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Differential regulation of Treg stability in human naïve and effector Treg subsets by TGFβ-signaling via ARKADIA-SKI axis

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Introduction: The human FOXP3⁺ regulatory T (Treg) cells, a subset of CD4⁺ T cells with immunosuppressive function, are essential for the maintenance of immune homeostasis and tolerance. Treg cells are a heterogeneous population, subdivided into a less stable “effector” subset and a more stable “naïve” subset. Under inflammatory conditions, Treg cells can lose their immunosuppressive properties, contributing to the development of autoimmune diseases. The TGFβ signaling is pivotal for the induction of Treg cells from naïve CD4 T cells and the thymic development of natural Treg cells. However, how TGFβ signaling regulates established naïve and effector Treg cells is not fully understood.

Methods: Human naïve and effector Treg cells were isolated from healthy donors using flow cytometry. Different subsets of Treg cells were treated with a TGFβ inhibitor or genetically modified to express SKI or ARKADIA via lentiviral transduction. Treg cell phenotype, stability and signaling regulation were analyzed using flow cytometry, western blotting, transcriptomic and meta-analyses. The functionality of Treg cells was analyzed by *in vitro* coculture assays.

Results: We find that the TGFβ signaling is differentially regulated in Treg subsets, with higher activity in the naïve Treg subset. Blockade of TGFβ pathway destabilizes both naïve and effector Treg cells, disrupting their immunosuppressive functions, with effector Treg cells being more susceptible. Further analysis shows that naïve Treg cells express lower levels of SKI protein, a negative regulator of TGFβ signaling suppressed by TGFβ-induced protein degradation. SKI overexpression destabilizes Treg cells and disrupts their immune suppressive function. Transcriptomic and meta-analyses reveal that TGFβ blockade and SKI overexpression commonly modulate pathways crucial for Treg to effector T cell conversion, downregulating Treg signature genes and upregulating effector T cell markers, which are validated as potential SKI targets. Importantly, overexpression of ARKADIA, an E3 ubiquitin ligase of SKI, efficiently reduces SKI levels, enhancing Treg cell stability and functionality under both TGFβ inhibition and chronic proinflammatory cytokine stimulation.

Conclusion: Our results identify a previously unrecognized role of the TGF β -ARKADIA-SKI axis in regulating the stability and functionality of human Treg subsets, highlighting novel strategies for harnessing TGF β -associated pathways to stabilize human Treg cells for clinical applications.

KEYWORDS

Treg, TGF β , autoimmune, naïve/effector Treg cell, Ski, Arkadia

1 Introduction

The CD4⁺FOXP3⁺ regulatory T (Treg) cells play a vital role in maintaining immune homeostasis and tolerance. Treg cells comprise only ~10% of CD4⁺ T cells, they are a heterogeneous population and can be further subdivided into a less stable “effector” subset and a more stable “naïve” subset (1). Naïve Treg cells, constituting only 0.2 to 3.3% of the total circulating CD4⁺ T cells, are thymus-derived and have not experienced antigen stimulation. In contrast, effector Treg cells are antigen-experienced and can originate either from the thymus or through conversion from CD4⁺ T cells. Naïve Treg cells can convert to effector Treg cells upon TCR stimulation (2, 3). Compared with effector Treg cells, naïve Treg cells exhibit less proliferation but greater stability in suppressive function and FOXP3 expression (4–6). One of the unique characteristics of Treg cells is their plasticity, which is defined as the ability to adapt to distinct inflammatory cues while retaining their suppressive capacity (7). Reports show that FOXP3⁺ Treg cells can co-express signature transcription factors of other T helper subsets, such as T-bet for Th1 cells, GATA-3 for Th2 cells, Bcl6 for T_{FH} cells and ROR γ t for Th17 cells. The co-expression of these transcription factors in Treg cells confers their advantage of lineage specific suppression, but also poses risks of Treg instability, which leads to the loss of immunosuppressive function and potentially contributing to autoimmunity (8–10). The study of regulatory mechanism of Treg plasticity and stability will provide valuable insights on the onset and pathogenesis of autoimmune diseases.

The immune suppression of Treg cells is achieved by generating an inhibitory environment, which involves the secretion of inhibitory cytokines (such as IL10, IL35 and TGF β), metabolites (such as adenosine), cytotoxic granzymes and perforin, in addition to consuming local IL2 (11). Treg cells can directly inhibit T cells or antigen presenting cells (APCs), through the expression of negative regulatory cell surface receptors such as cytotoxic T lymphocyte antigen 4 (CTLA-4) (11). With these unique features, adoptive Treg cell therapy has recently emerged as an innovative approach to treat severe autoimmune diseases and other immunological diseases, with its most recent pre-clinical and clinical practice summarized by Bluestone et al. (12). This therapy involves the isolation of Treg cells, expanding them *ex vivo*, engineering with Chimeric Antigen Receptor (CAR) or antigen specific TCR to enhance therapeutic specificity and potency, and eventually reinfusing them to restore immune tolerance

(12). One major challenge for Treg cell therapy is the concern of their stability, which is defined by the maintenance of three critical traits: (a) stable FOXP3 expression, (b) efficient suppressive activity, and (c) absence of effector activity (13). An increasing number of clinical trials are opting to use naïve Treg cells as a modality due to their greater stability compared to the total bulk Treg population, which was commonly used in previous studies. (6, 12). However, the underlying differences and mechanisms of regulating naïve Treg stability are largely unclear. Moreover, there is growing interest in exploring whether the stability of naïve Treg cells can be further enhanced via genetic engineering.

Transforming growth factor β (TGF β) plays important roles in Treg biology, including induced Treg differentiation, thymic Treg development and immune suppressive function (14). TGF β is secreted as a latent form, complexed with a latency-associated peptide (LAP), and is activated by the cleavage of LAP through various mechanisms, including integrins and proteases (15). TGF β binds its receptor, TGF β RII which then recruits TGF β RI. This triggers phosphorylation events that activate receptor-regulated SMADs (R-SMADs), including SMAD2 and SMAD3. These SMADs translocate to the nucleus and work together with the common-partner SMAD4 (co-SMAD) to modulate downstream gene expression (15). The TGF β signaling is negatively regulated by SKI proto-oncogene (SKI), its homolog SKI like proto-oncogene (SKIL/SNON) and Inhibitory SMADs (I-SMADs, including SMAD6 and 7). These elements act as negative regulatory factors by recruiting transcriptional corepressors, such as histone deacetylases (HDACs) (16, 17). The SKI and SNON protein can also be modulated by TGF β signaling via E3 ubiquitin ligase AKADIA mediated protein degradation (18–20). TGF β signaling is critical for the induction of FOXP3 expressing Treg cells from naïve CD4 T cells (21, 22) and thymic Treg development (23, 24). However, the role of TGF β in the maintenance of established Treg cells is less clear, with studies primarily conducted in mouse models indicating contentious and ambiguous findings (25–28). The study of TGF β signaling in human Treg cells is limited due to the heterogeneity of human Treg population and technical challenges in the isolation of Treg subsets. With rapid progress of the Treg cell therapies in clinical development, it is particularly beneficial to investigate how TGF β signaling regulates the stability and functionality of human Treg cells, especially different subpopulation.

In this study, we report a previously unappreciated role of TGF β signaling in human naïve and effector Treg populations and demonstrate that TGF β maintains the identity of human naturally occurring Treg cells through the ARKADIA-SKI axis, providing evidence that suggests novel therapeutic strategies for enhancing Treg stability.

2 Materials and methods

2.1 Human Treg cell isolation, culture and TGF β R1 inhibitor treatment

Leukopaks from healthy donors were obtained from StemExpress, BioIVT, or AllCells, with their full consent, under the approval of Institutional Review Board obtained by these companies. PBMCs were treated with ACK lysis to remove residual red blood cells, followed by positive selection for CD25⁺ cells (Miltenyi Biotec, 130092983). These enriched CD25⁺ cells were sorted by FACS to isolate CD4⁺CD25⁺CD127⁻CD45RO⁻ naïve Treg cells or CD4⁺CD25⁺CD127⁻CD45RO⁺ effector Treg cells on FACSARIATM II or FACSARIATM Fusion (BD Biosciences) with the indicated antibodies. The isolated primary cells were resuspended at 0.5×10^6 /ml in RPMI1640 (Gibco, A1049101), supplemented with 10% HI-FBS (Gibco, 10500064), 1x Penicillin-Streptomycin-Glutamine (Gibco, 10378016), 1x 2-Mercaptoethanol (Gibco, 21985023), 1x Gibco[®] MEM Non-Essential Amino Acids (Gibco, 11140050), 1000 IU/ml recombinant hIL-2 (Peprotech, 200-02-1MG), and 2nM rapamycin (Sigma, R8781). Activation was done using DynabeadsTM Human Treg Expander (Thermo Fisher, 11129D) in a cell-to-bead ratio of 1:4. Every 2–3 days, cells were passaged and media refreshed, with expansion continuing for a total of 14 days. Post-expansion, cells were collected by removing Dynabeads, then either cryopreserved or directly subjected to treatments. For transductions and most treatments, expanded Treg cells, whether ongoing or retrieved from cryo-stock, were maintained at 0.5×10^6 cells/ml in the same medium without rapamycin, with passaging every 2–3 days. Activation was done at a 1:1 cell-to-bead ratio every 14 days. TGF β R1 inhibitor treatments were done in the presence of 1nM TGF β R1 inhibitor SB431542 (Selleckchem, S1067), which was replaced every 3 days.

2.2 Flow cytometry

Fluorescence-conjugated antibodies for CD25 (BC96, 302606), CD127 (A019D5, 351310), CD45RO (UCHL1, 304234), FOXP3 (206D, 320114), HELIOS (22F6, 137214), IL17A (BL168, 512338), IFN γ (4S.B3, 502532), were purchased from Biolegend, and CD4 (L200, 552838), CD45RA (HI100, 568557) were from BD Biosciences. For FACS analysis, $0.5\text{--}1 \times 10^5$ cells were collected and subjected to surface staining, followed by intracellular staining using the eBioscienceTM Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher, 00-5523-00) according to the manufacturer's instructions. For intracellular cytokine staining, cells were stimulated

for 4 hours with 200 ng/ml PMA (Sigma, 19-144), 5 μ g/ml BFA (Sigma, B7651), and 1 μ M Ionomycin (Sigma, I0634). Stained cells were analyzed using BD[®] LSR II, LSRFortessaTM, FACSymphonyTM A3 or FACSymphonyTM A5 instruments (BD Biosciences). The FACS data were analyzed using FlowJo software (TreeStar).

2.3 Plasmids, lentivirus transduction and MACS enrichment

The coding sequence (CDS) of human SKI (NM_003036.4), or mutant (P35S) SKI (c.[97GGCGGCCCG>GGAGGATCC] on NM_003036.4), or ARKADIA/RNF111 (NM_001270528.2) was inserted upstream of the truncated NGFR sequence (29), flanked by T2A in a lentiviral vector driven by EF1 promoter. Subclones were generated by Genscript. Lentivirus was produced using the pPACKH1 packaging system (System Biosciences, cat# LV500A-1) according to the manufacturer's instructions. For transduction, 14-day expanded Treg cells, whether ongoing or retrieved from cryo-stock, were activated with DynabeadsTM Human Treg Expander (Thermo Fisher, 11129D) at a cell-to-bead ratio of 1:1 for one day. This was followed by a 2-hour spin inoculation of lentivirus in the presence of 10 μ g/mL protamine sulfate. The infected cells were incubated overnight with the virus in a 37 °C CO2 incubator before replacing with fresh media. 7 days after infection, the transduced cells were enriched for NGFR⁺ populations, using MACSelectTM LNGFR MicroBeads (Miltenyi Biotec, 130091330) as per the manufacturer's instructions. The enriched NGFR⁺ cells were assessed for enrichment efficiency by flow cytometry, activated and subjected to subsequent treatments for the desired duration.

2.4 Treg de-stabilization assay

Treg cells were cultured in media with or without the following cytokines: 20 ng/ml IL-1 β (Peprotech, 200-01B-10UG), 20 ng/ml IL-6 (R&D, 206-IL-010), and 40 ng/ml IL-23 (Biolegend, 574104). The culture duration spanned 3 to 5 weeks, with media being refreshed every 2–3 days. Treg identity was assessed weekly using flow cytometry.

