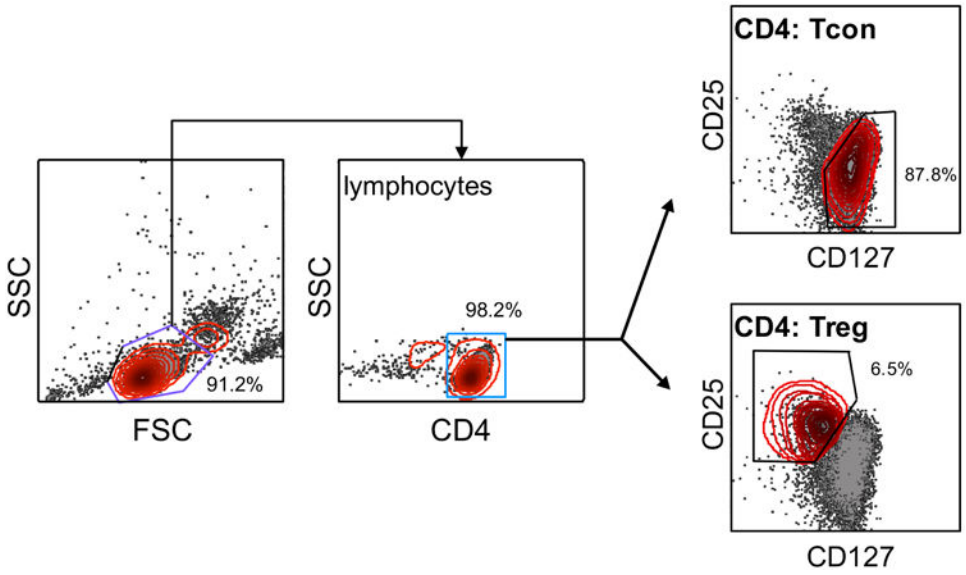

Figure 1. Gating strategy to sort human Tregs and Tcons

$CD4^+$ T cells were isolated from healthy human blood donors by negative selection and stained with CD4, CD25, and CD127 antibodies before sorting. Lymphocytes were delineated by forward/side scatter gating, and $CD4^+$ cells were further separated as $CD25^{hi}$ $CD127^{lo}$ Tregs or $CD25^{lo}$ $CD127^{hi}$ Tcons. A representative sort is shown; % of $CD4^+$ Tcons vs. Tregs are labeled for each gate.

