ORIGINAL RESEARCH

A Novel Role for Kruppel-like Factor 14 (KLF14) in T-Regulatory Cell Differentiation

Olga F. Sarmento,1 Phyllis A. Svingen,1 Yuning Xiong,1 Ramnik J. Xavier,2 Dermot McGovern,3 Thomas C. Smyrk,4 Konstantinos A. Papadakis,1 Raul A. Urrutia,1 and William A. Faubion1

1Epigenetics and Chromatin Dynamics Laboratory, Division of Gastroenterology and Hepatology and Translational Epigenomic Program, Center for Individualized Medicine, Mayo Clinic, Rochester, Minnesota; 2Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts; 3Cedars Sinai Hospital, Los Angeles, California; 4Laboratory and Medical Pathology, Mayo Clinic, Rochester, Minnesota

SUMMARY

KLF14 represses FOXP3 expression through an epigenetic mechanism involving the recruitment of the HP1 family of chromatin-modifying complexes to the promoter locus. The lack of KLF14 results in the differentiation of peripheral-induced T-regulatory cells and protection from colitis.

BACKGROUND & AIMS: Kruppel-like Factor 14 (KLF14) proteins function as epigenetic reprogramming factors during cell differentiation in many cell populations and in engineered induced pluripotent stem cells. In this study, we determined the function of KLF14 in the regulation of forkhead box protein 3 (FOXP3), a transcription factor critical for T regulatory (Treg) cell differentiation.

METHODS: We studied the effects of KLF14 on FOXP3 expression at the level of the protein and mRNA. We evaluated the functional relevance of KLF14 to FOXP3+ Treg cells in vitro and in vivo through suppression assays and two colitis models. Finally, we analyzed the effect of KLF14 on the epigenetic landscape of the FOXP3 promoter locus through chromatin immunoprecipitation (ChIP) assay.

RESULTS: KLF14, induced upon activation of naïve CD4+ T cells, segregates to the FOXP3+ population and is inversely associated with FOXP3 expression and Treg function. KLF14 knockout (KO) CD4+ cells differentiated into adaptive Tregs more readily in vitro and in vivo. KLF14 KO cells demonstrated an enhanced Treg suppressor function in vitro and in vivo. KLF14 repressed FOXP3 at the level of the mRNA and protein, and by ChIP assay KLF14 was found to bind to the Treg-specific demethylation region (TSDR) enhancer region of FOXP3. Furthermore, loss of KLF14 reduced the levels of H3K9me3, HP1, and Suv39H1 at the TSDR.

CONCLUSIONS: These results outline a novel mechanism by which KLF14 regulates Treg cell differentiation via chromatin remodeling at the FOXP3 TSDR. To our knowledge, this is the first evidence to support a role for KLF14 in maintaining the differentiated state of Treg cells, with an outline of a potential mechanism to modify the expression of immune genes such as FOXP3 that are critical to T-cell fate. (Cell Mol Gastroenterol Hepatol 2015;1:188–202; http://dx.doi.org/10.1016/j.ccmgh.2014.12.007)

Keywords: FOXP3; H3K9Me3; KLF14; T-regulatory cell; TSDR.

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Abbreviations used in this paper: CAR, Coxsackie adenovirus receptor; ChIP, chromatin immunoprecipitation; DSS, dextran sodium sulfate; FOXP3, forkhead box protein 3; KLF, Kruppel-like factor; KO, knockout; PCR, polymerase chain reaction; RAG, recombinase activating gene-1-deficient; S1P1, sphingosine-kinase-1; siRNA, small interfering RNA; TCR, T-cell receptor; TGFβ1, transforming growth factor β1; Treg, T regulatory; TSDR, Treg-specific demethylation region; WT, wild type.
susceptibility to colitis. The block in Treg differentiation was found to be Polycomb-mediated epigenetic silencing of the FOXP3 core promoter.\(^1\) Extending our investigations into the entire FOXP3 regulatory region, we now report a key role for KLF14 in the maintenance of FOXP3 expression, Treg cell function, and colitis susceptibility.

KLF14, the most recent member of the KLF family to be identified, is a rapidly evolving imprinted gene with a clear function in metabolism. Indeed, recently KLF14 has been found to play a key role in lipogenesis.\(^3\) This role in fatty acid metabolism has a profound impact in immune function, as the metabolic pathways driving T-cell differentiation fate are a novel, active area of research.\(^5\) To begin to address this intersection between metabolism and immune cell fate, we have for the first time ascribed a role for KLF14 in immune function. Specifically, combined cellular, biochemical, molecular analyses, and studies in genetically engineered mice revealed a novel KLF14-mediated function in Treg cell differentiation.

At the molecular level, KLF14 mediated this phenomenon, at least in part, by transcriptionally regulating FOXP3 via a mechanism that involves methylation and chromatin remodeling of the TSDR (Treg-specific demethylation region) within the promoter of this gene. At the cellular level, KLF14 inactivation rendered Treg cells in an abnormal state of differentiation marked by their hypersuppressive KLF14 inactivation rendered Treg cells in an abnormal state of differentiation marked by their hypersuppressive

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At the molecular level, KLF14 mediated this phenomenon, at least in part, by transcriptionally regulating FOXP3 via a mechanism that involves methylation and chromatin remodeling of the TSDR (Treg-specific demethylation region) within the promoter of this gene. At the cellular level, KLF14 inactivation rendered Treg cells in an abnormal state of differentiation marked by their hypersuppressive phenotype in vitro and in vivo. Congruently, KLF14 knockout (KO) mice were resistant to experimentally induced colitis.

These results assign for the first time a role for KLF14 in immune cell differentiation and chronic intestinal inflammation. These data also extend our understanding of molecular mechanisms that regulate Treg cell function, a population that is altered in many disease states.

**Materials and Methods**

**Transfection Assays**

For small interfering RNA (siRNA) experiments, 300 nM siRNA was used (ONTARGET plus siRNA; Dharmacon/Thermo Scientific, Lafayette, CO). The cell-stimulation conditions included plate-bound CD3 (2 μg/mL), soluble anti-CD28 (2 μg/mL; BD Biosciences, San Jose, CA), and human interleukin-2 (100 μM) and human transforming growth factor β1 (TGFβ1, 5 ng/mL) in complete RPMI media (both cytokines: PeproTech, Rocky Hill, NJ).