2.5 *In vitro* Treg suppression assay

Naïve CD25⁻CD4⁺ effector (Teff) cells were derived from the CD25⁻ PBMC remaining after Treg isolation, using negative selection to enrich CD4⁺ cells (Miltenyi Biotec, 130045101). These cells were cryo-reserved until usage. For evaluating Treg *in vitro* suppression ability, Teff cells were retrieved from cryo-stock and labeled with CellTraceTM Violet (CTV) (Invitrogen, C34557) as per the manufacturer's instructions. A fixed amount of 5×10^4 labeled Teff were mixed with varying amounts of Treg cells at different ratios, in Treg media excluding IL2, with the presence of human Treg Suppression Inspector (Miltenyi Biotec, 130092909) at a Teff-to-bead ratio of 1:1. The cell mixtures were cultured for 4

days at 37 °C CO₂ incubator. Following incubation, the proliferation of Teff cells was assessed by measuring CTV dilution via flow cytometry, analyzed using FlowJo software. Suppression efficiency was calculated as “% inhibition of Teff division,” determined by the percentage reduction in the division index of Teff cells compared to Teff cells cultured alone.

2.6 Western blot

Treg cell pellets were lysed in RIPA buffer (Thermo Scientific, 89900) containing 1x Halt™ Protease Inhibitor Cocktail (Thermo Scientific, 78430), in 4°C with rotation for 20min. The lysates were then centrifuged at 14,000 g for 10 minutes, and the supernatant was collected and were boiled in 1x Laemmli Sample Buffer sample buffer (Bio-rad, 1610747) with 1x NuPAGE™ Sample Reducing Agent (Invitrogen, NP0004) at 70°C for 10min. The prepared samples were subjected to electrophoresis using the Invitrogen™ NuPAGE™ gels and XCell SureLock Mini-Cell system (Invitrogen), followed by transfer using the iBlot™ 2 Transfer system (Invitrogen). The samples were then immunoblotted with the following primary and secondary antibodies: SKI (Santa Cruz, G8, sc-33693), RNF111 (Abnova, 1C4, H00054778-M05), GAPDH (Cell Signaling Technology, 5174), Anti-mouse IgG HRP-linked (Cell Signaling Technology, 7076S), and Anti-rabbit IgG HRP-linked (Cell Signaling Technology, 7074S). Protein levels were quantified using ImageJ. For the original raw gel images of western blot shown in figures, see [Supplementary Material](#).

2.7 Bulk RNA-seq sample preparation and analysis

For TGFβi treatment in freshly isolated naïve and effector Treg cells, cells from FACS were directly cultured in the absence of rapamycin, with or without 1nM SB431542 (Selleckchem, S1067) for 5 weeks before RNA extraction. For (P35S) SKI overexpression in naïve Treg cells, expanded naïve Treg cells were transduced, NGFR⁺ cells enriched, and cultured for 7 days prior to RNA extraction.

Total RNA was extracted using the MagBind Total RNA 96 kit (Omega Biotek) according to the manufacturer’s instructions. RNA quality and quantity were assessed with the Qubit HS RNA assay (ThermoFisher) and the TapeStation High Sensitivity RNA assay (Agilent). From each sample, 100 ng of total RNA was used for library preparation. Briefly, human ribosomal RNA was depleted using the Ribo-Zero Plus rRNA Depletion Kit (Illumina), followed by library generation with the NEBNext Ultra II RNA Directional Kit (New England Biolabs), according to the manufacturer’s instructions. Sequencing was performed on an Illumina NovaSeq 6000 sequencer with 2×150 bp paired-end reads. Following quality control and adapter trimming of the raw sequencing data, the reads were then aligned to the human reference genome (GRCh38) using STAR (version 2.7.11b) under default settings. Gene-level read

counts were extracted from STAR’s ‘ReadsPerGene’ output files, and the gene count matrix was prepared for downstream analysis.

Differential gene expression analysis was performed using the DESeq2 package (30) in R. The raw read count matrix obtained from STAR was imported into DESeq2 and subjected to normalization using the DESeq2 normalization method to account for differences in sequencing depth across samples. To assess the variance in gene expression profiles among samples, a Principal Component Analysis (PCA) was performed. Specifically, the DESeqDataSet object containing the raw read counts was transformed using the variance stabilizing transformation (VST) function to stabilize variance across the expression data. PCA was conducted on the transformed dataset, and the PCA plot was generated using the plotPCA function, which computes and plots the first two principal components, representing the greatest variance within the dataset. Paired comparisons between experimental groups were conducted to identify differentially expressed genes. A threshold of a fold change of 2 was used to define biologically significant changes in gene expression. Gene Set Enrichment Analysis (GSEA) was performed using the clusterProfiler package (31) in R to identify enriched biological pathways in differentially expressed genes. A ranked list of genes based on log₂ fold changes from DESeq2 was used as input for the analysis. Statistical significance was assessed using the Wald test, and p-values were adjusted for multiple comparisons using the Benjamini-Hochberg method to control the false discovery rate (FDR). Genes with an adjusted p-value of less than 0.05 were considered significantly differentially expressed. For the raw gene expression profiles, see [Supplementary Material](#).

2.8 Statistical analysis and bioinformatics

Unless otherwise indicated, statistical significance was determined by Student t test for two-sample analyses, analyzed using GraphPad Prism. P < 0.05 was considered as statistically significant and labeled with one asterisk.

GSEA and PCA analyses were done using R package “clusterProfiler”. Heatmaps were generated using R package “pheatmap” or a web tool Morpheus (<https://software.broadinstitute.org/morpheus>). Venn diagrams were plotted using R package “VennDiagram”. The SKI CHIP-seq data was retrieved from GSE107556 and visualized using IGV browser (32).

3 Results

3.1 Human Treg subsets demonstrate differential TGFβ signaling and stability

To investigate the role of TGFβ in different Treg subpopulations, we isolated human naïve (CD4⁺CD25⁺CD127^{low}CD45RA^{hi}CD45RO^{low}) Treg cells and effector (CD4⁺CD25⁺CD127^{low}CD45RA^{low}CD45RO^{hi}) Treg cells from human Leukopak samples by flow cytometry sorting ([Figure 1A](#)) and validated their purity post

sorting (Figure 1B). The isolated naïve and effector Treg cells were activated and cultured with or without TGF β signaling inhibition (TGF β i) by the small molecule inhibitor (SB-431542) that targets TGF β receptor 1 (33). We find that naïve Treg cells and effector Treg cells are transcriptionally distinct from each other even without TGF β i treatment (Figure 1C). Intriguingly, TGF β i shifted the transcriptional state of naïve Treg cells toward effector Treg cells, while further polarizing effector Treg cells away from the major Treg colonies (Figure 1C). Gene set enrichment (GSEA) revealed that naïve Treg cells inherently exhibit higher TGF β signaling activity upon expansion compared to effector Treg cells (Figure 1D, Supplementary Figure S1A). The increased TGF β signaling is consistent with the higher expression of TGF β receptor II in naïve Treg cells compared to effector Treg cells (Supplementary Figure S1B). We found that the higher expression of TGF β receptor II also exists in freshly isolated naïve Treg cells (Figure 1E, Supplementary Figures S1C, D). These findings underscore the importance of TGF β signaling in cell fate determination.

The loss of Treg cell identity is commonly associated with the gain of T helper (Th) cell function with increased proinflammatory cytokine production (10, 34). GSEA analysis revealed that TGF β inhibition destabilized both Treg subsets by suppressing Treg signature genes and promoting proinflammatory pathways (Figures 1F, G, Supplementary Figures S1E, F). Specifically, TGF β inhibition disrupted *FOXP3* RNA expression while increasing the expression of Th master regulators *TBX21* and *GATA3* (Figure 1H), as well as the expression of proinflammatory cytokines such as *IFNG*, *IL4*, *IL5*, *IL13* and *IL17A* (Supplementary Figure S1E). The TGF β inhibition-induced Treg destabilization is more severe in effector Treg cells compared to those in naïve Treg cells, which is consistent with the knowledge that naïve Treg cells are more stable than effector Treg cells (4–6). This observation is further supported by the loss of *FOXP3* protein expression and an increase in proinflammatory cytokine production, with effector Treg cells being more sensitive than naïve Treg cells (Figures 1I, J). Together, our findings highlight a critical role for TGF β signaling in maintaining the stability of human Treg cells and suggest the level of TGF β signaling might positively correlate with Treg stability in naïve and effector Treg subsets.

3.2 SKI expression negatively correlates with TGF β signaling in Treg subsets

We next sought to find the critical regulators of TGF β signaling responsible for the stability of naïve Treg and effector Treg subsets. Previous studies have shown that SKI, a major negative regulator of the TGF β signaling pathway, is crucial in the fate determination and functionality of mouse induced Treg (iTreg) and Th17 cells (17, 35–37). While SKI inhibits TGF β downstream transcriptional factors like SMAD proteins, it can also be suppressed by TGF β signaling via an E3 ligase ARKADIA (18). To test if TGF β stabilizes human naturally occurring Treg cells through SKI regulation, we measured the protein levels of SKI in the two subpopulations of human Treg cells. We found that naïve Treg cells harbor a significantly lower amount of SKI protein compared

with effector Treg cells (Figures 2A–C). However, we did not observe such a difference in the RNA level of SKI (Supplementary Figure S1A), indicating that the expression level of SKI is mainly regulated through post-translational modification (reviewed in (16)). Moreover, *in vitro* expansion increased the protein expression level of SKI in Treg cells, with a more pronounced upregulation observed in eTreg cells. As expected, blocking TGF β signaling by TGF β i treatment further enriched SKI protein in those cells (Figure 2C). Importantly, this pattern of SKI upregulation closely parallels the trend of Treg destabilization observed during culture (Figure 1J). Our results indicate SKI protein expression levels reflect the differential TGF β signaling in Treg subsets and is upregulated upon TGF β i treatment during Treg destabilization.

3.3 Ectopic expression of SKI destabilizes human Treg cells and impairs Treg suppressive function

To further investigate whether SKI is functionally relevant to Treg destabilization, we overexpressed SKI in Treg cells and monitored the stability of these cells in the presence or absence of TGF β blockade. Notably, overexpression of SKI significantly decreased the levels of both *FOXP3* and *HELIOS*—the latter widely considered a marker of stable human Treg cells (39) in both naïve Treg and effector Treg cells, with more severe Treg destabilization in effector Treg subsets (Figures 2D–F, Supplementary Figures S2A, B). Moreover, TGF β inhibition further exacerbated Treg destabilization in both populations with SKI overexpression (Figures 2D–F, Supplementary Figures S2A, B). This additive effect of TGF β inhibition is likely attributed to a further enhanced ectopic protein level by TGF β inhibition (Supplementary Figure S2C). In line with our transcriptomic observations from TGF β i-treated Treg cells (Supplementary Figure S1E), SKI overexpression also increased the protein level of a key proinflammatory cytokines, *IFN γ* , with the effects being more pronounced in effector Treg cells (Figures 2G–I, Supplementary Figures S2D, E). In addition, we observed the presence of a *FOXP3*⁺*IFN γ* ⁺ population upon treatment, especially in the effector Treg cells (Figure 2G). This phenotype suggests that TGF β inhibition and SKI overexpression could induce Treg cell intrinsic inflammatory cytokine production before *FOXP3* loss, highlighting the importance of Treg cell intrinsic TGF β signaling for their stability.

We next assessed the immune suppressive function of these cells, using an *in vitro* suppression assay. Consistent with the loss of Treg cell identity (Figures 2D–F), SKI overexpression dramatically dampened the immune suppression ability of these Treg cells, with a more dramatic effect on that of effector Treg cells, indicating that effector Treg cells are more sensitive to TGF β i-induced Treg function loss compared to naïve Treg cells (Figure 2J, Supplementary Figures S2F, G). These findings demonstrate that SKI destabilizes Treg cells, and that SKI expression levels potentially correlate with Treg instability upon TGF β i treatment. These findings also suggest that the comparatively lower stability of effector Treg cells may be attributed to their elevated endogenous SKI levels compared to naïve Treg cells.

