Kruppel-like Factor KLF10 Targets Transforming Growth Factor-β1 to Regulate CD4⁺CD25⁻ T Cells and T Regulatory Cells

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CD4⁺CD25⁻ regulatory T cells (T regs) play a major role in the maintenance of self-tolerance and immune suppression, although the mechanisms controlling T reg development and suppressor function remain incompletely understood. Herein, we provide evidence that Kruppel-like factor 10 (KLF10/TIEG1) constitutes an important regulator of T regulatory cell suppressor function and CD4⁺CD25⁻ T cell activation through distinct mechanisms involving transforming growth factor (TGF)-β1 and Foxp3. KLF10 overexpressing CD4⁺CD25⁻ T cells induced both TGF-β1 and Foxp3 expression, an effect associated with reduced T-Bet (Th1 marker) and Gata3 (Th2 marker) mRNA expression. Consistently, KLF10⁻/⁻ CD4⁺CD25⁻ T cells have enhanced differentiation along both Th1 and Th2 pathways and elaborate higher levels of Th1 and Th2 cytokines. Furthermore, KLF10⁻/⁻ CD4⁺CD25⁻ T cell effectors cannot be appropriately suppressed by wild-type T regs. Surprisingly, KLF10⁻/⁻ T reg cells have reduced suppressor function, independent of Foxp3 expression, with decreased expression and elaboration of TGF-β1, an effect completely rescued by exogenous treatment with TGF-β1. Mechanistic studies demonstrate that in response to TGF-β1, KLF10 can transactivate both TGF-β1 and Foxp3 promoters, implicating KLF10 in a positive feedback loop that may promote cell-intrinsic control of T cell activation. Finally, KLF10⁻/⁻ CD4⁺CD25⁻ T cells promoted atherosclerosis by ~2-fold in ApoE⁻/⁻/scid/scid mice with increased leukocyte accumulation and peripheral pro-inflammatory cytokines. Thus, KLF10 is a critical regulator in the transcriptional network controlling TGF-β1 in both CD4⁺CD25⁻ T cells and T regs and plays an important role in regulating atherosclerotic lesion formation in mice.

A subset of T cells known as CD4⁺CD25⁺ regulatory T cells (T regs) that express Foxp3 possess powerful immunosuppressive functions and play an important role in the maintenance of immunologic tolerance. T regs constitute ~5–10% of peripheral CD4⁺ T cells in healthy animals and humans; however, reduced numbers or function of T regs associate with the development of autoimmune diseases such as type I diabetes, multiple sclerosis, inflammatory bowel disease, and atherosclerosis, among others (1–5). Indeed, mutation of Foxp3 associates with a reduction of T regs in mice and leads to a fatal X-linked autoimmune disorder known as scurfy, whereas in humans it associates with the development of immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (6, 7). Recent studies provide evidence that CD4⁺CD25⁺ T regs may arise in the periphery from CD4⁺CD25⁻ T cells in response to a variety of stimuli both in vitro and in vivo, an effect, in part, dependent upon induced Foxp3 expression (8–16). Thus, identification of novel molecular mechanisms underlying the development and suppressor function of T regs in the periphery holds considerable scientific and therapeutic interest.

Transforming growth factor (TGF)-β1, a pleiotropic mediator, plays an important role in cell growth, differentiation, and activation in a number of immune and non-immune cell types (17–19). TGF-β1 participates in the maintenance of self-tolerance and homeostasis of several T cell effectors including T regs (12, 14, 20). Indeed, disruption of TGF-β1 or its receptors in T

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3 The abbreviations used are: T reg, CD4⁺CD25⁺ regulatory T cells; TGF-β1, transforming growth factor-β1; KLF10, Kruppel-like factor 10; EV, empty virus; ChIP, chromatin immunoprecipitation; WT, wild-type; SEAP, secreted form of human placental alkaline phosphatase; FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; APC, antigen presenting cell; MMP, macrophage inflammatory protein; MCP, —; SDF, stromal cell derived factor; mAb, monoclonal antibody; IL, interleukin; IFN, interleferon; RANTES, regulated on activation normal T cell expressed and secreted; TCR, T cell receptor; MCP, monocyte chemoattractant protein-1; MMP, matrix metalloproteinase.
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EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Primary CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells were isolated from mouse spleens by using a magnetic-based isolation kit (Miltenyi Biotech), followed by FACs sorting (>99% pure), and grown in RPMI 1640 medium (ATCC) supplemented with 10% fetal bovine serum, 0.02 mM β-mercaptoethanol. Jurkat cells were obtained from American Type Culture Collection (ATCC) and cultured as recommended.