**Isolation of Primary T Cells**

Male mice were used for all experiments. Murine naïve CD4\(^+\) splenocytes were isolated using a combination of magnetic separation kits (Miltenyi Biotec, Bergisch Gladbach, Germany). Sequential use of the CD4\(^+\)CD25\(^+\) regulatory T cell isolation kit and the CD4\(^+\)CD62L\(^-\) T cell isolation kit resulted in naïve FOXP3-negative T cells used for in vitro induction of FOXP3.

**Cell Stimulation**

In vitro activation of the isolated T cells followed similar conditions among the different cell types. Anti-CD3, 145-2C11 (BD Biosciences) for the mouse T cells was plate-bound at 2 μg/mL. Soluble anti-CD28 (BD Biosciences) at 2 μg/mL plus 100 units/mL interleukin-2 was added to the cultures throughout the incubation period. Human TGFβ1 recombinant (PeproTech) at a concentration of 5 ng/mL was used to generate adaptive Treg cells. The media were refreshed every 3 days with cells plated into new wells coated with anti-CD3 as described previously.

**Suppression Assays**

For the suppression assays, CD4\(^+\)CD25\(^+\) cells were sorted from splenocytes, using anti-CD4/anti-CD25 conjugated beads (Miltenyi Biotec), as previously described elsewhere.\(^3\) Mixed leukocyte reactions were performed using 1 × 10\(^4\) CD4\(^+\)CD25\(^-\) T responder cells and 1 × 10\(^5\) irradiated (3300 rads) T-cell-depleted antigen-presenting cells isolated from the same animals. Treg cells were added to the cell culture at titrations of 1:12 to 1:1. The culture medium was complete RPMI supplemented with 10% fetal bovine serum and 2.5 μg/mL anti-CD3 (UCHT1) and anti-CD28 (BD Biosciences) at 2 μg/mL. Proliferation was read at 4d upon addition of 1 μCi tritiated thymidine for the last 18 hours of culture.

**RNA Isolation, cDNA Synthesis, and Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was isolated using the manufacturer protocol in the RNeasy Mini Kit (Qiagen, Valencia, CA). The cDNA was synthesized from 0.5–1 μg of total RNA with random primers using the SuperScript kit III First-Strand (Invitrogen/Life Technologies, Carlsbad, CA). We used 2 μL of reverse-transcription products for each real-time polymerase chain reaction (PCR). The PCRs were in 20 μL of total volume that contained primers and 10 μL of Express SYBR green ER quantitative PCR Supermaster mixes (Invitrogen). Of note, KLF14 is an intron-less GC rich gene not optimal for real-time PCR. Thus, for semiquantitative real-time PCR, genes of interest were amplified under the following conditions: initial denaturation, 95°C for 3 minutes, followed by 34 cycles with denaturation at 95°C for 30 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 60 seconds. All the PCR products were visualized by running 1.5% agarose gels electrophoresis and ethidium bromide staining for the pictures.

The following primers were used. The Foxp3 core promoter chromatin immunoprecipitation (ChiP) primers for gels were 5’-TTCAGATGACTTTGAAGGGGAAAAAG and 3’-GAGAAGAAAAACCCGCGGTGGGAG and 3’-GAGAAGAAAAACCCGCGGTGGGAG and 3’-GGCCGGATGACCTGATGGTTCA. The KLF14 primers were 5’-TCAACTGCTGTCGAGGC and 3’-ACGACCTCCGTTACCTGCATCA. The Foxp3 primers were 5’-TTCAGATGACTTTGTA AAGGGCAAAG and 3’-GAGAAGAAAAACCCGCGGTGGGAG.

**Western Blot Analysis**

Protein was extracted from whole-cell lysates derived from freshly isolated Treg cells and non-Treg cells. The cells were lysed Laemmli sample buffer. Protein (20 μg) was run
on 10% gel over 1 hour at 100 Amps. Upon transfer to nitrocellulose, the membrane was incubated with anti-Foxp3 (Abcam, Cambridge, MA) in 5% milk for 1 hour. Foxp3 protein was detected with 1/1000 dilution of anti-rabbit-horseradish peroxidase conjugate and an electrochemiluminescence detection kit (SuperSignal West Femto Chemiluminescent Kit; Pierce Biotechnology, Rockford, IL). The exposed films were scanned, and the digitized images were analyzed using the software VisionWorks (UVP, Upland, CA). The protein signals were indexed by measuring their relative mean grey value per area.

**Chromatin Immunoprecipitation Assays**

The ChIP assays were performed using a ChIP isolation kit (Millipore, Billerica, MA). We treated 1 x 10^5 to 5 x 10^5 murine naïve T cells with 1% formaldehyde to cross-link histones to DNA. The fixed cells were sonicated to yield chromatin fragments of 200–500 base pairs (bp). The antibodies used in the ChIP assays included: H3Ac (Millipore), H4Ac (Upstate Biotechnology, Lake Placid, NY), H3K9Ac (Active Motif, Carlsbad, CA), H3K4Me3 (Millipore), H3K4Me2 (Abcam), H3K4Me (Millipore), H3K9Me3 (Abcam) H3K27me2 (Abcam) H3K27me3 (Millipore), p300 (Abcam), PCAF (Abcam), SUV39H1 (Millipore), CBP (Abcam), ESET/SetDB1 (Millipore), Ezh2 (Cell Signaling Technology, Beverly, MA), G9a/EHMT2 (C6H3) (Abcam), HP1α (Millipore), HP1β (Active Motif), and HP1γ (Millipore). DNA was recovered by a Chelex 10% slurry and sample boiling method, or using the IP-STAR (Diagenode, Denville, NJ) direct chip protocol followed by the IPPURE DNA purification. 1% Pre-enriched chromatin (input) served as the percentage input for sample quantification, confirmed by fold over IgG changes. The UV-exposed 4% agarose gels were imaged, and the digitized images were analyzed using the software VisionWorks (UVP). Protein signals were indexed by measuring their relative mean grey value per area.