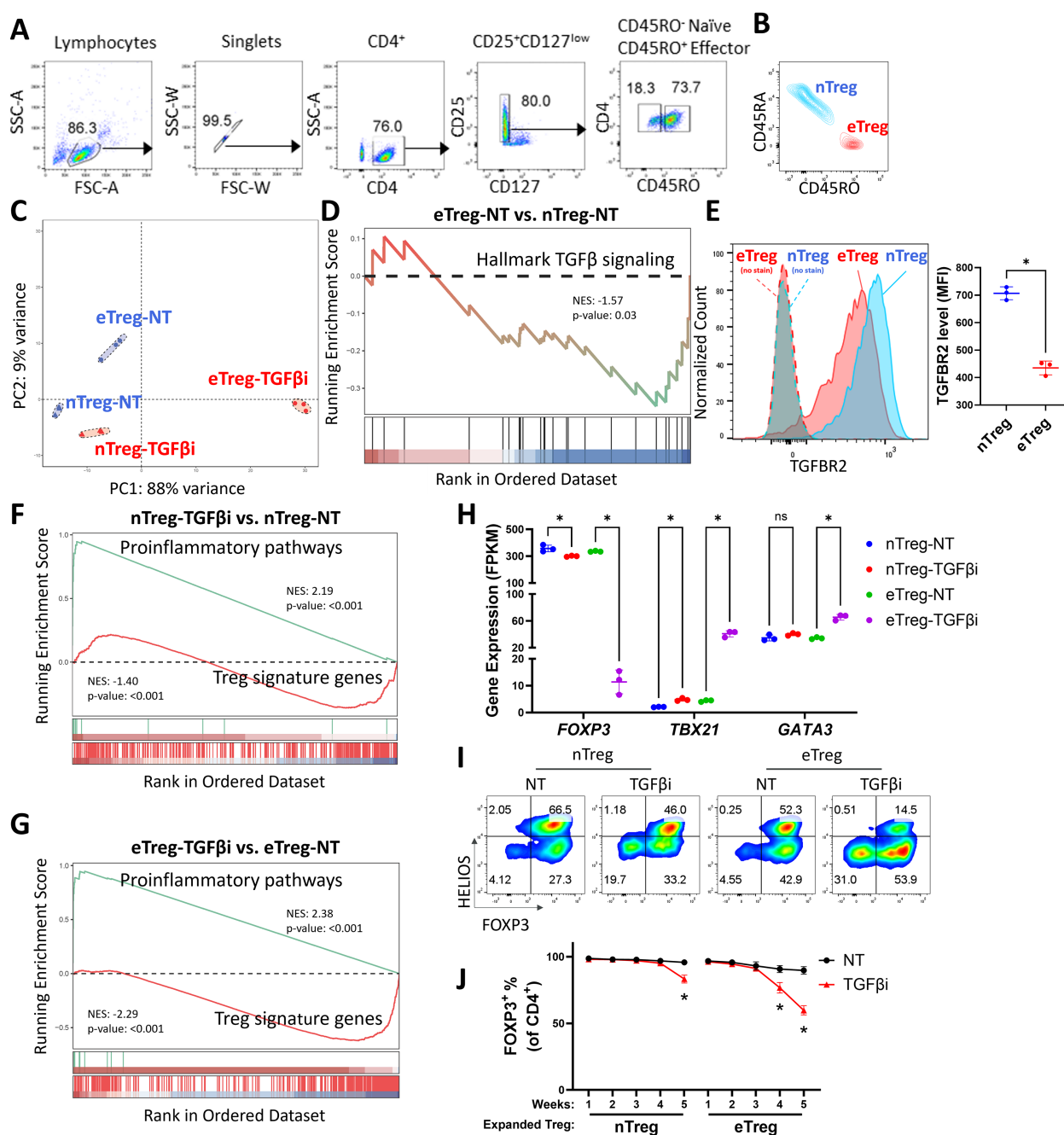


FIGURE 1

$TGF\beta$ signaling blockade destabilizes human Treg cells. **(A)** A diagram of the gating strategy to isolate naïve Treg ($nTreg$, $CD4^+CD25^+CD127^-CD45RO^-$) cells and effector Treg ($eTreg$, $CD4^+CD25^+CD127^-CD45RO^+$) cells from $CD25$ beads enriched PMBC in healthy donors by FACS. The flow cytometry profiles are representative of more than three donors. **(B)** The expression of $CD45RO$ and $CD45RA$ in freshly isolated $nTreg$ and $eTreg$ cells were assessed by flow-cytometry. The flow cytometry profiles are representative of more than three donors. **(C)** Principal Component Analysis (PCA) of whole genome transcriptome data in $nTreg$ and $eTreg$ cells with ($TGF\beta i$) or without (NT) $TGF\beta R1$ inhibitor treatment. $n = 3$ replicates of one donor. **(D, F, G)** Gene set enrichment analysis (GSEA) was performed on the indicated sample groups and gene collections. Normalized Enrichment Scores (NES), and p-values were calculated using permutation tests and are labeled accordingly. **(E)** The expression of $TGFBR2$ in freshly isolated $nTreg$ and $eTreg$ cells were assessed by flow-cytometry (Left panel). The flow cytometry profiles are representative of three donors. Quantifications of MFI of $TGFBR2$ level in $nTreg$ and $eTreg$ cells (Right panel). Data are presented as Mean $\pm$ SD. * $p < 0.05$, two-sided t test. $n = 3$ replicates of one donor. This data is representative of experiments conducted in three donors. **(H)** A scatter plot illustrates gene expression levels of the indicated genes from whole genome transcriptome dataset. Data are presented as Mean $\pm$ SD. * $p < 0.05$, two-sided t test. $n = 3$ replicates of one donor. **(I)** Representative flow cytometry profiles showing $FOXP3$ and $HELIOS$ levels in the $nTreg$ and $eTreg$ cells with ($TGF\beta i$) or without (NT) $TGF\beta R1$ inhibitor treatment for five weeks. This data is representative of experiments conducted in three donors. **(J)** Line charts summarizing the percentage of $FOXP3^+$ population in $nTreg$ and $eTreg$ cells at indicated time points. Data are presented as Mean $\pm$ SEM. * $p < 0.05$, two-sided t test. $n = 3$ donors.

3.4 Degradation-resistant SKI further destabilizes Treg stability and functionality

It has been well-established that TGF β regulates SKI through protein degradation (reviewed in (16)). We found that TGF β i stabilizes SKI protein in Treg cells while further disrupting Treg stability in the combination of SKI overexpression. To further

evaluate the role of TGF β -mediated SKI degradation in Treg stability, we generated a mutant of SKI (P35S) that is resistant to TGF β -induced degradation (40). The SKI mutant was verified in naïve Treg cells, where its protein level remained steady regardless of the presence of TGF β i. In contrast, the wildtype SKI protein level was significantly increased by TGF β inhibition (Figures 3A, B). Functionally, the mutated SKI was more potent in destabilizing

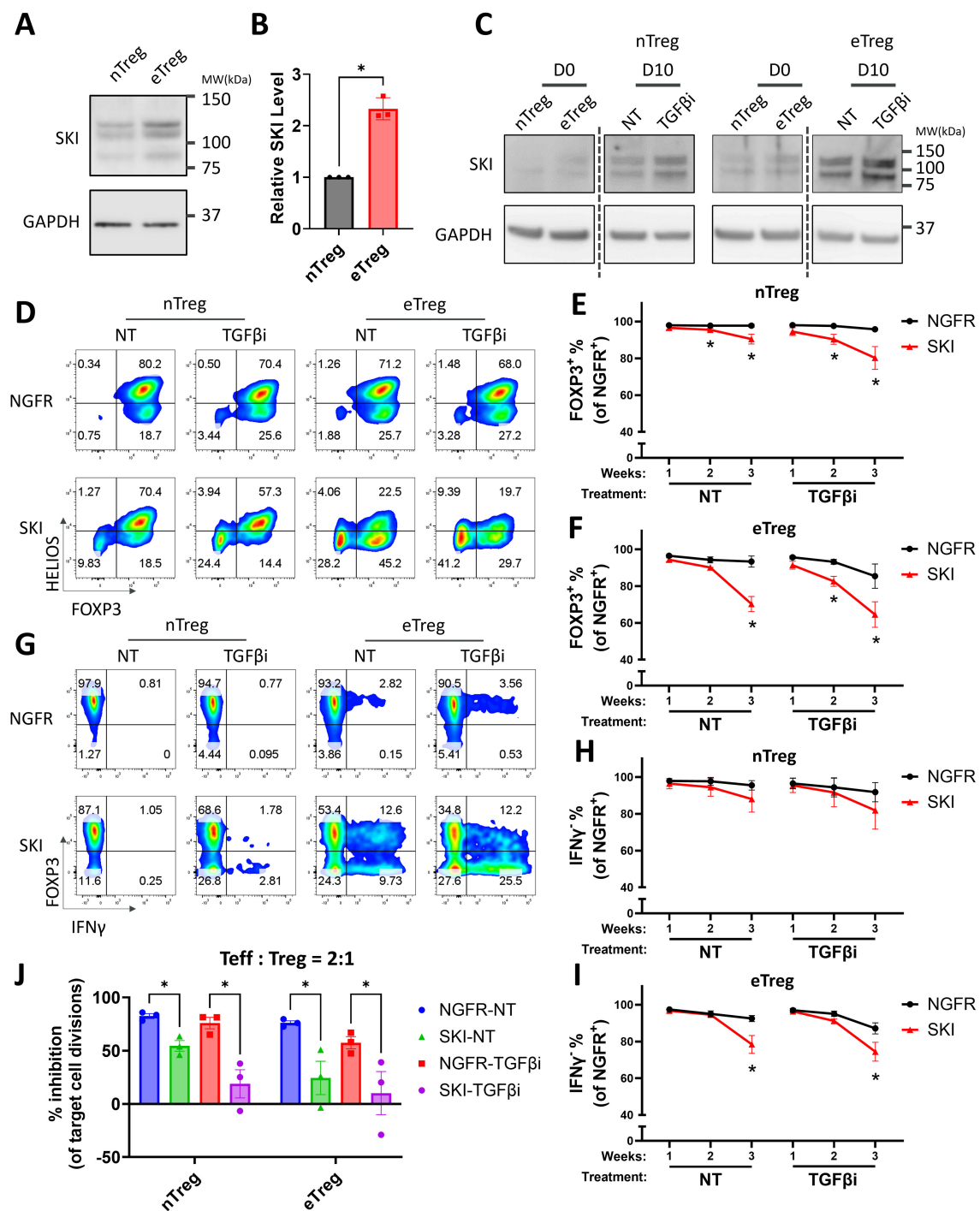


FIGURE 2 (Continued)

FIGURE 2 (Continued)

Overexpression of SKI destabilizes human Treg cells and further promotes TGF β i induced Treg destabilization. **(A)** The expression level of SKI protein in expanded naïve Treg (nTreg) cells and effector Treg (eTreg) cells analyzed by immunoblots with GAPDH as loading control. The blots are representative of three donors. Note: The endogenous SKI shows multiple bands due to post-translational modifications, a pattern previously reported before using the same antibody clone (38). **(B)** The expression level of SKI protein was quantitated and normalized to GAPDH to calculate normalized protein levels and then compared to nTreg cells to determine relative protein levels. Data are presented as Mean $\pm$ SEM. * $p < 0.05$, two-sided t test. $n = 3$ donors. **(C)** The expression level of SKI and GAPDH protein in naïve Treg cells treated with (TGF β i) or without (NT) TGF β R1 inhibitor for 0 and 10 days, analyzed by immunoblots. Blots from the same gels are separated by dotted lines. The blots are representative of three donors. **(D–I)** Expanded nTreg and eTreg cells transduced with NGFR or SKI-T2A-NGFR (SKI) were treated with (TGF β i) or without (NT) TGF β R1 inhibitor for three weeks. Levels of FOXP3, HELIOS, and IFN γ within the NGFR $^{+}$ population were assessed weekly via flow cytometry. Representative flow cytometry profiles showing FOXP3 and HELIOS levels **(D)** and FOXP3 and IFN γ levels **(G)** are shown. The flow cytometry profiles are representative of experiments conducted in four donors. Line charts summarizing the percentage of FOXP3 $^{+}$ **(E, H)** and IFN γ $^{+}$ cells **(H, I)** within the NGFR $^{+}$ population in nTreg cells **(E, H)** and eTreg cells **(F, I)** at indicated time points are shown. Data are presented as Mean $\pm$ SEM. * $p < 0.05$, two-sided t test. $n = 4$ donors. **(J)** nTreg or eTreg cells carrying SKI or the empty vector (NGFR) were treated with (TGF β i) or without (NT) TGF β R1 inhibitor for three weeks and then subjected to an *in vitro* suppression assay. Treg cells were cultured with Teff cells at a 2:1 ratio (Treg: Teff) for four days. Following incubation, Teff proliferation was assessed by CTV dilution and flow cytometry. Suppression efficiency was calculated as “% inhibition of Teff division,” determined by the percentage reduction in the division index of Teff cells compared to Teff cells cultured alone. Data are presented as Mean $\pm$ SEM. * $p < 0.05$, two-sided t test. $n = 3$ donors.