Mice—KLF10−/− mice, originally from the laboratory of Dr. Thomas Spelsberg (Mayo Clinic), and ApoE−/−/scid/scid mice have been described (32, 36). Mouse genotypes were determined by PCR. 8–12-Week-old mice were used for most experiments.

In Vitro Suppression Assays—CD4⁺CD25⁻ and CD4⁺CD25⁺ T cell populations were isolated from WT and KLF10−/− mice spleens or mesenteric lymph nodes. CD4⁺CD25⁻ responder cells (5 × 10⁶) were co-cultured with various ratios of CD4⁺CD25⁺ T cells as indicated in the presence of 1 μg/ml anti-CD3 (clone 2C11) in 96-well microplates for 72 h. Cultures were pulsed with 1 μCi of [³H]thymidine per well for the last 18 h. After the 72-h incubation, the cultures were harvested and [³H]thymidine incorporation was measured by scintillation counting.

Northern and Western Analyses—Total RNA was isolated from cultured cells using TRIzol methods as previously described (30). Cellular protein extraction and Western blot analyses were performed as described (30).

Retroviral and Adenoviral Transduction—For retroviral studies, the indicated cDNA (mouse KLF10) was cloned into the retroviral vector GFP-RV (gift from K. Murphy) and retrovirus was generated as described (30). For retroviral infection of primary CD4⁺CD25⁻ T cells, cells were first activated overnight with plate-bound anti-CD3 antibodies (3 μg/ml) at 2 × 10⁶ cells per well, infected the next day with empty vector (EV) and KLF10 retroviral supernatants generated from phoenix cells, and centrifuged at 1800 × g for 45 min. Retroviral supernatant and culture medium (10% fetal calf serum/Dulbecco’s modified Eagle’s medium + 8 μg/ml Polybrene) were mixed at a 1:1 ratio. The next day, cell media were changed with fresh cell culture RPMI medium. GFP positive cells were then sorted 48 h after infection. For adenoviral transduction, 100 multiplicity of infection of the Ad-TGF-β1-promoter-SEAP reporter (kindly provided by P. Gonzalez, Duke University) was used to infect WT or KLF10−/− CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells in the presence or absence of TGF-β1 (1 ng/ml). Activation of the TGF-β1 promoter was quantified by determining the amount of SEAP released to the culture medium using the Great EscAPE™ SEAP chemiluminescence detection kit 2.0 (Clontech).

ELISAs Using Searchlight Multiplex Protein Arrays—Primary CD4⁺CD25⁻ Jurkat EcoR cells were retrovirally infected with EV or KLF10 for 48 h, followed by stimulation with phorbol 12-myristate 13-acetate (20 ng/ml)/ionomycin (3.5 μg/ml) for 6 h. The supernatants were collected for ELISA analysis using Searchlight Multiplex protein arrays (Pierce). Cell culture media from CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells and the plasma from adoptive transferred ApoE−/−/scid/scid mice were also collected for ELISA analysis.
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Atherosclerosis Induction by Adoptive Transfer of CD4+ CD25+ T Cells in ApoE−/−/scid/scid Mice—Wild-type and KLF10−/− CD4+ CD25+ cells were isolated and injected into retro-orbital veins of 8–10-week-old ApoE−/−/scid/scid mice. Recipient mice were provided with a high-fat diet after injection for 4 weeks. Mice were sacrificed and plasma samples were collected for ELISA and cholesterol measurements. Mouse hearts were embedded in optimal cutting temperature compound and the sections were stained with Oil red O, Mac-3, and CD4 for lesion characterization.

Quantitative Real-time PCR—Total RNA from purified CD4+ CD25+ and CD4− CD25− cells was isolated using TRIzol reagent (Invitrogen). The real-time PCR was performed in triplicate with Brilliant SYBR green mixture using the Mx3000P Real-time PCR system (Stratagene). 

Flow Cytometric Analysis and Cell Sorting—For FACS analysis, APC, phycoerythrin, or fluorescein isothiocyanate-conjugated antibodies specific for CD4, CD25, and Foxp3 were used to label CD4+ CD25+ and CD4− CD25− cells as described by the manufacturer’s protocol (eBioscience). FACS analysis was performed on a FACSCalibur flow cytometer and analyzed with CellQuest (BD Biosciences). Retrovirally infected CD4+ CD25− cells with EV and KLF10 were also sorted for GFP-positive cells and used in suppression assays. Cell culture media were collected for ELISA analysis.