**Adenoviral Transduction**

Naïve T cells isolated from the Coxsackie adenovirus receptor (CAR) transgenic Balb/cj[Tg],CARdelta1-[Tg] D011.10 mice were activated for 48 hours with either empty vector (EV) or KLF14 at a multiplicity of infection of 250. The cells were activated under the typical stimulation conditions for 2 days and processed through mRNA expression.

**Mouse Strains**

C57BL/6/J mice and C57BL/6J RAG-1 mice (recombinase activating gene-1-deficient) were initially purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in conventional housing in the Mayo Clinic animal facility (Rochester, MN). The CAR transgenic mouse was obtained through the National Institute of Allergy and Infectious Diseases Exchange Program of the National Institutes of Health: Balb/cj[Tg]CARdelta1-[Tg]D011.10 mouse line 4285.12 The CAR mouse expresses the Coxsackie adenovirus receptor transgene and is optimal for adenoviral transduction studies in resting lymphocytes. The KLF14^−/− mice were kindly provided by Dr. Raul Urrutia (Mayo Clinic, Rochester, MN).12 All the mice used in experiments were males of 4–20 weeks in age. The mice were age-matched in experiments comparing wild type (WT) with KLF14^−/−. All animal work was done in accordance with the Mayo Clinic Institutional Animal Care and Use Committee.

**Dextran Sodium Sulfate Colitis**

The mice were given water supplemented with 3% dextran sulfate sodium salt for 5 days. The water was then replaced with normal drinking water for 3 more days before the mice were sacrificed for tissue removal for histologic analysis. Flow cytometry was used to look at levels of FOXP3 expression within the CD4^+ population. Intracellular staining procedures for FOXP3 were followed using the application notes from Alexa Fluor 488 anti-mouse/rat/human FOXP3 (BioLegend, San Diego, CA).

The mice were weighed every other day, and their colon lengths were determined during autopsy. The degree of colitis was quantified using three outcome variables: weight loss, colon histology, and a disease activity index. The disease activity index is an established clinical index of colitis severity encompassing clinical signs of colitis (wasting and hunching of the recipient mouse and the physical characteristics of stool) and an ordinal scale of colonic involvement (thickness and erythema).13 We adapted an existing histology damage score for the dextran sodium sulfate (DSS) colitis model.14 This score assesses eight parameters, including the extent of crypt loss, depth of erosions/ulcers, and semiquantitative assessment of inflammatory cells. We added one additional parameter: extent of re-epithelialization when erosions/ulcers were present/expressed as ratio of re-epithelialized ulcer to nonepithelialized ulcer.

**T-Cell Transfer Model of Chronic Colitis**

Chronic colitis was induced via adoptive transfer of naïve CD4^+CD45RB<sup>high</sup> T cells derived from WT donors using our previously published method.15 Briefly, spleens were removed from donor WT C57BL/6/J mice and macerated between the frosted ends of two microscopy glass slides to produce single-cell suspensions in phosphate-buffered saline (PBS) supplemented with 0.1% fetal bovine serum. The CD4^+ T cells were enriched by negative selection using magnetic beads (Miltenyi Biotec). The enriched T cells were labeled with anti-CD45RB and anti-CD25 (BioLegend) and sorted for CD45RB<sup>high</sup>CD25<sup>low</sup> with a FACSR Aria (fluorescence-activated cell sorting; BD Biosciences). We injected 3 x 10^5 cells suspended in 100 µL of PBS intraperitoneally into C57BL6/J RAG-1 KO animals. After 14 days, the CD25<sup>+</sup> cells were isolated using CD4^+CD25<sup>+</sup> regulatory T cell isolation kit (Miltenyi Biotec) as described by the manufacturer's suggested protocol from the spleens of either WT C57BL/6/J or KLF14 KO mice. These cells were subjected to a second magnetic column and washing step at the CD25<sup>+</sup> step to further purify the regulatory T-cell population (greater than 95% FOXP3<sup>+</sup> in
WT or KLF14 KO mice, data not shown). We suspended 7.5 × 10⁴ cells in 100 μl of PBS, which was injected intraperitoneally into animals that had undergone adoptive transfer of CD4⁺CD45RB⁹⁹ WT T cells 2 weeks earlier. The animals were switched to nonirradiated chow, and their weight was recorded every other day. The Mayo Clinic Animal Facility, unless specifically directed, only uses autoclaved and irradiated chow, but nonirradiated chow frequently is required to induce robust colitis in this model.¹⁶ The histologic disease activity was assessed by our participating gastrointestinal pathologist who was blinded to the study grouping. We adapted an existing histology damage score for the T-cell transfer colitis model.¹⁵ This score assesses eight parameters, including extent of crypt loss, depth of erosions/ulcers, and semiquantitative assessment of inflammatory cells.

RNAseq of Human Samples

The isolation of lamina propria CD4⁺ lymphocytes has previously been described elsewhere.⁹ Briefly, resection specimens or mucosal biopsy samples of the terminal ileum were obtained from six patients with Crohn’s disease or age/sex-matched healthy controls. The tissue initially underwent mechanical disruption in the presence of 1 mM EDTA in a 37°C CO₂ incubator for 30 minutes followed by a collagenase (1 mg/ml), DNase (1 mg/ml), and trypsin inhibitor (1 mg/ml) overnight at 4°C in complete RPMI supplemented with 10% human serum. After passage through a 70-μm cell strainer, the buffy coat was isolated using Ficoll gradient centrifugation. The CD4⁺ lamina propria cells were isolated using magnetic bead sorting (CD4⁺ T Cell Isolation kit, 130–091–155; Miltenyi Biotec) and two passes through the LS column on the MACS magnetic separator. Purification was confirmed by flow cytometry.

Sequencing data was analyzed using the processed paired-end mRNA sequencing analysis using the MAPRSeq v1.2.1.3 workflow (Mayo Clinic, Rochester, MN). This analysis provided gene and exon level expression. We performed differential expression analysis using edgeR (Bioconductor, Bioconductor.org) for the following disease versus control.