Treg cells compared to the wildtype SKI. Its destabilization effect could not be further increased upon TGF β inhibition, as measured by decreased FOXP3 level (Figures 3C, D) and impaired suppression ability (Figure 3E, Supplementary Figure S3). Therefore, our findings demonstrated that the expression of SKI drives the Treg cell destabilization and the degradation of SKI by TGF β signaling is essential to maintain Treg stability and functionality.

3.5 SKI destabilizes human Treg cells by suppressing Treg signature genes and promoting pro-inflammatory pathways

To investigate how SKI regulates Treg stability and functionality, we overexpressed the SKI mutant (SKI (P35S)) in Treg cells and evaluated the global transcriptome changes by mRNA-sequencing. We chose the degradation resistant SKI mutant as it can minimize the effect of TGF β -mediated downregulation of SKI (Figure 3). We found that the ectopic expression of the SKI mutant significantly altered the Treg transcriptome (Figure 4A), revealing 1788 differentially expressed genes (Supplementary Figure S4A). The results aligned with the knowledge that the target genes of SKI/SNON such as SKIL (43) and SMAD7 (44) appeared as top downregulated genes by mutant SKI overexpression (Supplementary Figure S4A). The transcriptional level data also aligned with the protein level data from both wildtype (Figures 2D–I) and mutant SKI (Figures 3C, D). SKI mutant expression destabilizes naïve Treg cells by reducing Treg signature genes expression, including FOXP3, while increasing the expression of Th master regulators TBX21 and GATA3, as well as pro-inflammatory cytokines (Figures 4B, C). Furthermore, we observed that SKI influences the secretion and signaling of pro-inflammatory cytokines through unsupervised GSEA analysis using KEGG and MSigDB_Hallmarks data sets. Notably, pathways mediated by IL17, IL6, and IFN were enhanced (Figures 4D, E). Additionally, SKI activated pathways that undermine Treg stability and functionality. These pathways include the PI3K-AKT-mTORC

pathway, which influences Treg vs. Teff lineage commitment through metabolic regulations (45, 46), the WNT pathway, which disrupts FOXP3 transcriptional activity (47), and glycolysis, whose unrestrained upregulation impairs Treg function (48).

3.6 TGF β -SKI axis regulates key target genes responsible for Treg to effector T cells conversion

We aimed to determine the most significant components causing Treg destabilization that are commonly regulated by TGF β -SKI axis. Since TGF β -inhibition-increased endogenous SKI and overexpressed SKI both destabilized Treg cells and disrupted Treg functionality (Figure 2), we overlapped differentially expressed genes (DEGs) from mutant SKI overexpression in naïve Treg cells with those resulting from TGF β inhibition in both naïve Treg and effector Treg cells (Supplementary Figures S4B, C). This analysis yielded 195 upregulated and 123 downregulated genes. By comparing these DEGs with Treg signatures defined by the Human Protein Atlas (41), we found 16 Treg signature genes downregulated and 3 regulated (Figure 4F left panel), supporting our GSEA findings that SKI overexpression (Figure 4C) and TGF β inhibition (Figures 1F, G) disrupt Treg identity. A similar analysis using a recently defined pan-Th versus Treg signature gene set (42) revealed 15 downregulated Treg signature genes and 19 upregulated Th signature genes (Figure 4F right panel). Among DEGs tied to Treg or Teff signatures, we found key genes critical for Treg function were downregulated, including DUSP4 (49), FANK1 (50, 51), MEOX1 (52), and INPP5F (53, 54). In contrast, we found that Treg negative regulators like ID2 (55), IL7R (56) and pro-inflammatory molecules CD40LG (57) and OSM (58) were upregulated. These genes indicate the shift from Treg to Teff facilitated by SKI or TGF β inhibition, detailed in (Figure 4G). Although FOXP3, the Treg master regulator, was not prominent in our analysis due to a modest fold change ($|FC| < 2$) under TGF β inhibition (Figure 1G), it remains part of our list. Similarly, TBX21, the Th1 master regulator, consistently upregulated by both SKI (Figure 4B) and TGF β i (Figure 1G), was included due to its relevance despite not

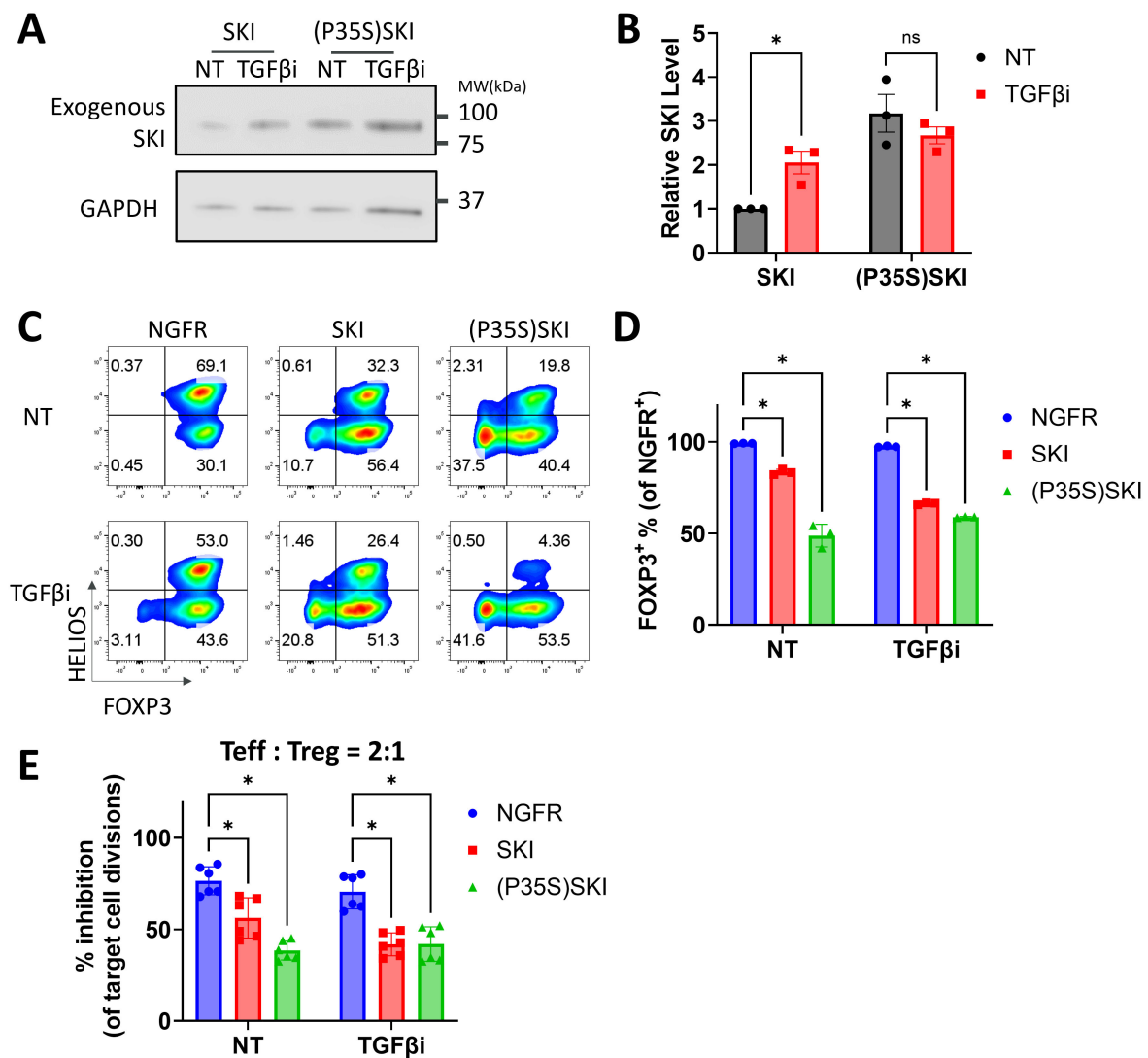


FIGURE 3

A degradation resistant SKI further destabilizes human Treg cells. (A, B) Naïve Treg cells transduced with SKI or (P35S) SKI were treated with (TGFβi) or without (NT) TGFβR1 inhibitor for three days, followed by immunoblots targeting SKI and GAPDH. (A) Representative immunoblots of three donors. (B) SKI levels were quantitated and normalized to GAPDH to calculate normalized protein levels and then compared to the NT-SKI samples to determine relative protein levels. Data are presented as Mean ± SD. **p* < 0.05, two-sided *t* test. *n* = 3 donors. (C–E) Naïve Treg cells transduced with NGFR or SKI-T2A-NGFR (SKI) or (P35S)SKI-T2A-NGFR ((P35S)SKI) were treated with (TGFβi) or without (NT) TGFβR1 inhibitor for three weeks. (C) Levels of FOXP3 and HELIOS within the NGFR⁺ population were assessed via flow cytometry. Representative flow cytometry profiles showing FOXP3 and HELIOS levels. *n* = 3 donors. (D) A bar graph summarizing the level of FOXP3 within the NGFR⁺ population. Data are presented as Mean ± SD. **p* < 0.05, two-sided *t* test. Data present *n* = 3 technical replicates from a single donor. This experiment was independently repeated with cells from three donors, yielding similar results. (E) These Treg cells were then subjected to an *in vitro* suppression assay and cultured with Teff cells at a 2:1 ratio (Treg: Teff) for four days. Following incubation, Teff proliferation was assessed by CTV dilution and flow cytometry. Suppression efficiency was calculated as “% inhibition of Teff division,” determined by the percentage reduction in the division index of Teff cells compared to Teff cells cultured alone. Data are presented as Mean ± SD. **p* < 0.05, two-sided *t* test. *n* = 6 replicates from two donors.

being in the pan-Th signature genes (42). SKI is known as a transcriptional coregulator functioning in either the activation or repression of its target genes (17). In line with this, we identified several genes from our list, including *DUSP4*, *FANK1*, *INPP5F*, *ID2*, *IL7R*, and *OSM*, as potential SKI targets due to SKI’s known occupation of their regulatory domains (59, 60) (Figure 4H). Collectively, TGFβ-SKI signaling regulates Treg stability by regulating multiple signaling and metabolic pathways that are responsible for Treg to effector T cells conversion.