Antibodies—Polyclonal KLF10 antibody was used as described (32). Antibodies to Smad2 (number 3103), phosphorlated-Smad2 (number 3108), and β-actin (number 4967L) were from Cell Signaling. IgG (sc-14967L) were from Santa Cruz Biotechnology. Allophycocyanin-labeled anti-CD4 (RM4-5), phycoerythrin-labeled anti-CD25 (pc61.5), and fluorescein isothiocyanate-labeled anti-Foxp3 (FJK-16a) and Foxp3 (eBio7979) were purchased from eBioscience.

Identification of KLF10 in CD4+ T Cells—KLF10 targets TGF-β1 in CD4+ CD25+ T cells and T regulatory cells (supplemental Fig. S1A). In contrast, other KLFs, such as KLF2, KLF4, and KLF5, either did not change or were decreased in CD4+ CD25+ T regulatory cells (supplemental Fig. S1B and data not shown). One of the hallmarks of peripheral CD4+ CD25+ T regulatory cells is their dependence upon TGF-β1 for maintenance and survival (20, 38). CD4+ CD25− T cells can be differentiated into CD4+ CD25+ T regulatory cells that express Foxp3 in vitro in the presence of TGF-β1 and anti-CD3 mAbs (12). CD4+ CD25− T cells had markedly induced KLF10 mRNA: by 27-fold at 6 h, and 7.5-fold at 24 h of TGF-β1 treatment (supplemental Fig. S1C). Remarkably, from at least two independent experiments. Site-directed mutagenesis (Stratagene) was performed to generate the Foxp3 promoter with the mutant KLF site. The PCR primer sequences used to generate this mutant Foxp3 promoter were: forward, 5′-AACATATGAAACCCTTTGCAATTGATTATCACGCCG-3′ and reverse, 5′-GGCGTGATACCGAGATCAGCGCC-3′. Cells were subjected to chromatin immunoprecipitation (ChIP) assays using antibodies to IgG or KLF10 as described (30). Briefly, DNAs isolated from ChIP assays were analyzed in triplicate by quantitative real-time PCR using the Brilliant SYBR green mixture (Stratagene) and the Mx3000P real-time PCR system. Values were presented as relative to DNA input. The upper and lower primers were 5′-GCCGTCGCACTTCTCTCTCG-3′ and 5′-GCAACTGGCTCCTCATCTCG-3′, corresponding to −341 and −166 bp, respectively, of the TGF-β1 promoter.

Th1 and Th2 Cell Skewing Experiments—CD4+ T cells were purified using anti-CD4 beads (Milenyi) and further sorted into naïve CD4+ CD25− CD62L hi T cells. Th1 and Th2 cell differentiation was performed as described (8). Cells were stimulated with anti-CD3 and anti-CD28 Abs for 2–3 days in the presence of recombinant IL-12 (10 ng/ml; R&D Systems) and neutralizing antibodies to IL-4 (10 µg/ml, 11B11; R&D Systems) for Th1 cell differentiation and in the presence of recombinant IL-4 (10 ng/ml, R&D Systems) and neutralizing antibodies to IFN-γ (10 µg/ml, XMG1.2; R&D Systems) for Th2 cell differentiation.

Cholesterol Measurements—The plasma samples from adoptive transferred ApoE−/−/scid/scid mice were used to measure total cholesterol (C7510-120), triglyceride (T7532-120), and high density lipoprotein cholesterol (H7511-60) levels by kits from Pointe Scientific Inc. The low density lipoprotein cholesterol level was calculated based on the values of total cholesterol, triglycerides, and high density lipoprotein.

Statistical Analyses—Values are expressed as mean ± S.D. Differences between values were examined using the two-tailed Student’s t test and were considered significant at p < 0.05.