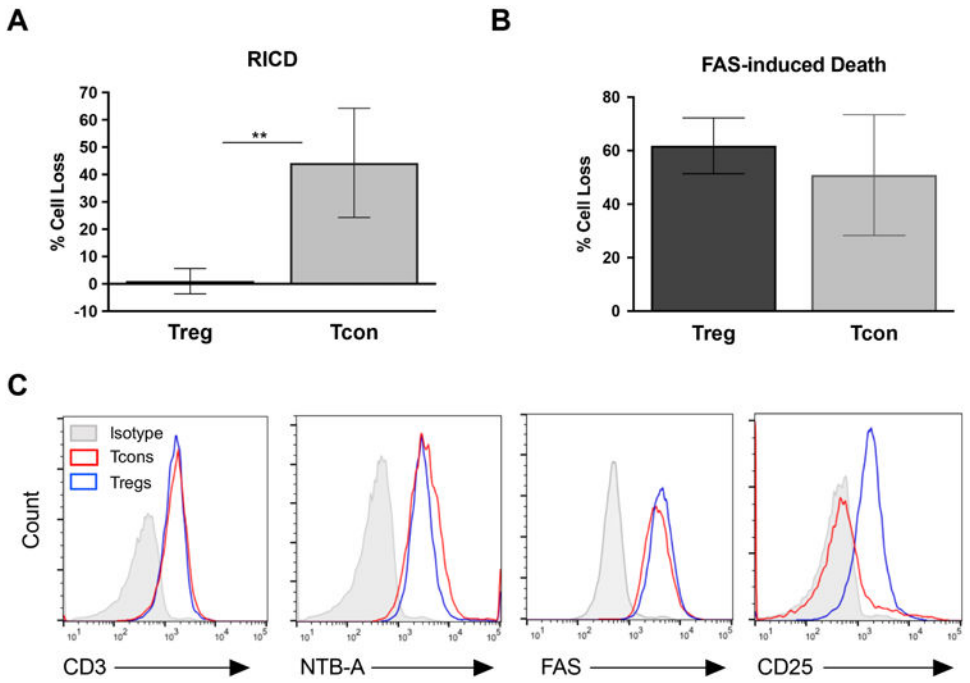

Figure 2. Human Tregs are resistant to RICD, but not FAS-induced death

(A) Sorted Treg and Tcons were activated with anti-CD2/3/28 beads and expanded in culture with IL-2. Cells were then restimulated with OKT3 antibody (1 μg/ml) plus Protein A (2 μg/ml) for 24 hours in triplicate. Cell death was measured by flow cytometry using TO-PRO-3 live/dead staining. Data are average ± SD of 4 independent experiments using separate donors; significance was assessed by an unpaired t-test. **(B)** Cells cultured as in (A) were treated with 200 ng/ml APO1.3 antibody plus Protein A for 24 hours. Cell death was measured as above; results data are average ± SD of 3 independent experiments. **(C)** Cells were stained with antibodies against CD3, NTB-A, FAS, and CD25, or an isotype control antibody, and analyzed by flow cytometry. Results are representative of 3 independent experiments.

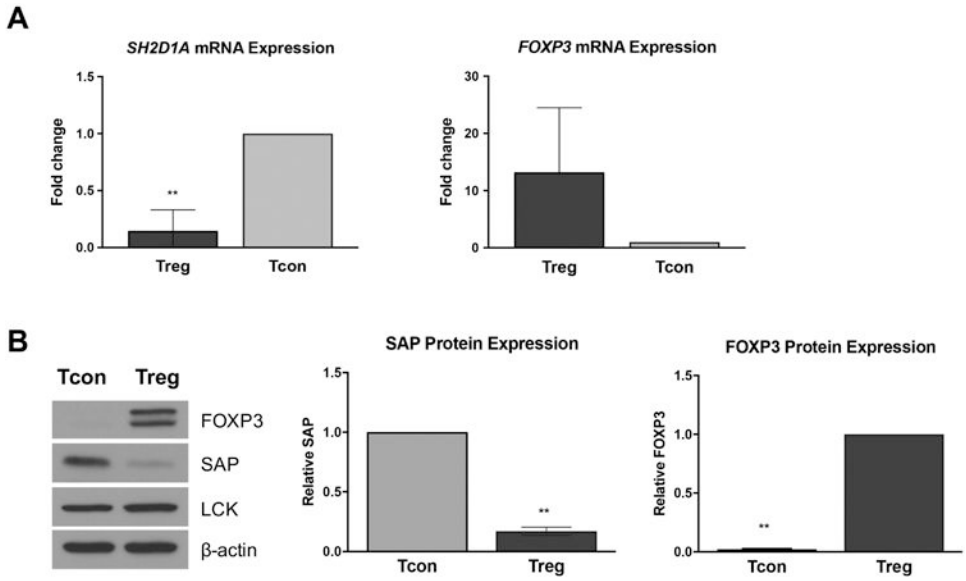

Figure 3. Inverse correlation between FOXP3 and SAP expression in human Tregs

(A) RNA was extracted from activated cells, and qRT-PCR was performed with primers specific for *FOXP3* and *SAP* mRNA. *RPL30* was used as a housekeeping gene for normalization. Data are average ± SD of 3 independent experiments; significance was assessed by unpaired t tests. **(B)** Protein lysates were subjected to SDS-PAGE and Western blot analysis for FOXP3, SAP, and LCK. β-actin was used as a loading control. One representative blot is shown (left). Results from 3 independent experiments were quantified by spot densitometry (right) and assessed for significance by two-tailed t tests.