3.7 E3 ubiquitin ligase ARKADIA protects human Treg cells stability by degrading SKI

It is well defined that TGFβ signaling degrades SKI and its analog SNON via the E3 ubiquitin ligase ARKADIA in T cells as well as other cell types (18, 36, 61). Specifically, it has been reported that Arkadia degrades Ski and SnoN to enhance TGFβ-induced Treg differentiation from naïve CD4 T cells in mouse models (36). Another study further demonstrated that both SKI and SNON are

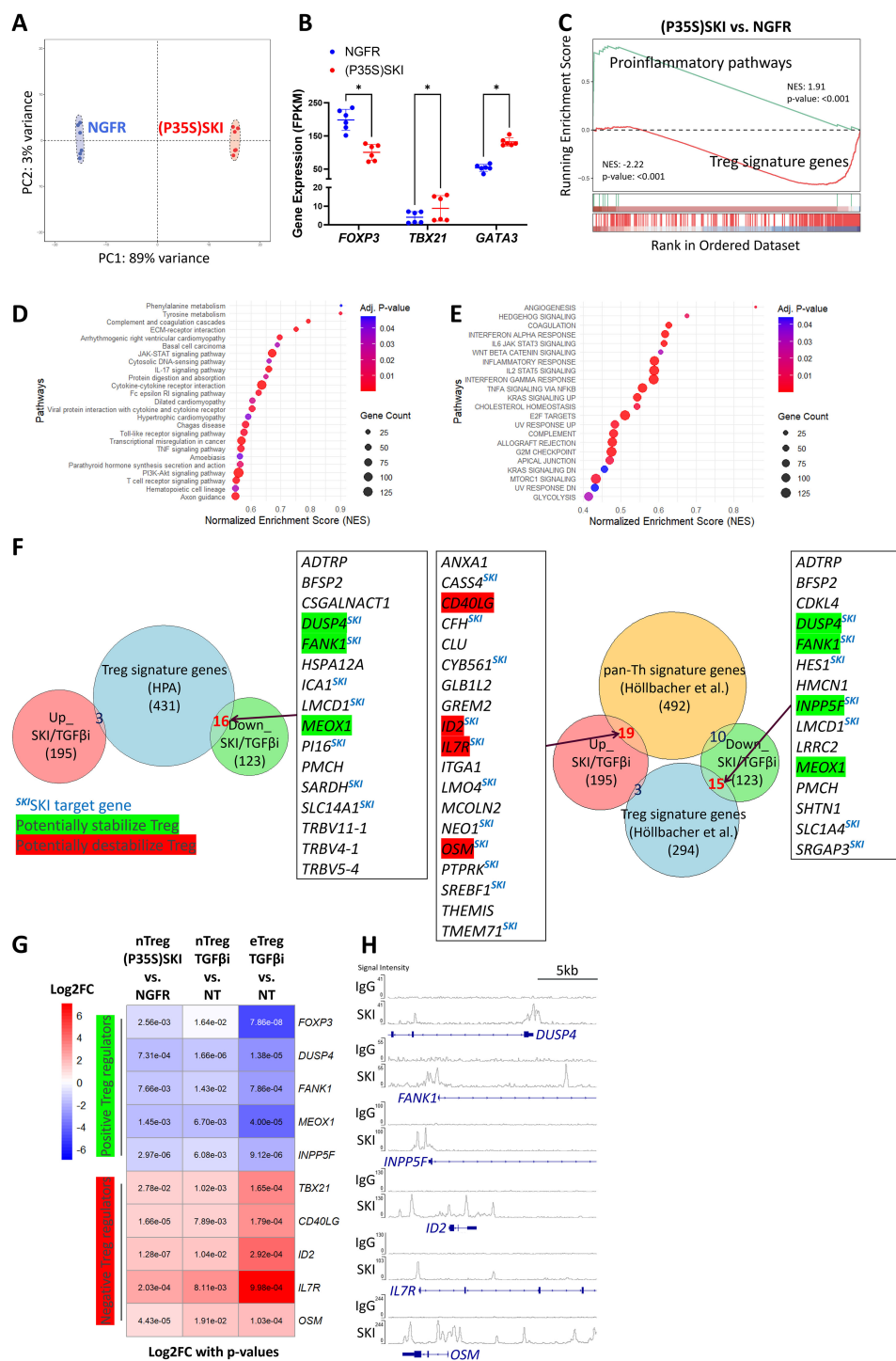


FIGURE 4
Upregulation of SKI transcriptionally disrupts Treg cell identity. **(A)** Principal Component Analysis (PCA) of whole genome transcriptome data in naive Treg cells transduced with NGFR or (P35S)SKI. $n = 6$ samples from two donors. **(B)** A scatter plot illustrates gene expression levels of the indicated genes in naive Treg cells transduced with NGFR or (P35S)SKI. Data are presented as Mean $\pm$ SD. * $p < 0.05$, two-sided t test. $n = 6$ samples from two donors. **(C)** Gene set enrichment analysis (GSEA) was performed on the indicated sample groups and gene collections. Normalized Enrichment Scores (NES) and p-values were calculated using permutation tests and are labeled accordingly. **(D, E)** KEGG **(D)** and MSigDB Hallmarks **(E)** enrichment analysis for differently expressed genes (DEGs) ((P35S)SKI vs. NGFR, adjusted $p < 0.05$, $|FC| > 2$) using the GSEA method. **(F)** Overlap analysis of commonly up- and down-regulated genes by (P35S)SKI overexpression and TGFβi in Treg cells (Up_SKI/TGFβi and Down_SKI/TGFβi, shared Differentially Expressed Genes across three RNA-seqs, adjusted $p < 0.05$, $|FC| > 2$) with gene sets of Treg signature genes (41) (left panel) or with Th/ Treg signature genes (42) (Right panel). Important overlapping genes are listed, with known positive regulators of Treg cells highlighted in green and negative regulators in red. SKI target genes are also noted. **(G)** A heatmap illustrating important Up_SKI/TGFβi and Down_SKI/TGFβi, which are known to modulate Treg identity or functionality. Log2FC values are depicted by colors, with p values labeled. **(H)** CHIP-seq profiles showing SKI occupancy on the indicated genes. Data were obtained from GSE107556 and visualized using the IGV browser.

targets of ARKADIA in response to TGF β (20). We also observed the functional redundancy of SKI and SNON (36), as single knockout of SKI failed to prevent TGF β i induced Treg destabilization (data not shown). We hypothesized that TGF β degrades SKI via ARKADIA to stabilize Treg cells. We thus overexpressed ARKADIA in naïve Treg cells and confirmed the following degradation of SKI (Figures 5A, B). We then evaluated its effect on Treg stability with or without TGF β inhibition and found that overexpression of ARKADIA prevented the FOXP3 loss and prevented the production of IFN γ in naïve Treg cells under TGF β i treatment (Figures 5C–F). Moreover, overexpression of ARKADIA preserved the Treg immune suppressive function in naïve Treg cells with TGF β i treatment (Figures 5G–H, Supplementary Figure S5).

In autoimmune patients, chronic inflammation could destabilize Treg cells and disrupt their immunosuppressive function (10, 62, 63). Since inflammatory cytokines could suppress TGF β signaling (64), we hypothesized that ARKADIA could block inflammatory-cytokine-induced Treg destabilization as well. To do so, we employed an inflammatory condition to destabilize Treg cells with or without ARKADIA overexpression. We found the inflammatory cytokine combination of IL-6, IL-1 β and IL-23 destabilized naïve Treg cells and converted them into Th1- and Th17- like cells (Figure 6A). ARKADIA overexpression significantly prevented the FOXP3 loss and suppressed the production of IFN γ and IL-17A cytokines in naïve Treg cells (Figures 6B–E), underscoring its ability to maintain Treg identity even under inflammatory conditions. Importantly, overexpression of ARKADIA notably preserved the suppressive ability of naïve Treg cells in response to inflammatory stimuli (Figures 6F, G, Supplementary Figure S5). In summary, our data suggests that ARKADIA is essential to prevent Treg destabilization and engineering ARKADIA has the potential to enhance Treg cell stability and functionality.

4 Discussion

Treg cells play an essential role in the maintenance of immune homeostasis and tolerance. Their unique characteristics, heterogeneity and plasticity, are critical for adapting to distinct inflammatory cues while retaining their suppressive capacity and effectively managing different types of inflammatory scenarios (13). Meanwhile, Treg cells must maintain their identity and functionality to prevent acquiring pathogenic phenotype. This is achieved via mechanisms like epigenetic remodeling to promote FOXP3 stability and regulation of their capacity to sense inflammatory cytokines, primarily by modulating cytokine receptors and intracellular regulators (65). The TGF β signaling is not only instrumental for induction of Treg cells from naïve CD4 T cells, but also regulates the thymic development of natural Treg cells (14). However, it remains unclear whether and how TGF β signaling contributes to the stability of established Treg cells. In this study, we demonstrate that the blockade of TGF β signaling destabilizes human Treg cells and converts them into Th-like cells expressing pro-inflammatory cytokines. Furthermore, we show that the TGF β signaling is

differentially regulated between naïve Treg cells and effector Treg cells, and that naïve Treg cells are more stable upon TGF β blockade treatment compared to that of effector Treg cells. Mechanically, we show that TGF β regulates human Treg stability through a TGF β -ARKADIA-SKI axis. By leveraging this pathway, we have developed novel strategies to stabilize human naïve Treg cells by overexpressing ARKADIA, which enhances Treg stability even under inflammatory conditions. Overall, this study broadens our understanding of the molecular regulation of human Treg cell stability and functionality.

While effector Treg cells are generally considered to have enhanced suppression against specific conventional T cells—mediated by the adoptive expression of corresponding transcription factors (e.g., TBET⁺ Treg cells controlling Th1 cells, GATA3⁺ Treg cells controlling Th2 cells, and ROR γ T⁺ Treg cells controlling Th17 cells) (8)—effector Treg cells are typically less stable than naïve Treg cells, presenting challenges for Treg cell therapies (4–6). Our research shows that TGF β dependency for stability is more significant in human effector Treg cells (CD45RO⁺) than in naïve Treg cells (CD45RO⁻), potentially due to higher SKI protein levels. Aligning with this, we provide evidence that ectopic SKI expression increases TGF β dependency in both naïve Treg and effector Treg cells. Additionally, our transcriptome analysis reveals an intrinsic reduction of TGF β signaling in effector Treg cells, which we preliminarily attribute to a lower *TGFB2* RNA level, a pattern consistent across multiple datasets. This also aligns with findings that TCR stimulation reduces *TGFB1* protein levels, affecting TGF β signaling, despite reductions in RNA levels for both *TGFB1* and *TGFB2* (66). Our findings indicate that TGF β signaling is differentially regulated in Treg subsets and the increased TGF β signaling in naïve Treg cells contributes to enhanced stability.

In this work, we explore the mechanisms of human Treg regulation by SKI and TGF β , by examining transcriptome changes following TGF β inhibition and SKI overexpression in Treg cells. We anticipate that both modulations, which increase SKI levels in human Treg cells, would reveal common patterns of transcriptional regulation that could potentially disturb Treg stability. By overlapping differentially expressed genes (DEGs) from both approaches across naïve Treg and effector Treg cells, we found a significant proportion of shared DEGs, particularly in TGF β -inhibited naïve Treg samples, which exhibited the mildest transcriptomic disturbance (195/425 in Up_DEGs and 123/365 in Down_DEGs). In this study, we focused on these common DEGs to identify genes known to regulate Treg cells and targeted by SKI. This approach allowed us to pinpoint several key target genes regulated by SKI. It is also important to note the existence of SMAD-/SKI- independent noncanonical signaling pathways triggered by TGF β ligand (15), as well as TGF β -/SMAD-independent signaling modulated by SKI (67, 68). These pathways might explain the large number of unique DEGs in TGF β -inhibited effector Treg samples (724/1303 in Up_DEGs and 1525/2186 in Down_DEGs) and the mutant SKI-overexpressed naïve Treg samples (363/850 in Up_DEGs and 387/961 in Down_DEGs), respectively. Therefore, we cannot rule out the possibility that additional mechanisms might contribute to the TGF β i-mediated and SKI overexpression-mediated Treg

