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Research paper

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

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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.

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 non-specific 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 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

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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 min 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 Fig. 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 Biotec) in complete RPMI (RPMI 1640 (ThermoFisher Scientific) + 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 h. Cells were stained with 50 nM 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 min. 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 min on ice. Lysates were cleared by centrifugation and boiled in 2x 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 iScript 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 min, 40 cycles of 95 °C for 15 s and 60 °C for 60 s, 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′.

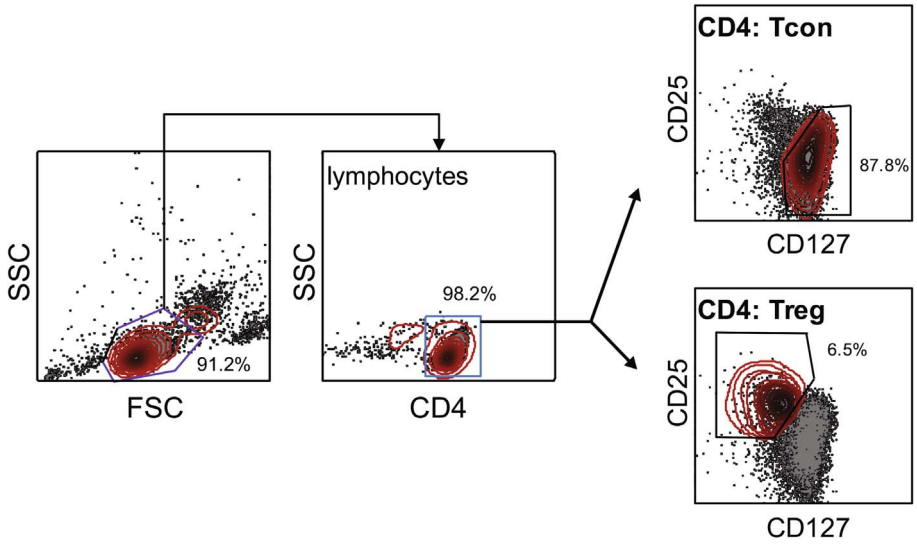

Fig. 1. Gating strategy to sort human Tregs and Tcons. CD4$^+$ T cells were isolated from healthy human blood donors by negative selection and stained with CD4, CD25, and CD127 antibodies before sorting. Lymphocytes were delineated by forward/side scatter gating, and CD4$^+$ cells were further separated 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.