RESULTS

Identification of KLF10 in CD4+ CD25− T Regulatory Cells and Responsiveness to TGF-β1—In light of the acknowledged role for KLFs in other aspects of hematopoietic and immune cell biology, we hypothesized that members of this family may also play a role in T regulatory cell biology. We screened a panel of 1–17 KLFs and found that freshly isolated peripheral CD4+ CD25− T regulatory cells robustly express KLF10 (−6-fold) compared with CD4+ CD25+ T cells (supplemental Fig. S1A). In contrast, other KLFs, such as KLF2, KLF4, and KLF5, either did not change or were decreased in CD4+ CD25+ T regulatory cells (supplemental Fig. S1B and data not shown). One of the hallmarks of peripheral CD4+ CD25+ T regulatory cells is their dependence upon TGF-β1 for maintenance and survival (20, 38). CD4+ CD25− T cells can be differentiated into CD4+ CD25+ T regulatory cells that express Foxp3 in vitro in the presence of TGF-β1 and anti-CD3 mAbs (12). CD4+ CD25− T cells had markedly induced KLF10 mRNA: by −23-fold at 1 h, −27-fold at 6 h, and −7.5-fold at 24 h of TGF-β1 treatment (supplemental Fig. S1C). Remarkably,
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FIGURE 1. KLF10 overexpression induces TGF-β1 expression in CD4+ CD25− T cells. CD4+ CD25− T cells were isolated as described under “Experimental Procedures” and transduced with retrovirus GFP-RV-EV (EV, control) or GFP-RV-KLF10 for 72 h, then FACS sorted for GFP positivity. Transduced cells were subjected to qPCR and cell culture supernatants were subjected to ELISA for TGF-β1 (A) or IFN-γ (B) expression. C, effects of KLF10-overexpressing CD4+ CD25− T cells on T-bet (left) and Gata3 (right) mRNA expression by qPCR analysis. D, the growth rate of EV or KLF10-infected CD4+ CD25− T cells counted over 5 days. E, suppression of co-cultured CD4+ CD25− T cells by KLF10. EV or KLF10-overexpressing cells (5 × 10^4) were co-cultured with CD4+ CD25− T cell effectors (5 × 10^5), α-CD3 Ab (1 µg/ml), and APCs for 72 h and proliferation was assessed by thymidine incorporation during the last 18 h. Error bars indicate S.D.

TGF-β1 also induced the expression pattern of the T regulatory marker Foxp3 in an analogous pattern. Finally, KLF10 responsiveness to TGF-β1 was also examined in the Jurkat T cell line. KLF10 mRNA was rapidly induced after 1 h in Jurkat cells and induced in a dose-dependent manner (supplemental Fig. S1, A and E). Collectively, these observations indicate that KLF10 expression is enriched in CD4+ CD25− T regulatory cells and can be rapidly induced in response to TGF-β1.

KLF10 Induces a T Regulatory Phenotype in CD4+ CD25− T Cells by Inducing Both TGF-β1 and Foxp3 Expression—To determine whether KLF10 participates directly in T regulatory cell differentiation, we retrovirally infected CD4+ CD25− T cells with either full-length KLF10 or an EV control and analyzed the cells for the T regulatory marker Foxp3 4 days later. In comparison to EV-infected cells, we noticed a marked induction of Foxp3 mRNA and protein expression by flow cytometry (intracellular staining) (supplemental Fig. S2, A and B). In addition, KLF10-overexpressing cells markedly induced TGF-β1 mRNA and protein levels, whereas it suppressed the elaboration of Th1 cytokines including IFN-γ (Fig. 1, A and B). Consistent with a T regulatory phenotype, KLF10-transduced cells also expressed lower levels of the Th1 master regulator T-bet and the Th2 master regulator Gata3 (Fig. 1C), had lower proliferative growth rates (Fig. 1D), and markedly repressed co-cultured wild-type CD4+ CD25− T responder cells activated in the presence of anti-CD3 mAb and APCs (Fig. 1E and supplemental Fig. S5A). Similarly, overexpression of KLF10 in Jurkat cells induced cells with a quiescent phenotype. In comparison to EV control cells, KLF10-overexpressing cells decreased cell growth by ∼2-fold over 5 days, induced p21^WAF1^, and inhibited a range of pro-inflammatory cytokines and growth factors, including IL-2 and IFN-γ, in response to phorbol 12-myristate 13-acetate/ ionomycin (supplemental Fig. S3). These findings in Jurkat and CD4+ CD25− T cells were associated with a ∼3-fold increase in exogenous KLF10 mRNA levels in comparison to EV control (supplemental Fig. S2 and data not shown). Taken together, these data indicate that KLF10 can promote the acquisition of a T regulatory cell phenotype by inducing both Foxp3 and TGF-β1 expression.