Statistical Methodology

Statistical analyses were performed using JMP version 9.0 (SAS Institute, Cary, NC). Nonparametric unpaired t test was performed using the Mann-Whitney t test, and P < .05 was considered statistically significant. For multiple comparisons, statistical significance was determined using the Holm-Sidak method, with alpha = 5.000% (P < .05). Each variable was analyzed individually, without assuming a consistent standard deviation.

Figure 1. KLF14 is present in the immune compartment and induced by transforming growth factor β1 (TGFβ) in vitro in FOXP3-negative lymphocytes. (A) DNA gel for polymerase chain reaction (PCR) analysis of the expression of KLF14 in mouse tissues. The data are a representative figure of three independent experiments. (B) DNA gel for PCR analysis of the expression of KLF14 in splenocyte subsets. Note the enhanced expression of KLF14 in CD4⁺ lymphocytes (right four panels) and CD4⁺ lymphocytes stimulated with TGFβ (far right column). The housekeeping gene gapdh is provided as loading control (lower row). The data are representative of three independent experiments. (C, D) DNA gel for PCR analysis of the expression of KLF14 in CD4⁺ cells activated to induce FOXP3. Note that KLF14 expression segregates to GFP (surrogate for FOXP3) negative cells at all time points. (D) Histogram representing the optical density of gel bands. Note that time 0 (grey bar) represents unstimulated cells. The data are representative of three independent experiments. Statistical significance was determined using the Holm-Sidak method of multiple comparisons, with alpha = 5.000% (P < .05). Each time point was analyzed individually, without assuming a consistent standard deviation.
individually, without assuming a consistent standard deviation (SD).

**Results**

**KLF14, Present in Immune Cells, Is Inversely Associated With FOXP3**

The expression pattern of KLF14 in immune tissue is currently unknown, precluding us from inferring any potential function of this protein in the immune system. Consequently, we performed an expression analysis from various murine tissue extracts to gain insight into the spatial distribution of this transcription factor. KLF14 mRNA was ubiquitous in murine tissues, with particularly strong expression in primary and secondary lymphatic organs (Figure 1A).

Within the spleen, KLF14 expression segregated to CD4+ lymphocytes, a finding that suggested a putative role for KLF14 in the differentiation of CD4+ T helper phenotypes (Figure 1B). We stimulated naïve CD4+ lymphocytes into adaptive Treg cells (see Materials and Methods) and measured KLF14 expression (Figure 1B). After 72 hours of activation, KLF14 was induced by TGFβ1 and up-regulated within the entire population of CD4+ cells (right column, Figure 1B). Upon separation of CD4+ cell subsets based upon FOXP3 expression, we demonstrated an inverse relationship between FOXP3 and KLF14 (Figure 1C and D). Together, these studies demonstrate that KLF14, enriched in immune organs and cells is induced during the initial differentiation of naïve lymphocytes to adaptive Treg cells; however, KLF14...
segregates to the FOXP3 population in vitro. These results led us subsequently to investigate the impact of KLF14 gene deletion on the biological responses of differentiated Treg cells.

Expression of KLF14 Is Inversely Correlated With FOXP3 Expression and Function In Vivo

A survey of the principal immune components of the KLF14 KO mouse demonstrates a normal CD4:CD8 ratio and normal populations of CD19⁺ and CD11b⁺ cells (B cell and myeloid lineage, respectively) within the spleen when compared with age- and sex-matched control mice (Supplementary Figure 1). We did observe numerically higher but not statistically significantly different percentages of FOXP3⁺ cells (expressed as a percentage of CD4⁺ lymphocytes) in mucosal-associated lymphoid tissue (mesenteric lymphatic nodes [MLN] and Peyer’s patches [PP], Figure 2A).

Upon stimulation of naïve CD4⁺ splenocytes to differentiate into adaptive Treg cells (see Materials and Methods), we observed a higher frequency of FOXP3⁺ cells in vitro in the absence of KLF14 (Figure 2B). Furthermore, intracellular expression of FOXP3 is enhanced as assessed by mean fluorescent intensity in KLF14 KO Treg cells (Figure 2C). Enhanced differentiation into FOXP3⁺ T cells in the absence of KLF14 was not due to altered kinetics of cellular proliferation, as naïve lymphocytes from WT or KLF14 KO mice proliferated equally as assessed by fluorescence-activated cell sorting and intracellular dye dilution (Supplementary Figure 2). Similar proliferation between WT and KLF14 KO cells was seen in both FOXP3⁺ and FOXP3⁻ cell populations (Supplementary Figure 3).

To study the functional relevance of this in vitro finding, we evaluated mucosal immune response to DSS. Although not specific for adaptive immune cell function, the DSS-induced colitis model is a well-established tool to study mucosal inflammation in genetically engineered mice, including those deficient in KLF proteins. Thus, we assessed the development and severity of colitis by DSS in KLF14 KO mice by measuring weight loss, colon length, disease activity index, and histopathology. Interestingly, we found that upon DSS treatment, KLF14 KO mice displayed statistically significantly less weight loss (28.0 ± 1.1 g vs 23.5 ± 0.99 g, P = .001), reduced colonic shortening (6.8 ± 0.6 cm vs 4.9 ± 0.5 cm, P = .0007), and lower clinical disease activity scores as compared with the WT control animals (0.69 ± 0.73 vs 2.13 ± 0.86, P = .02) (Figure 3A–C).

Figure 3. KLF14 KO animals are resistant to dextran sodium sulfate (DSS) colitis. Tabulation of (A) weight change, (B) colon length, (C) clinical disease activity scores, and (D) histologic disease in DSS-exposed mice demonstrates significantly less severe colitis in KLF14-deficient (closed circles) versus wild type (WT) (open circles) animals. (D) Representative histologic section from the colon of a KLF14-deficient mouse compared with the significant inflammation and ulceration demonstrated in the colon of a WT mouse (D). Statistical significance for weight change was determined using the Holm-Sidak method, with alpha = 5.000% (P < .05). Each time point was analyzed individually, without assuming a consistent standard deviation. For colon length and disease activity index, significance was determined using a nonparametric, unpaired t test of statistical significance (Mann-Whitney), P < .05. The data are representative of three experiments, n = 5 mice per group.