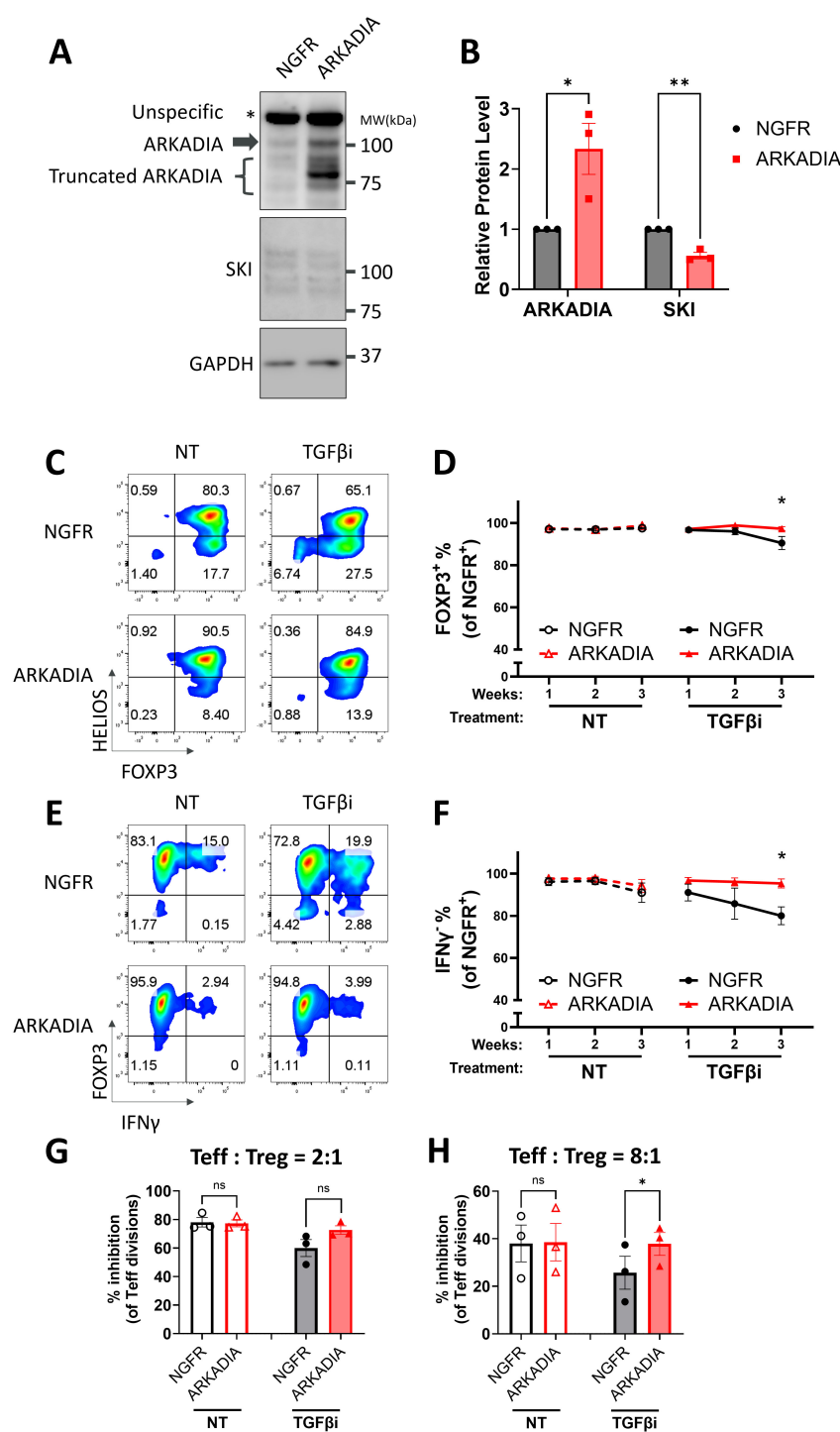


FIGURE 5

ARKADIA prevents Treg destabilization upon TGFβ inhibition. (A, B) Naïve Treg cells carrying NGFR or ARKADIA-T2A-NGFR (ARKADIA) were cultured for seven days, followed by immunoblot targeting ARKADIA, SKI, and GAPDH. (A) Representative immunoblots of three donors. Note: ARKADIA shows multiple bands due to truncation, a pattern previously reported using the same antibody clone (20). (B) ARKADIA and SKI levels were quantitated and normalized to GAPDH to calculate normalized protein levels and then compared to the NGFR samples to determine relative protein levels. Data are presented as Mean ± SEM. **p* < 0.05, two-sided *t* test. *n* = 3 donors. (C–H) Naïve Treg cells carrying NGFR or ARKADIA-T2A-NGFR (ARKADIA) were treated with (TGFβi) or without (NT) TGFβR1 inhibitor for three weeks. Levels of indicated proteins within the NGFR⁺ population were assessed weekly via flow cytometry. Representative flow cytometry profiles showing FOXP3 and HELIOS levels (C) or FOXP3 and IFNγ levels (E) in transduced Treg cells after three weeks of treatment. The flow cytometry profiles are representative of experiments conducted in three donors. Line charts summarize the percentage of FOXP3⁺ cells (D) or IFNγ⁺ cells (F) within the NGFR⁺ population in the Treg cells. Data are presented as Mean ± SEM. **p* < 0.05, two-sided *t* test. *n* = 3 donors. After the three-week treatment, those Treg cells were subjected to an *in vitro* suppression assay by being cultured with Teff cells at a 2:1 ratio (Treg: Teff) (G) or 8:1 ratio (Treg: Teff) (H) for four days. Following incubation, Teff proliferation was assessed by CTV dilution and flow cytometry. Suppression efficiency was calculated as “% inhibition of Teff division,” determined by the percentage reduction in the division index of Teff cells compared to Teff cells cultured alone. Data are presented as Mean ± SEM. **p* < 0.05, two-sided *t* test. *n* = 3 donors.

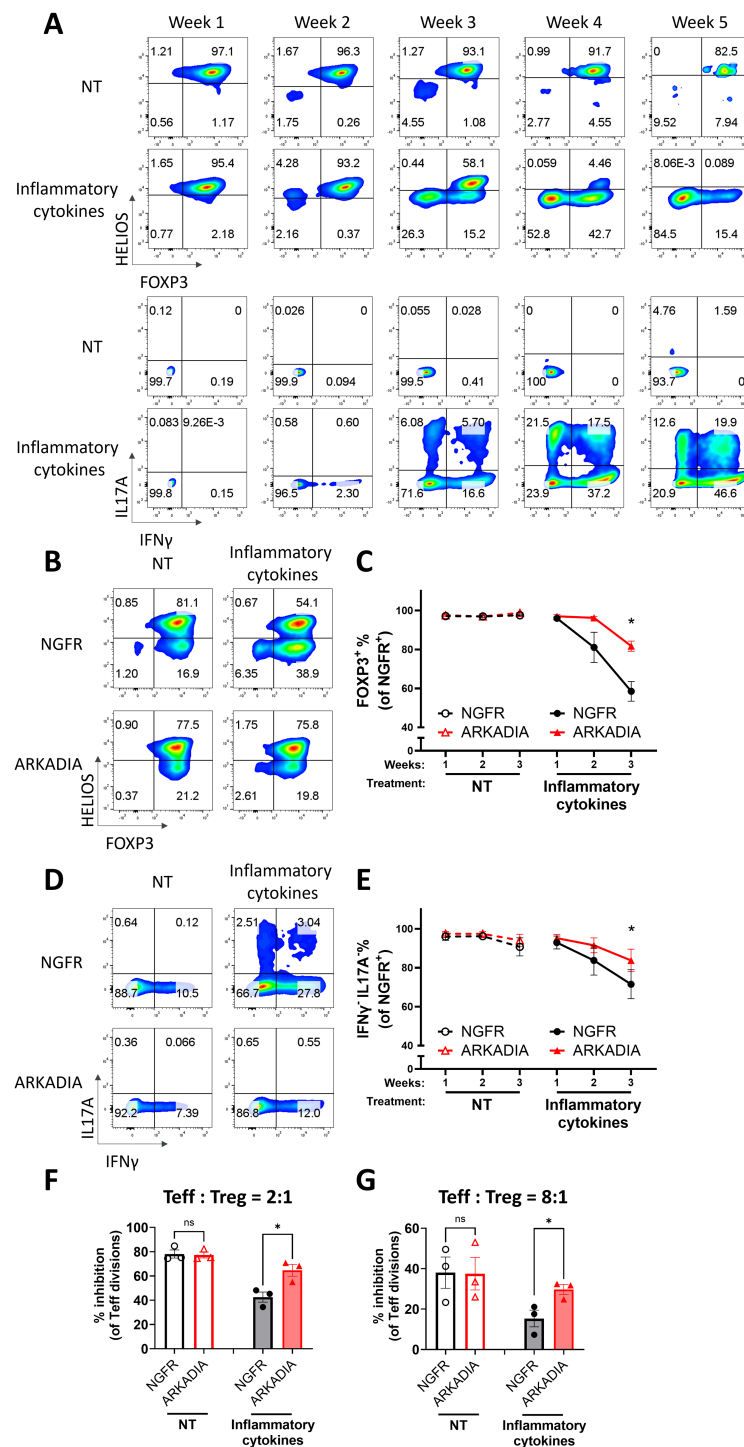


FIGURE 6

ARKADIA prevents Treg destabilization and preserves Treg function in the presence of inflammatory stimulation. **(A)** Naive Treg cells were treated with (inflammatory cytokines) or without (NT) pro-inflammatory cytokines for five weeks. FOXP3, HELIOS, IL17A and IFN γ levels were assessed weekly via flow cytometry. The flow cytometry profiles are representative of experiments conducted in more than three donors. **(B–G)** Naive Treg cells transduced with NGFR or ARKADIA-T2A-NGFR (ARKADIA) were treated with (inflammatory cytokines) or without (NT) pro-inflammatory cytokines for three weeks. Levels of indicated proteins within the NGFR⁺ population were assessed weekly via flow cytometry. Representative flow cytometry profiles showing FOXP3 and HELIOS levels **(B)** or IL17A and IFN γ levels **(D)** in transduced Treg cells after three weeks of treatment. The flow cytometry profiles are representative of experiments conducted in three donors. Line charts summarize the percentage of FOXP3⁺ cells **(C)** or IFN γ IL17A⁺ cells **(E)** within the NGFR⁺ population in the Treg cells. Data are presented as Mean $\pm$ SEM. * p < 0.05, two-sided t test. n = 3 donors. After the three-week treatment, those nTreg cells were subjected to an *in vitro* suppression assay, by being cultured with Teff cells at a 2:1 ratio (Treg: Teff) **(F)** or 8:1 ratio (Treg: Teff) **(G)** for four days. Following incubation, Teff proliferation was assessed by CTV dilution and flow cytometry. Suppression efficiency was calculated as “% inhibition of Teff division,” determined by the percentage reduction in the division index of Teff cells compared to Teff cells cultured alone. Data are presented as Mean $\pm$ SEM. * p < 0.05, two-sided t test. n = 3 donors.

destabilization. In summary, our transcriptional analysis has identified a novel role of SKI-driven transcriptional regulation of the stability and functionality of human Treg cells. We believe these findings lay the groundwork for future, more detailed mechanistic investigations into this axis.

TGF β is central to Treg not only because Treg cells are among the primary producers of latent TGF β 1 and possess specific activation mechanisms, but also due to their high surface expression of the TGF β receptor (69). At the molecular level, TGF β signaling—particularly through SMAD3—can directly enhance FOXP3 expression by binding to the conserved non-coding sequence CNS1 within the FOXP3 locus (70). While this axis is essential for the generation of peripherally-derived Treg (pTreg) cells (71, 72) and iTreg cells (21, 22), but largely dispensable for thymus-derived (tTreg) cell generation under non-inflammatory conditions (73, 74). However, the role of TGF β in the maintenance of Treg cells, especially regarding sustained FOXP3 expression, remains controversial, with prior studies in mouse models reporting mixed results (25–28). HELIOS cooperates with FOXP3 to reinforce the functionality and stability of Tregs and is widely recognized as a stable marker of Treg (39, 75, 76). Intriguingly, here we observed that SKI overexpression not only interrupts FOXP3 but also HELIOS levels in Treg cells. This observation is consistent with what others have observed in mice where Arkadia deficiency in Cd4 T cells increases the percentage of Helios⁺ Treg cells (36). The regulation of both FOXP3 and HELIOS by the TGF β -ARKADIA-SKI axis highlights their central roles in maintaining human Treg stability and underscores the need for further investigation into this pathway.

Ski has been previously shown to prevent the differentiation of Th17 cells from naïve CD4 T cells in *in vitro* Th17 polarizing cytokine conditions (35, 37). In our study, we found that destabilization of SKI through ectopic ARKADIA overexpression protected human Treg cells from transitioning into a Th1-/Th17-like phenotype under similar conditions. This discrepancy may arise from differences in the regulatory mechanisms governing Th17 differentiation from Treg cells versus naïve CD4 T cells, as well as from variations in these mechanisms between humans and mice. Additionally, it may be attributed to the dose-dependent regulation by SKI (16), where ARKADIA overexpression only destabilizes the inherently low-dose endogenous SKI in Treg, which is insufficient to modulate Th17 differentiation from Treg cells. This discrepancy highlights the need for further investigation into the distinct signaling contexts under which TGF β and SKI operate within human immune cells. Another limitation remains as our findings are primarily based on *in vitro* experiments. As we move forward, integrating insights from *in vitro* studies with *in vivo* experimentation to evaluate SKI-mediated Treg destabilization, and especially ARKADIA-mediated Treg stabilization, will further benefit our knowledge in Treg biology and Treg cell therapies.