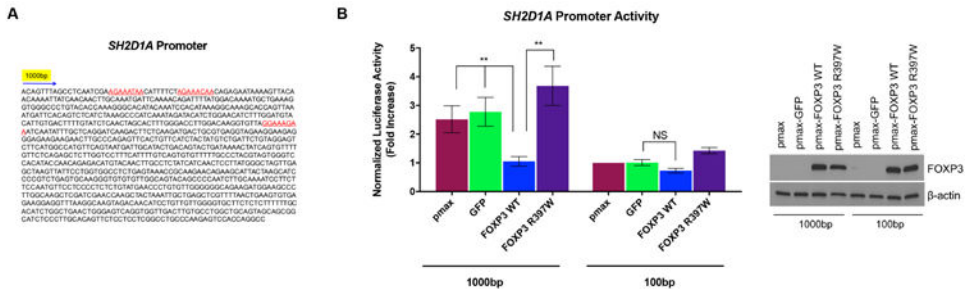

Figure 4. FOXP3 represses *SH2D1A* promoter activity

(A) Sequence of the proximal *SH2D1A* promoter (~1000 bp) cloned into the pGL3 luciferase vector. Putative FOXP3 binding sites are highlighted in red. **(B)** Jurkat T cells were triple transfected with pmax-GFP or FOXP3 expression vectors, firefly luciferase *SH2D1A* promoter constructs, (1000bp vs. 100bp), and a renilla luciferase control plasmid. Promoter activity was measured using a dual luciferase assay at 24 hours post-transfection. Luciferase activity (firefly/renilla ratio) was normalized to pmax empty vector control cells (left). Expression of FOXP3 was confirmed by Western blot analysis (right); β-actin served as a loading control. Data are average ± SD of 3 independent experiments. Significance was determined by ordinary one-way ANOVA with Tukey's multiple comparisons test.

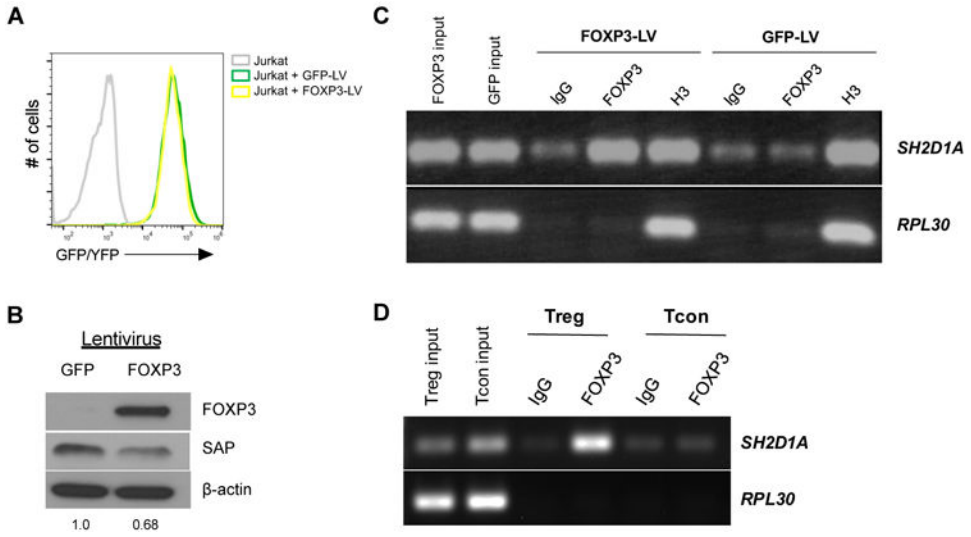

Figure 5. FOXP3 binds directly to the *SH2D1A* promoter

(A) Jurkat T cells were transduced with GFP or YFP-2A-FOXP3 lentiviral (LV) vectors; stable expression was monitored over time by GFP/YFP fluorescence. **(B)** FOXP3 and SAP expression was measured in LV-transduced Jurkat T cell lysates by Western blot. Numbers represent quantitation of SAP expression by spot densitometry, normalized to LV-GFP transduced cells. **(C)** LV-transduced Jurkat T cells were lysed for chIP using antibodies specific for FOXP3, histone H3, or an isotype IgG control. Immunoprecipitated DNA was then subjected to PCR using primers specific for the *SH2D1A* promoter, or the *RPL30* housekeeping gene. Results are representative of 3 independent experiments. **(D)** ChIP analysis was performed on activated human Tregs and Tcons using FOXP3 or IgG isotype antibodies. Immunoprecipitated DNA was then subjected to PCR using primers specific for the *SH2D1A* promoter, or the *RPL30* housekeeping gene. Data are representative of 2 independent experiments.