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Figure 4. KLF14-deficient T regulatory (Treg) cells demonstrate enhanced suppression in vitro. (A) Histogram representing thymidine incorporation of T-responder cells in coculture with T-regulatory cells isolated from wild-type (WT) (white columns) or KLF14 knockout (KO) mice (black columns). Note the enhanced suppression of proliferation across all titrations, significantly so upon higher titration ratios (WT vs KLF14 KO: 7595 ± 906 vs 5099 ± 647, 1:3; 5948 ± 1488 vs 3158 ± 716, 1:2; 4804 ± 1833 vs 1349 ± 223, 1:1, P < .05). Presented are the mean and standard deviation (SD) of thymidine counts conducted in triplicate. Data are representative of three independent experiments. Statistical significance was determined using the Holm-Sidak method, with alpha = 5.000% (P < .05). Each titration was analyzed individually, without assuming a consistent SD. (B) Enhanced in vitro suppression of KLF14-deficient Treg cells associated with greater expression of FOXP3. Demonstrated is the immunoblot analysis (upper row) and DNA gel (lower row) for FOXP3 protein (upper) and mRNA (lower) in Treg cells after 3 days of suppression assay culture. Note the heightened expression of FOXP3 in KLF14 KO cells compared with WT. Data are representative of three independent experiments.

Figure 5. KLF14 knockout (KO) T-effector cells induce colitis. Tabulation of (A) weight change, (B) colon length, and (C) clinical disease activity scores in CD45Rbhigh transferred RAG recipients. Results demonstrate equivalently severe colitis in RAG recipients of KLF14 KO (closed circles or histogram) and wild-type (WT) (open circles or histogram) CD45Rbhigh T cells. (D) Representative histologic sections from the colon of a recipient of KLF14 KO T cells (top panel) compared with the colon of a recipient of WT T cells (lower panel). Immunohistochemical analysis for FOXP3 demonstrates the presence of FOXP3+ lymphocytes in recipients of KLF14 KO T cells. Data from n = 10 mice, 5 per treatment group (mean and standard deviation). A nonparametric, unpaired t test of significance between genotypes (Mann-Whitney) demonstrated no statistically significant difference.
The development of colonic inflammation was confirmed by blinded histologic examination of the colons. In comparison with the WT control animals, the colon of the DSS-treated KLF14 KO mice contained fewer inflammatory cells, reduced ulceration (11.4% ± 4.17% vs 30.7% ± 8.8%, $P = .02$, for $n = 7$ animals), and enhanced re-epithelialization (83.3% ± 28.8% vs 36.2% ± 6.5%, $P = .02$, for $n = 7$ animals; Figure 3D). These observations demonstrate that genetic inactivation of KLF14 in the germ line confers protection from experimental colitis. We recognize the

Figure 6. KLF14-deficient T regulatory (Treg) cells demonstrate enhanced suppression in vivo. Tabulation of (A) weight change, (B) clinical disease activity scores, and (C) histologic disease activity index in CD45Rb$^{high}$ transferred RAG recipients after treatment with WT or KLF14 KO Treg cells. The results demonstrate significantly less severe colitis in recipients of KLF14-deficient (closed circles or histogram) versus wild-type (WT) (open circles or histogram) Treg cells. (D) Representative histologic sections from the colon of a recipient of KLF14 KO Treg cells compared with the significant inflammation and ulceration demonstrated in the colon of a recipient of WT Treg cells. Statistical significance for weight change was determined using the Holm-Sidak method, with alpha = 5.000% ($P < .05$). Each time point was analyzed individually, without assuming a consistent standard deviation (SD). For the disease activity index and histologic activity index, statistical significance was determined using a nonparametric, unpaired $t$ test of significance (Mann-Whitney), $P < .05$. Data from $n = 10$ mice, 5 per treatment group (mean and SD, *$P < .05$).

Figure 7. SP6 (KLF14) is inversely associated with Foxp3 in CD4$^+$ cells isolated from patients with Crohn’s disease. We performed gene expression analysis on CD4$^+$ cells isolated from affected (CD, closed circles) and unaffected tissue (CTRL, open circles). Note the inverse relationship between FOXP3 and KLF14 ($n = 10$ subjects, $P < .05$). A differential expression analysis using edgeR for disease versus control at a false-discovery rate of <0.05 was used.
shortcomings of the DSS acute colitis model, particularly in its lack of specificity for adaptive immune cells, so we focused further study of KLF14 and colitis in T-cell-dependent assays.

We next tested the in vitro suppressor function of isolated Treg cells from both WT and KLF14 KO animals against titrated T-responder cells (Figure 4A and Supplementary Figure 4). The results of these experiments demonstrated an enhanced KLF14 KO Treg cell suppressor function (counts per minute 1349 ± 223.2 vs 4804 ± 1833.2, P = .03; KL14 KO vs WT at 1:1 ratio). The suppression assay was performed using alternatively syngeneic T-responder cells (Figure 4A) and a common T-responder cell population (Supplementary Figure 4) with equivalent results. As the cellular abundance of FOXP3 correlates with function17,18 we quantified FOXP3 in WT and KLF14 KO Treg cells in cell culture conditions established for the suppression assay. Figure 4B shows that deletion of KLF14 increased the expression of FOXP3 at both the protein (top row, Figure 4B) and the mRNA level (bottom row, Figure 4B).

Subsequently, we tested the in vivo suppressor function of KLF14 KO Treg cells. We used the CD4⁺CD45RB⁺ transfer model of colitis. To rule out an effect of KLF14 in the T-effector cells in vivo, we first performed a Rab transfer using naïve cells from WT or KLF14 KO donor mice. The transfer of naïve CD4⁺ T cells from both WT and KLF14 KO donor mice resulted in equally severe colitis (Figure 5). In support of our previous in vitro data of enhanced conversion of naïve T cells to Treg cells in the absence of KLF14, we did detect more FOXP3⁺ cells within the colon of recipients of KLF14 KO T cells when compared to WT by immunohistochemical analysis (Figure 5).