Treg cell-based therapies are actively investigated to treat autoimmune diseases, organ transplantation rejection and GvHD (12). Techniques such as Chimeric Antigen Receptor (CAR) and antigen specific TCR are employed to enhance the specificity and

potency of these therapies. However, a critical concern in using Treg cells as modality is their stability, defined by the stable maintenance of FOXP3 expression and suppressive activity, without the production of pro-inflammatory cytokines. While the stable FOXP3 expression has traditionally been viewed as the primary determinant of Treg stability, it is now clear that additional mechanisms play important roles, such as epigenetic modifications, metabolism changes and genetics factors (12).

Our investigation into the TGF β -ARKADIA-SKI axis highlights a previously underappreciated mechanism governing the stability of naturally occurring human Treg cells—the major population used in Treg and CAR-Treg cell therapy clinical trials. We demonstrate for the first time in primary human Treg cells that TGF β regulates SKI protein stability via the E3 ligase ARKADIA, with SKI negatively impacting Treg functionality. Though important differences exist between human and murine Treg biology and immune responses (77, 78), our findings extend and validate observations from murine models, in which Arkadia is required for murine Treg cell differentiation and the control of microbiota-induced mucosal inflammation (36). Importantly, by confirming this evolutionarily conserved pathway specifically in human natural Tregs—not just murine Treg cells and murine inflammation models—our work directly addresses a critical question in the field and provides mechanistic and translational insights with direct application for Treg cell-based therapies. Furthermore, we provide the first evidence that ARKADIA overexpression can enhance the stability of human Tregs in a cell-therapy-compatible setting, supporting its future exploration as a molecular target for improving Treg-based immunotherapies in inflammatory diseases.

In summary, our study uncovers a novel role of TGF β -ARKADIA-SKI axis in human Treg cell stability and functionality. It also provides potential targets to either limit Treg stability to improve immunotherapies for tumor or enhance Treg stability of Treg cell therapies for inflammatory diseases.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/[Supplementary Material](#).

Ethics statement

The studies involving humans were approved by the independent Institutional Review Boards responsible for overseeing donor consents and protocols at StemExpress (WIRB-Copernicus Group), BioIVT (WIRB-Copernicus Group), and AllCells (Alpha Independent Review Board). The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from StemExpress, BioIVT, or AllCells. Written informed consent was obtained from all donors by the respective vendors prior to sample collection.

Author contributions

FY: Validation, Resources, Visualization, Conceptualization, Data curation, Methodology, Formal Analysis, Writing – review & editing, Software, Writing – original draft, Investigation. AY: Writing – review & editing, Methodology. ML: Formal Analysis, Software, Data curation, Methodology, Investigation, Writing – review & editing. PH: Methodology, Writing – review & editing. IL: Methodology, Writing – review & editing. MS: Writing – review & editing, Methodology. YS: Methodology, Writing – review & editing. TO: Project administration, Conceptualization, Supervision, Writing – review & editing. KB: Project administration, Supervision, Writing – review & editing, Conceptualization. YI: Investigation, Supervision, Conceptualization, Resources, Funding acquisition, Writing – review & editing, Project administration. ZG: Methodology, Supervision, Writing – review & editing, Investigation, Conceptualization, Funding acquisition, Formal Analysis, Project administration, Resources.

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Conflict of interest

All authors were employees of AstraZeneca when this work was conducted and own stock and stock options in the company.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1636434/full#supplementary-material>

References

1. Sakaguchi S, Mikami N, Wing JB, Tanaka A, Ichiyama K, Ohkura N. Regulatory T cells and human disease. *Annu Rev Immunol.* (2020) 38:541–66. doi: 10.1146/annurev-immunol-042718-041717
2. Valmori D, Merlo A, Souleimanian NE, Hesdorffer CS, Ayyoub M. A peripheral circulating compartment of natural naive cd4+ Tregs. *J Clin Invest.* (2005) 115:1953–62. doi: 10.1172/JCI23963
3. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human cd4+ T cells expressing the foxp3 transcription factor. *Immunity.* (2009) 30:899–911. doi: 10.1016/j.immuni.2009.03.019
4. Hoffmann P, Eder R, Boeld TJ, Doser K, Pishesha B, Andreesen R, et al. Only the cd45ra+ Subpopulation of cd4+cd25high T cells gives rise to homogeneous regulatory T-cell lines upon *in vitro* expansion. *Blood.* (2006) 108:4260–7. doi: 10.1182/blood-2006-06-027409
5. Booth NJ, Mcquaid AJ, Sobande T, Kissane S, Agius E, Jackson SE, et al. Different proliferative potential and migratory characteristics of human cd4+ Regulatory T cells that express either cd45ra or cd45ro. *J Immunol.* (2010) 184:4317–26. doi: 10.4049/jimmunol.0903781
6. Wendering DJ, Amini L, Schlickeiser S, Farrera-Sal M, Schulenberg S, Peter L, et al. Effector memory-type regulatory T cells display phenotypic and functional instability. *Sci Adv.* (2024) 10:Eadn3470. doi: 10.1126/sciadv.adn3470
7. Raffin C, Vo LT, Bluestone JA. Treg cell-based therapies: challenges and perspectives. *Nat Rev Immunol.* (2020) 20:158–72. doi: 10.1038/s41577-019-0232-6
8. Campbell DJ, Koch MA. Phenotypic and functional specialization of foxp3+ Regulatory T cells. *Nat Rev Immunol.* (2011) 11:119–30. doi: 10.1038/nri2916
9. Honing DY, Luiten RM, Matos TR. Regulatory T cell dysfunction in autoimmune diseases. *Int J Mol Sci.* (2024) 25(13):7171. doi: 10.3390/ijms25137171
10. Miyao T, Floess S, Setoguchi R, Luche H, Waldmann H, Huehn J, et al. Plasticity of foxp3+ T cells reflects promiscuous foxp3 expression in conventional T cells but not reprogramming of regulatory T cells. *Immunity.* (2012) 36:262–75. doi: 10.1016/j.immuni.2011.12.012
11. Schmidt A, Oberle N, Krammer PH. Molecular mechanisms of treg-mediated T cell suppression. *Front Immunol.* (2012) 3:51. doi: 10.3389/fimmu.2012.00051
12. Bluestone JA, McKenzie BS, Beilke J, Ramsdell F. Opportunities for treg cell therapy for the treatment of human disease. *Front Immunol.* (2023) 14:1166135. doi: 10.3389/fimmu.2023.1166135
13. Overacre AE, Vignali DA. T(Reg) stability: to be or not to be. *Curr Opin Immunol.* (2016) 39:39–43. doi: 10.1016/j.coi.2015.12.009
14. Chen W. Tgf-beta regulation of T cells. *Annu Rev Immunol.* (2023) 41:483–512. doi: 10.1146/annurev-immunol-101921-045939