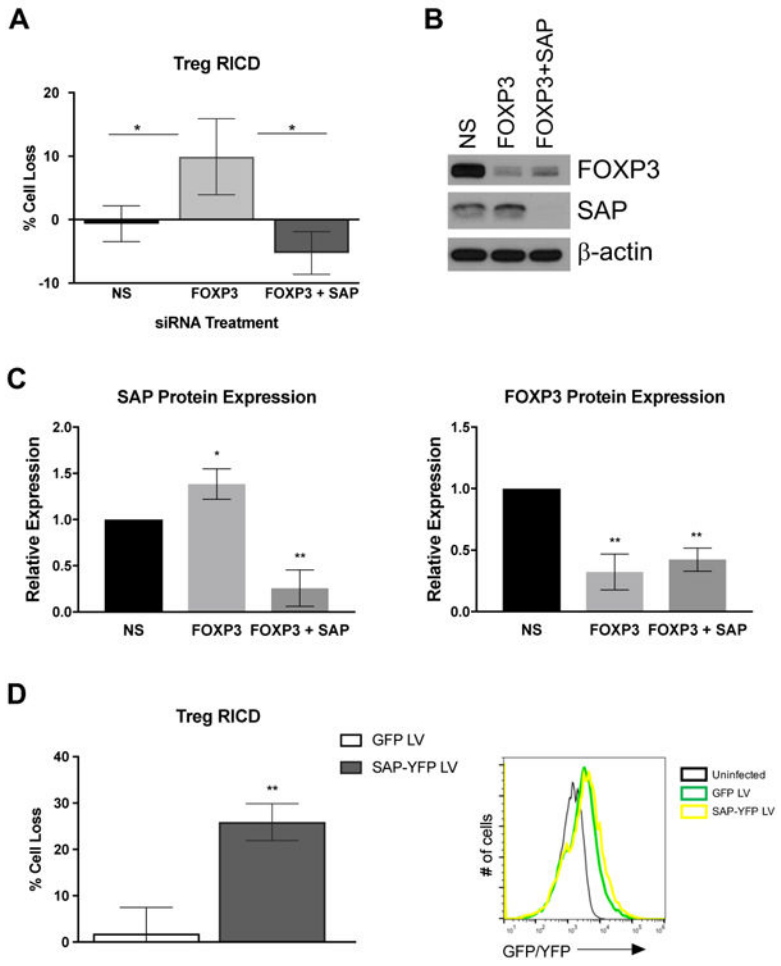

Figure 6. FOXP3 confers RICD resistance in Tregs by a SAP-dependent mechanism

(A) Activated Tregs were transfected with non-specific (NS), FOXP3-specific, or SAP-specific siRNAs. At 4 days post-transfection, cells were restimulated with OKT3 antibody (1 μg/ml) plus Protein A (2 μg/ml) for 24 hours in triplicate. Cell death was measured by flow cytometry using TO-PRO-3 live/dead staining. Data are average ± SD of 3 independent experiments using separate donors. Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparisons test. **(B)** Changes in FOXP3 and/or SAP expression in siRNA-transfected cells from (A) were assessed by Western blot. β-actin served as a loading control. Data from 4 independent experiments were quantified and assessed for statistical significance by one-way ANOVA with Tukey's multiple comparisons test (right). **(C)** Activated Tregs were transduced with GFP or YFP-2A-SAP LVs during activation-induced expansion. Cells were then restimulated 24 hours later with OKT3 (1 μg/ml) plus Protein A for an additional 24 hours. Cell loss was determined by TO-PRO-3 live/dead staining within GFP/YFP^{+} gated cell populations. Data are average ± SD of 2 independent experiments. Transduction efficiency of Tregs was assessed 24 hours post infection by flow cytometry compared to uninfected control cells (right).