Next, to directly test the in vivo function of KLF14 KO Treg cells, we performed adoptive transfer of WT or KLF14 KO Treg cells into mice with established disease. Treg cells were isolated from the spleens of WT or KLF14 KO mice after CD25 magnetic bead selection (see Materials and Methods). Two weeks after the CD45Rb transfer of WT naïve T cells, 75,000 WT or KLF14 KO Treg cells were transferred into RAG KO recipient animals to stringently test the extraordinary in vivo Treg function. We assayed colitis in the mice by measuring weight loss, colon length, disease activity index, and histopathology. Mice rescued with KLF14 KO Treg cells experienced statistically significantly more...
KLF14 KO Treg cells had statistically significant immunoprecipitation for H3K27me3 and G9a in naïve T cells. Analysis of the expression of FOXP3 in cell fractions after polymerase chain reaction chromatin immunoprecipitation gion) in relationship to KLF14 genotype.

- Figure 9: KLF14 Associates with H3K9me3 Histone Marks and the Corresponding Histone Methyltransferase Complex at the TSDR. Semiquantitative polymerase chain reaction chromatin immunoprecipitation analysis of the expression of FOXP3 in cell fractions after immunoprecipitation for H3K9me3, HP1γ, SUV39H1, and HP1β in naïve T cells at t = 0 hours and 24 hours after transforming growth factor β1/T regulatory (TGFβ-Treg) stimulating conditions. Note the association of H3K9me3, HP1γ, HP1β, and SUV39H1 with the wild-type (WT) but not KLF14 knockout (KO) Treg cells. The data are representative of three independent experiments.

- Figure 10: Lack of Association with Either G9a or H3K27me3 at the TSDR (Treg-Specific Demethylation Region) in Relationship to KLF14 Genotype. Semiquantitative polymerase chain reaction chromatin immunoprecipitation analysis of the expression of FOXP3 in cell fractions after immunoprecipitation for H3K27me3 and G9a in naïve T cells at t = 0 hours and 24 hours after transforming growth factor β1/T regulatory (TGFβ-Treg) stimulating conditions. Note there are no differences in the levels of the methyltransferase G9a or the Polycomb-associated mark H3K27me3. The data are representative of three independent experiments.

weight gain (121.5% original weight ± 14.9% vs 103.5% original weight ± 8.1%, P = .03), reduced colonic shortening (8.21 ± 0.43 cm vs 7.05 ± 0.34 cm, P = .0004), and lower clinical disease activity scores as compared with the WT Treg-injected control animals (2.25 ± 0.80 vs 5.50 ± 1.45 P = .0008) (Figure 6). Furthermore, animals injected with KLF14 KO Treg cells had statistically significantly less cellularity of their mesenteric lymph nodes (4.4 × 10^6 ± 1.9 × 10^6 vs 2.2 × 10^6 ± 9.6 × 10^5, P = .03) than the WT Treg injected animals. The development of colonic inflammation was confirmed by blinded histologic examination of the colons. In comparison with the WT Treg-injected control animals, the colon of the KLF14 KO Treg-injected mice contained fewer inflammatory cells and reduced ulceration/erosion and crypt loss, resulting in a statistically significantly reduced histologic index of colitis (11.6 ± 3.29 vs 1.8 ± 0.45, P = .0002, n = 10, n = 5 animals per treatment group) (Figure 6).

Combined, our in vivo and in vitro experiments demonstrated that deletion of KLF14 results in an abnormal increase in the intracellular levels of FOXP3 and enhanced suppressive function of Treg cells in vitro and in vivo. Moreover, gene expression analysis of CD4+ lymphocytes isolated from Crohn’s disease intestinal lesions demonstrated KLF14 (alias SP6) to be inversely associated with FOXP3 and differentially regulated between Crohn’s patients and healthy controls (Figure 7). This finding suggests a potential role for KLF14 in human inflammatory disease as well. As stable FOXP3 expression is necessary to maintain the fully differentiated state of Treg cells,\(^{20}\) and epigenetic modifications of the TSDR are associated with stable FOXP3 expression, we subsequently studied the epigenetic state of the TSDR.

KLF14 Is a Transcriptional Repressor of FOXP3 Acting via the Treg-Specific Demethylated Region

Our experiments in genetically engineered KLF14 KO Treg cells revealed an inverse correlation between KLF14 and maintenance of FOXP3 expression, the master regulator of Treg cell differentiation. To rule out the possibility that the up-regulation of FOXP3 is not simply a compensatory response to the deletion of KLF14 in the germ line, we performed complimentary experiments overexpressing and knocking down KLF14. We performed acute depletion of the KLF14 mRNA using siRNA-based knockdown. The results of these experiments, shown in Figure 8A, demonstrated that acute depletion of KLF14 by siRNA increased FOXP3 expression.

Reciprocal overexpression experiments were performed using adenoviral transduction of KLF14 into primary naïve CD4+ lymphocytes. Figure 8B shows that overexpression of KLF14 decreased Foxp3 expression in both resting (Naïve Ts-TGFβ1) and stimulating conditions (Naïve Ts+TGFβ1). Thus, both genetic approaches—in vivo knock out (Figure 8C) and in vitro knock down of KLF14—link the function of this transcription factor to the regulation of FOXP3 gene expression and maintenance.

To test whether KLF14 regulated FOXP3 directly, we performed ChIP assays in primary naïve T cells. Upon adenoviral transduction of KLF14-His into primary naïve CD4+ lymphocytes, we demonstrated that KLF14 bound to the FOXP3 promoter locus, and specifically within the TSDR (Figure 8D). Together these experiments demonstrated that KLF14 is normally bound to the TSDR during cell
differentiation from the naïve state to adaptive Treg cells. In the absence of KLF14, FOXP3 expression and Treg suppressor capacity were enhanced; thus, we subsequently evaluated the potential for KLF14 to regulate chromatin modification of the TSDR.

Regulation of the FOXP3 Locus by KLF14 Involves Chromatin Remodeling of the Treg-Specific Demethylated Region

It has recently been demonstrated that stable Foxp3 expression, like the one that is observed in thymus-derived, terminally differentiated Treg cells, is marked by epigenetic modifications of the Treg-specific demethylated region (TSDR), a CpG-rich, noncoding sequence within the first intron of the Foxp3 gene locus. Based on this knowledge, we reasoned that KLF14 potentially influences FOXP3 expression by mediating changes in the levels of both histone marks and CpG island methylation within this regulatory module. Consequently, we examined whether KLF14 changes the levels of histone marks associated with nucleosomes present on the TSDR.