15. Deng Z, Fan T, Xiao C, Tian H, Zheng Y, Li C, et al. Tgf- β Signaling in health, disease, and therapeutics. *Signal Transduct Target Ther.* (2024) 9:61. doi: 10.1038/s41392-024-01764-w
16. Wang J, Zhao X, Wan YY. Intricacies of tgf- β Signaling in treg and th17 cell biology. *Cell Mol Immunol.* (2023) 20(9):1002–22. doi: 10.1038/s41423-023-01036-7
17. Tecalco-Cruz AC, Rios-Lopez DG, Vazquez-Victorio G, Rosales-Alvarez RE, Macias-Silva M. Transcriptional cofactors ski and snon are major regulators of the tgf-beta/smad signaling pathway in health and disease. *Signal Transduct Target Ther.* (2018) 3:15. doi: 10.1038/s41392-018-0015-8
18. Nagano Y, Mavrikis KJ, Lee KL, Fujii T, Koinuma D, Sase H, et al. Arkadia induces degradation of snon and C-ski to enhance transforming growth factor- β Signaling. *J Biol Chem.* (2007) 282:20492–501. doi: 10.1074/jbc.M701294200
19. Le Scolan E, Zhu Q, Wang L, Bandyopadhyay A, Javelaud D, Mauviel A, et al. Transforming growth factor- β Suppresses the ability of ski to inhibit tumor metastasis by inducing its degradation. *Cancer Res.* (2008) 68:3277–85. doi: 10.1158/0008-5472.CAN-07-6793
20. Laigle V, Dingli F, Amhaz S, Perron T, Chouchène M, Colasse S, et al. Quantitative ubiquitylome analysis reveals the specificity of rnf111/arkadia E3 ubiquitin ligase for its degradative substrates ski and skil/snon in tgf- β Signaling pathway. *Mol Cell Proteomics.* (2021) 20:100173. doi: 10.1016/j.mcpro.2021.100173
21. Chen W, Jin W, Hardegen N, Lei K-J, Li L, Marinos N, et al. Conversion of peripheral cd4+cd25- naive T cells to cd4+cd25+ Regulatory T cells by tgf- β Induction of transcription factor foxp3. *J Exp Med.* (2003) 198:1875–86. doi: 10.1084/jem.20030152
22. Wan YY, Flavell RA. Identifying foxp3-expressing suppressor T cells with A bicistronic reporter. *Proc Natl Acad Sci.* (2005) 102:5126–31. doi: 10.1073/pnas.0501701102
23. Liu Y, Zhang P, Li J, Kulkarni AB, Perruche S, Chen W. A critical function for tgf- β Signaling in the development of natural cd4+ cd25+ Foxp3+ Regulatory T cells. *Nat Immunol.* (2008) 9:632–40. doi: 10.1038/ni.1607
24. Ouyang W, Beckett O, Ma Q, Li MO. Transforming growth factor- β Signaling curbs thymic negative selection promoting regulatory T cell development. *Immunity.* (2010) 32:642–53. doi: 10.1016/j.immuni.2010.04.012
25. Li MO, Sanjabi S, Flavell RA. Transforming growth factor- β Controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and-independent mechanisms. *Immunity.* (2006) 25:455–71. doi: 10.1016/j.immuni.2006.07.011
26. Marie JC, Letterio JJ, Gavin M, Rudensky AY. Tgf- β 1 maintains suppressor function and foxp3 expression in cd4+ cd25+ Regulatory T cells. *J Exp Med.* (2005) 201:1061–7. doi: 10.1084/jem.20042276
27. Zhang N, Bevan MJ. Tgf- β Signaling to T cells inhibits autoimmunity during lymphopenia-driven proliferation. *Nat Immunol.* (2012) 13:667–73. doi: 10.1038/ni.2319
28. Ślodzińska A, Hemmers S, Mair F, Gorka O, Ruland J, Fairbairn L, et al. Tgf- β Signalling is required for cd4+ T cell homeostasis but dispensable for regulatory T cell function. *PLoS Biol.* (2013) 11:E1001674. doi: 10.1371/journal.pbio.1001674
29. Bonini C, Grez M, Traversari C, Cicceri F, Marktel S, Ferrari G, et al. Safety of retroviral gene marking with A truncated ngf receptor. *Nat Med.* (2003) 9:367–9. doi: 10.1038/nm0403-367
30. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for rna-seq data with deseq2. *Genome Biol.* (2014) 15:1–21. doi: 10.1186/s13059-014-0550-8
31. Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z, et al. Clusterprofiler 4.0: A universal enrichment tool for interpreting omics data. *Innovation.* (2021) 2. doi: 10.1016/j.xinn.2021.100141
32. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, et al. Integrative genomics viewer. *Nat Biotechnol.* (2011) 29:24–6. doi: 10.1038/nbt.1754
33. Inman GJ, Nicolás FJ, Callahan JF, Harling JD, Gaster LM, Reith AD, et al. Sb-431542 is a potent and specific inhibitor of transforming growth factor- β Superfamily type I activin receptor-like kinase (Alk) receptors alk4, alk5, and alk7. *Mol Pharmacol.* (2002) 62:65–74. doi: 10.1124/mol.62.1.65
34. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martínez-Llordella M, Ashby M, et al. Instability of the transcription factor foxp3 leads to the generation of pathogenic memory T cells *in vivo*. *Nat Immunol.* (2009) 10:1000–7. doi: 10.1038/ni.1774
35. Zhang S, Takaku M, Zou L, Gu A-D, Chou W-C, Zhang G, et al. Reversing ski-smad4-mediated suppression is essential for th17 cell differentiation. *Nature.* (2017) 551:105–9. doi: 10.1038/nature24283
36. Xu H, Wu L, Nguyen HH, Mesa KR, Raghavan V, Episkopou V, et al. Arkadia-ski/snon signaling differentially regulates tgf- β -induced itreg and th17 cell differentiation. *J Exp Med.* (2021) 218:E20210777. doi: 10.1084/jem.20210777
37. Li P, Guo Z, Wan YY. Ski expression suppresses pathogenic th17 cell response and mitigates experimental autoimmune encephalomyelitis. *Front Immunol.* (2021) 12:707899. doi: 10.3389/fimmu.2021.707899
38. Marcelain K, Hayman MJ. The ski oncoprotein is upregulated and localized at the centrosomes and mitotic spindle during mitosis. *Oncogene.* (2005) 24:4321–9. doi: 10.1038/sj.onc.1208631
39. Kim H-J, Barnitz RA, Kreslavsky T, Brown FD, Moffett H, Lemieux ME, et al. Stable inhibitory activity of regulatory T cells requires the transcription factor helios. *Science.* (2015) 350:334–9. doi: 10.1126/science.aad0616
40. Gori I, George R, Purkiss AG, Strohbuecker S, Randall RA, Ogradowicz R, et al. Mutations in ski in shprintzen-goldberg syndrome lead to attenuated tgf- β Responses through ski stabilization. *Elife.* (2021) 10:e63545. doi: 10.7554/eLife.63545
41. Uhlen M, Karlsson MJ, Zhong W, Tebani A, Pou C, Mikes J, et al. A genome-wide transcriptomic analysis of protein-coding genes in human blood cells. *Science.* (2019) 366:Eaax9198. doi: 10.1126/science.aax9198
42. Höllbacher B, Duhen T, Motley S, Klicznik MM, Gratz IK, Campbell DJ. Transcriptomic profiling of human effector and regulatory T cell subsets identifies predictive population signatures. *Immunohorizons.* (2020) 4:585–96. doi: 10.4049/immunohorizons.2000037
43. Tecalco-Cruz AC, Sosa-Garrocho M, Vázquez-Victorio G, Ortiz-García L, Domínguez-Hüttlinger E, Macías-Silva M. Transforming growth factor- β /smad target gene skil is negatively regulated by the transcriptional cofactor complex snon-smad4. *J Biol Chem.* (2012) 287:26764–76. doi: 10.1074/jbc.M112.386599
44. Tabata T, Kokura K, Ten Dijke P, Ishii S. Ski co-repressor complexes maintain the basal repressed state of the tgf- β Target gene, smad7, via hdac3 and prmt5. *Genes Cells.* (2009) 14:17–28. doi: 10.1111/j.1365-2443.2008.01246.x
45. Gerriets VA, Kishton RJ, Johnson MO, Cohen S, Siska PJ, Nichols AG, et al. Foxp3 and toll-like receptor signaling balance treg cell anabolic metabolism for suppression. *Nat Immunol.* (2016) 17:1459–66. doi: 10.1038/ni.3577
46. Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, Xiao B, et al. The mtor kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity.* (2009) 30:832–44. doi: 10.1016/j.immuni.2009.04.014
47. Van Loosdregt J, Fleskens V, Tiemessen MM, Mokry M, Van Boxtel R, Meerding J, et al. Canonical wnt signaling negatively modulates regulatory T cell function. *Immunity.* (2013) 39:298–310. doi: 10.1016/j.immuni.2013.07.019
48. Angelin A, Gil-De-Gómez L, Dahiya S, Jiao J, Guo L, Levine MH, et al. Foxp3 reprograms T cell metabolism to function in low-glucose, high-lactate environments. *Cell Metab.* (2017) 25:1282–1293.E7. doi: 10.1016/j.cmet.2016.12.018
49. Sugimoto N, Liu Y-J. Dusp4 stabilizes foxp3 expression in human regulatory T cells. *Blood.* (2013) 122:3473. doi: 10.1182/blood.V122.21.3473.3473
50. Wang H, Song W, Hu T, Zhang N, Miao S, Zong S, et al. Fank1 interacts with jab1 and regulates cell apoptosis via the ap-1 pathway. *Cell Mol Life Sci.* (2011) 68:2129–39. doi: 10.1007/s00018-010-0559-4
51. Ogawa C, Tone Y, Tsuda M, Peter C, Waldmann H, Tone M. Tgf- β -mediated foxp3 gene expression is cooperatively regulated by stat5, creb, and ap-1 through cns2. *J Immunol.* (2014) 192:475–83. doi: 10.4049/jimmunol.1301892
52. Baßler K, Schmidleithner L, Shakiba MH, Elmzahi T, Köhne M, Floess S, et al. Identification of the novel foxp3-dependent treg cell transcription factor meox1 by high-dimensional analysis of human cd4+ T cells. *Front Immunol.* (2023) 14:1107397. doi: 10.3389/fimmu.2023.1107397
53. Kim HS, Li A, Ahn S, Song H, Zhang W. Inositol polyphosphate-5-phosphatase F (Inpp5f) inhibits stat3 activity and suppresses gliomas tumorigenicity. *Sci Rep.* (2014) 4:7330. doi: 10.1038/srep07330
54. Laurence A, Amarnath S, Mariotti J, Kim YC, Foley J, Eckhaus M, et al. Stat3 transcription factor promotes instability of ntreg cells and limits generation of itreg cells during acute murine graft-versus-host disease. *Immunity.* (2012) 37:209–22. doi: 10.1016/j.immuni.2012.05.027
55. Hwang S-M, Sharma G, Verma R, Byun S, Rudra D, Im S-H. Inflammation-induced id2 promotes plasticity in regulatory T cells. *Nat Commun.* (2018) 9:4736. doi: 10.1038/s41467-018-07254-2
56. Waickman AT, Keller HR, Kim T-H, Luckey MA, Tai X, Hong C, et al. The cytokine receptor il-7 α Impairs il-2 receptor signaling and constrains the *in vitro* differentiation of foxp3+ Treg cells. *Iscience.* (2020) 23. doi: 10.1016/j.isci.2020.101421
57. Sharma MD, Hou D-Y, Baban B, Koni PA, He Y, Chandler PR, et al. Reprogrammed foxp3+ Regulatory T cells provide essential help to support cross-presentation and cd8+ T cell priming in naive mice. *Immunity.* (2010) 33:942–54. doi: 10.1016/j.immuni.2010.11.022
58. Wolf CL, Pruett C, Lighter D, Jorczyk CL. The clinical relevance of osm in inflammatory diseases: A comprehensive review. *Front Immunol.* (2023) 14:1239732. doi: 10.3389/fimmu.2023.1239732
59. Yevshin I, Sharipov R, Valeev T, Kel A, Kolpakov F. Gtrd: A database of transcription factor binding sites identified by chip-seq experiments. *Nucleic Acids Res.* (2016), Gkw951. doi: 10.1093/nar/gkw951
60. Feld C, Sahu P, Frech M, Finkernagel F, Nist A, Stiewe T, et al. Combined cistrome and transcriptome analysis of ski in aml cells identifies ski as a co-repressor for runx1. *Nucleic Acids Res.* (2018) 46:3412–28. doi: 10.1093/nar/gky119
61. Levy L, Howell M, Das D, Harkin S, Episkopou V, Hill CS. Arkadia activates smad3/smad4-dependent transcription by triggering signal-induced snon degradation. *Mol Cell Biol.* (2007) 27:6068–83. doi: 10.1128/MCB.00664-07

62. Kleinewietfeld M, Hafler DA. The plasticity of human treg and th17 cells and its role in autoimmunity. *Semin Immunol.* (2013) 25(4):305–12. Elsevier. doi: 10.1016/j.smim.2013.10.009
63. Komatsu N, Okamoto K, Sawa S, Nakashima T, Oh-Hora M, Kodama T, et al. Pathogenic conversion of foxp3+ T cells into th17 cells in autoimmune arthritis. *Nat Med.* (2014) 20:62–8. doi: 10.1038/nm.3432
64. Yu J, Wei M, Becknell B, Trotta R, Liu S, Boyd Z, et al. Pro- and antiinflammatory cytokine signaling: reciprocal antagonism regulates interferon-gamma production by human natural killer cells. *Immunity.* (2006) 24:575–90. doi: 10.1016/j.immuni.2006.03.016
65. Contreras-Castillo E, Garcia-Rasilla VY, Garcia-Patino MG, Licona-Limon P. Stability and plasticity of regulatory T cells in health and disease. *J Leukoc Biol.* (2024) 116:33–53. doi: 10.1093/jleuko/qiae049
66. Tu E, Chia CPZ, Chen W, Zhang D, Park SA, Jin W, et al. T cell receptor-regulated tgf- β Type I receptor expression determines T cell quiescence and activation. *Immunity.* (2018) 48:745–759.E6. doi: 10.1016/j.immuni.2018.03.025
67. Dai P, Shinagawa T, Nomura T, Harada J, Kaul SC, Wadhwa R, et al. Ski is involved in transcriptional regulation by the repressor and full-length forms of gli3. *Genes Dev.* (2002) 16:2843–8. doi: 10.1101/gad.1017302
68. Rashidian J, Le Scolan E, Ji X, Zhu Q, Mulvihill MM, Nomura D, et al. Ski regulates hippo and taz signaling to suppress breast cancer progression. *Sci Signaling.* (2015) 8:Ra14–4. doi: 10.1126/scisignal.2005735
69. Moreau JM, Velegraki M, Bolyard C, Rosenblum MD, Li Z. Transforming growth factor- β 1 in regulatory T cell biology. *Sci Immunol.* (2022) 7:Eabi4613. doi: 10.1126/sciimmunol.abi4613
70. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding dna elements in the foxp3 gene in regulatory T-cell fate. *Nature.* (2010) 463:808–12. doi: 10.1038/nature08750
71. Coombes JL, Siddiqui KR, Arancibia-Cárcamo CV, Hall J, Sun C-M, Belkaid Y, et al. A functionally specialized population of mucosal cd103+ Dcs induces foxp3+ Regulatory T cells via A tgf- β -and retinoic acid-dependent mechanism. *J Exp Med.* (2007) 204:1757–64. doi: 10.1084/jem.20070590
72. Sun C-M, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, et al. Small intestine lamina propria dendritic cells promote *de novo* generation of foxp3 T reg cells via retinoic acid. *J Exp Med.* (2007) 204:1775–85. doi: 10.1084/jem.20070602
73. Josefowicz SZ, Niec RE, Kim HY, Treuting P, Chinen T, Zheng Y, et al. Extrathymically generated regulatory T cells control mucosal th2 inflammation. *Nature.* (2012) 482:395–9. doi: 10.1038/nature10772
74. Josefowicz SZ, Lu L-F, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol.* (2012) 30:531–64. doi: 10.1146/annurev.immunol.25.022106.141623
75. Getnet D, Grosso JF, Goldberg MV, Harris TJ, Yen HR, Bruno TC, et al. A role for the transcription factor helios in human cd4(+)Cd25(+) regulatory T cells. *Mol Immunol.* (2010) 47:1595–600. doi: 10.1016/j.molimm.2010.02.001
76. Thornton AM, Shevach EM. Helios: still behind the clouds. *Immunology.* (2019) 158:161–70. doi: 10.1111/imm.13115
77. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Nat Acad Sci.* (2013) 110(9):3507–12. doi: 10.1073/pnas.1222878110
78. Medetgul-Ernar K, Davis MM. Standing on the shoulders of mice. *Immunity.* (2022) 55(8):1343–53. doi: 10.1016/j.immuni.2022.07.008