We performed ChIP-based analyses of this region upon differentiation of naïve CD4\(^+\) lymphocytes to induce FOXP3. Of interest, stimulation over 24 hours in conditions to induce FOXP3 led to H3K9me3 marks deposited on the TSDR of WT but not KLF14 knockout (KLF14 KO CD4\(^+\)) Treg cells (Figure 9). Congruently, the H3K9me3 mark was associated with the responsible histone methyltransferase (SUV39H1, Figure 9) and the HP1 family of epigenetic transcriptional repressors recruited through binding to the H3K9me3 mark (HP1\(\alpha\), HP1\(\gamma\), Figure 9). Control experiments demonstrated that this variance in H3K9me3 levels between WT and KLF14 KO cells was accompanied by the expected inverse relationship with the preceding H3K9 dimethylation mark (Supplementary Figure 5).

Subsequent experiments evaluating additional chromatin repressor complexes demonstrated no specific
association with the H3K9 methyltransferase G9a or additional repressor marks (H3K27me3, Figure 10). We performed confirmatory analyses of freshly isolated Treg cells from WT and KLF14 KO donor mice. Using ChIP assay and quantitative real-time PCR, we confirmed the association of the H3K9me3 marks, the HMT SUV39H1, and HP1g with the TSDR of WT but not KLF14 KO freshly isolated Treg cells (CD25+; Figure 11). It is established that H3K9me3 marks may lead to DNA methylation. We performed pyrosequencing of the TSDR in Treg cells from WT and KLF14 KO animals demonstrating variance in methylation of critical CpG residues (Figure 12). We conclude, that congruent with a role as a transcriptional repressor of FOXP3, deletion of KLF14 results in higher levels of expression for this target gene via a mechanism that involves, at least in part, chromatin remodeling at the TSDR.

Discussion

Kruppel-like transcription factors orchestrate complex physiologic and pathologic phenotypes by regulating selective gene expression networks through DNA binding and coupling to chromatin-remodeling enzymes. These biochemical properties are critically linked to the epigenetic reprogramming of gene expression that must occur during the functional capacitation of most cell types. During the generation of induced pluripotent stem cells, KLF4 best illustrates these KLF family properties. However, many members of the KLF family support cell differentiation in a large variety of tissues.

Relevant to our current study, several recent investigations have indicated an important role for KLF proteins in the functional regulation of immune cell types. Indeed, KLF2, KLF13, and KLF10 have been implicated in the development of T cells, B cells, and Treg cells, respectively. However, the full repertoire of KLF proteins regulating immune cell differentiation and function has yet to be established. To begin filling this knowledge gap, our laboratory and others have been studying the role of KLF proteins in FOXP3-mediated differentiation of Treg cells, the proper execution of which is critical for the maintenance of immune self-tolerance. We observed that KLF14 is expressed at the time that induced Treg cells commence their
differentiation program. Of note, KLF14 has been associated with human inflammatory diseases, including type 2 diabetes and atherosclerosis, but until now has not been specifically found to act in the immune compartment. We found KLF14 to be highly expressed within primary and secondary lymphatic organs, segregating within the CD4+ lymphocytes, which suggests a putative role for KLF14 in T-helper phenotypes. One such phenotype, the FOXP3+ Treg cell, is of great interest within the field of immune-mediated disease.

Mutations in Foxp3 result in the devastating and lethal immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome, which is characterized by an overwhelming systemic autoimmune reaction in the first year of life. Indeed, point mutations of FOXP3 have been recently identified in cases of familial refractory IBD-like disease. Therefore, understanding the epigenetic mechanisms regulating FOXP3 is of upmost importance.

We found KLF14 to be a repressor of FOXP3 through the epigenetic regulation of the TSDR region of the promoter locus. Incomplete data exist regarding the negative regulation of FOXP3. Genetic mutant mouse lines have suggested T-cell receptor (TCR)-dependent signaling pathways (tyrosine kinase Itk, and cytokine responsive genes [IRF-1]), to repress FOXP3 gene transcription; however, little mechanistic information particularly relating to epigenetic events was reported. We examined the role for KLF14 in chromatin-dependent mechanisms leading to epigenetic regulation at this locus. KLF14 strongly bound the TSDR in ChIP assays; in its absence, Foxp3 expression was enhanced. Thus, we looked for its effect on the chromatin landscape at the TSDR.

Compelling evidence has established demethylation at the TSDR to be required for long-term maintenance of FOXP3 expression in Tregs. However, the preceding signaling events, specifically the chromatin-mediated events leading to TSDR methylation, remain unknown. Loss of KLF14 results in reduced levels of H3K9me3 and hypomethylation at the TSDR. Interestingly, Polansky et al reported of the 15 CpG motifs in the TSDR that only 6, 7, 11, and 13 are essential for FOXP3 transcriptional activity as determined by luciferase reporter analysis. In our pyrosequencing comparison of pooled KLF14 KO and WT naïve cells taken at 0 and 48 hours, 5 days, and 9 days of Treg-inducing conditions, we found numerical variance in CpG motifs 7 and 11. Further work into the kinetics of this process is warranted. Therefore, deletion of KLF14 at the promoter region results in higher levels of Foxp3 gene expression via a mechanism that involves chromatin remodeling at the TSDR.

Interestingly, H3K9 methylases have been previously described to repress FOXP3 through epigenetic modification of the core promoter region under the direction of the SUMO E3 ligase, PIAS-1. This function was found to be relevant to natural Treg cells. Recently, the methyltransferase G9A was found to regulate T-cell differentiation during intestinal inflammation, causing a similar promotion in Treg differentiation program in its absence similar to what we find with KLF14 deficiency. Our study is the first to define a role for H3K9 methylase complexes in TGFβ1-inducible adaptive Treg cells through epigenetic silencing at the TSDR. Thus, a common epigenetic regulatory complex may function to regulate both natural and adaptive Treg cells, and further research is warranted into the mechanisms of specificity of recruitment to precise FOXP3 promoter domains.

Sphingosine-kinase-1 (S1P1) has been shown to be activated by KLF14 in endothelial cells and to play important roles in T-cell trafficking and thymus egress. Of particular interest, Liu et al found that S1P1 blocks the differentiation of Treg cells and that this difference was not due to proliferation of Treg cells, as we have demonstrated here. Although we cannot rule out synergistic effects of KLF14 acting indirectly through S1P1 in our system, we do show that KLF14 directly acts upon the Foxp3 locus through an epigenetic mechanism.

Given our interest in adaptive Treg cells and the TGFβ1-rich milieu of the intestine, we previously identified the mechanism leading to colitis susceptibility in KLF10 KO mice to result from Polycromb-mediated epigenetic silencing of FOXP3. As opposed to the block in adaptive Treg generation in the KLF10 KO mouse, the lymphocytes from the KLF14 KO mouse readily convert to adaptive Treg cells and demonstrate enhanced suppressor function in vitro and in vivo. KLF14 deficiency is associated with protection from colitis, and interestingly KLF14 is also inversely associated with FOXP3 in T cells isolated from patients with Crohn’s disease. KLF10 and KLF14, members of the same family and both induced by TGFβ, recruit different histone-modifying complexes and, as demonstrated in our current and previous publications, bind to different elements of the FOXP3 promoter locus. These data suggest that the KLF network-regulating T-cell differentiation has relevance to intestinal homeostasis and colitis.

In conclusion, we previously demonstrated that KLF10 directly regulates the promoter of FOXP3 in vivo and that alteration of this process impairs Treg cellular differentiation, leading to the development of autoimmunity. As a direct extension of these studies, we now report that KLF14, a protein structurally and functionally related to KLF10, also regulates the differentiation and functional specialization of Treg cells. Our findings demonstrate, for the first time, that 1) KLF14 is highly expressed in the immune system, in particular in Treg cells, where it functions as a transcriptional repressor of the FOXP3 gene; 2) KLF14 KO mice, which carry hypersuppressive Treg cells, are protected from experimentally induced colitis; and 3) the increase in FOXP3 levels observed in KLF14 KO Treg cells involves epigenetic modifications at the TSDR, the intrinsic enhancer region associated with stable expression of this gene. This work furthers our knowledge into how FOXP3 is repressed and/or silenced during Treg cell differentiation and lineage determination, and/or in conditions of immunopathogenesis.

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Correspondence
Address correspondence to: William A. Faubion, MD, 200 First Street SW, Rochester, Minnesota 55905. e-mail: faubion.william@mayo.edu; fax: (507) 255-6318.

Conflicts of interest
The authors disclose no conflicts.

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Supplementary Figure 1. KLF14 KO mice demonstrate normal CD4:CD8 ratio and normal populations of CD19+ and CD11b+ cells within the spleen when compared to age- and sex-matched control mice. The histogram represents results from flow cytometric analysis of splenocytes for CD4, CD8, B cell (CD19), and myeloid (CD11b) lineage cells. Note no demonstrable differences in above subsets (WT open histogram, KLF14 KO closed histogram). The data represent the mean/standard error of the mean of three independent experiments. A nonparametric, unpaired t test of significance between lymphoid organs (Mann-Whitney) demonstrated no statistically significant difference.
Supplementary Figure 2. KLF14 KO CD4\(^+\) T cells demonstrate normal kinetics of proliferation. Histograms represent fluorescent intensity of CellTrace Violet in naïve KLF14 KO (upper panels) and WT (second row) CD4\(^+\) T cells after stimulation with TCR and TGF\(\beta\) for 24 (left column) or 48 hours of stimulation. Note the similar profiles of fluorescent dilution with subsequent cell proliferative cycles between KLF14 KO versus WT. Quantitation of lymphocytes present in flow cytometric gates in triplicate experiments is presented in histograms below. Note no significant differences between KLF14 KO (open histogram) and WT (closed histogram) cells represented within each cell division. The experiment was performed in triplicate and histograms represent mean/standard error of the mean of all data. Multiple t test analysis was performed using the Holm-Sidak method, with alpha = 5.000\% (\(P < .05\)). Each time point was analyzed individually, without assuming a consistent standard deviation. No statistically significant difference between genotypes was evident.
Supplementary Figure 3. KLF14 KO CD4⁺ FOXP3⁺ T cells demonstrate normal kinetics of proliferation. Dot plots represent fluorescent intensity of CellTrace Violet in naïve KLF14 KO (upper panels) and WT (lower panels) CD4⁺ T cells after stimulation with TCR and TGFβ for 24 (left column), 48 (middle column), or 72 (right column) hours of stimulation. FOXP3⁺ cells are GFP⁺ cells on the y axis, and the box represents FOXP3⁺ cells expressed as a percentage of CD4⁺ cells. Note the similar profiles of fluorescent dilution with subsequent cell proliferative cycles between KLF14 KO versus WT FOXP3⁺ cells and, as observed previously, the higher percentage of FOXP3⁺ cells in the KLF14 KO cells. The experiment was performed in triplicate, and all data are shown.
Supplementary Figure 4. KLF14-deficient T-regulatory (Treg) cells demonstrate enhanced suppression in vitro. Histogram represents thymidine incorporation of T responder cells (WT) in coculture with Treg cells isolated from WT (black column) or KLF14 KO mice (grey column) normalized to WT. 1:1 = 60% reduction, $P = .0002$; 1:2 = 41% reduction, $P = .002$; 1:3 = 40% reduction, $P = .004$; 1:4 = 24% reduction, $P = .03$. Presented are the mean and standard deviation (SD) of thymidine counts conducted in triplicate. Data are representative of three independent experiments. Statistical significance was determined using the Holm-Sidak method, with alpha = 5.000% ($P < .05$). Each titration was analyzed individually, without assuming a consistent SD.

Supplementary Figure 5. KLF14 KO associates with H3K9me2 histone marks at the TSDR. Semiquantitative PCR ChIP analysis of the expression of FOXP3 in cell fractions after immunoprecipitation for H3K9me2 shows higher levels of the modification in KLF14 KO naive cells stimulated in T regulatory (Treg) conditions for 24 hours in comparison with WT cells, inverse to the H3K9me3 mark. Data are representative of three independent experiments.