KLF10 Targets the TGF-β1 and Foxp3 Promoters—To define the mechanism(s) underlying the ability of KLF10 to induce expression of Foxp3 and TGF-β1, we performed transient transfection studies using the Foxp3 and TGF-β1 promoters. We observed a ∼1.5-fold induction of the Foxp3 promoter by KLF10 (supplemental Fig. S2C). Members of the Kruppel-like family bind to specific DNA elements (5′-CNCCC-3′) to exert their function. Mutation of an evolutionarily conserved CACCC KLF DNA-binding site abolished the KLF10-mediated induction of the Foxp3 promoter. Consistently, TGF-β1 induced the Foxp3 promoter by ∼2.4-fold, and mutation of the KLF10 DNA-binding site also prevented TGF-β1-mediated induction of the Foxp3 promoter (supplemental Fig. S2C). Interestingly, we observed a ∼5.6-fold induction of the TGF-β1 promoter by KLF10. This induction was significantly reduced to ∼1.8-fold after co-transfection of either a 5′-deletion TGF-β1 promoter construct (∼175 bp) that lacked a KLF DNA-binding site (at −212 bp) or a 2-bp mutation of the KLF site (Fig. 2A). Consistently, in response to TGF-β1, the TGF-β1 promoter construct bearing a mutated KLF site failed to transactivate in WT CD4+ CD25− T cells (Fig. 2A). Furthermore, TGF-β1 responsiveness completely disappeared on both the wild-type and mutated TGF-β1 promoter constructs in KLF10−/− CD4+ CD25− T cells (Fig. 2B). In addition, real-time quantitative PCR (qPCR) of chromatin immunoprecipitation studies verified that KLF10 is capable of binding to the KLF site of the TGF-β1 promoter, an effect that is markedly attenuated.
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A

B

C

D

E
in the presence of a construct bearing a mutated KLF site (Fig. 2C). Finally, to assess the effect of constitutive overexpression of the TGF-β1 promoter in WT or KLF10−/− CD4+CD25− T cells and Tregs, we adenovirally infected these cells with a TGF-β1-promoter-SEAP reporter at 100 multiplicity of infection followed by treatment with TGF-β1 or vehicle control. As shown in Fig. 2D, KLF10−/− CD4+CD25− T cells had ~47 and ~38% reductions in TGF-β1 promoter activity in the presence or absence of TGF-β1, respectively, compared with WT cells. Similarly, as demonstrated in Fig. 2E, KLF10−/− CD4+CD25+ Tregs had ~55 and ~59% reductions in TGF-β1 promoter activity in the presence or absence of TGF-β1, respectively, compared with WT Tregs. Collectively, these data implicate that in response to TGF-β1, KLF10 dynamically regulates both Foxp3 and TGF-β1 promoter activity.

KLF10 Deficiency Impairs TGF-β1 and Foxp3 Expression and Alters Th1 and Th2 Differentiation—Because overexpression of KLF10 alone promoted T regulatory cell differentiation in primary CD4+CD25− T cells, we hypothesized that KLF10 deficiency may reduce the induction of TGF-β1 and Foxp3 expression and possibly modulate Th1 and Th2 differentiation. To assess this, CD4+CD25− T cells were isolated from KLF10−/− mice and treated with TGF-β1 for 6 h. KLF10−/− CD4+CD25− T cells had impaired induction of TGF-β1 and Foxp3 mRNA in response to TGF-β1 compared with WT CD4+CD25− T cells (Fig. 3A and supplemental Fig. S2D). Consistently, KLF10−/− mice had ~50 and ~67% reductions in peripheral CD4+CD25− T cells and CD4+CD25+Foxp3+ T regulatory cells, respectively, compared with WT mice (supplemental Fig. S2E). Previous reports demonstrate that relative TGF-β1 or Foxp3 deficiency may alter the balance along CD4+Th1 or Th2 differentiation pathways (2, 39). Treating WT or KLF10−/− CD4+CD25− T cells under Th1 or Th2 skewing conditions as described (8) enabled exploration into whether KLF10-deficient cells have enhanced skewing along these pathways. KLF10−/− CD4+CD25− T cells had markedly enhanced differentiation along Th1 and Th2 pathways, including increased expression of Th1 markers T-bet and IFN-γ and Th2 markers Gata3 and IL-5 (Fig. 3, C and D). Collectively, these observations indicate that the presence of KLF10 figures importantly in modulating TGF-β1 and Foxp3 expression levels and CD4+CD25− Th1 and Th2 differentiation pathways.

KLF10−/− T Regulatory Cells Have Impaired Suppressor Function That Exogenous TGF-β1 Can Restore—To assess whether the absence of KLF10 alters T regulatory cell function, we isolated equal numbers of CD4+CD25+ T regulatory cells from WT or KLF10−/− mice and performed suppression assays with co-cultured wild-type CD4+CD25− T cell responders.

FIGURE 2. KLF10 transactivates the TGF-β1 promoter. A, transient transfection experiments were performed with either pcDNA3 (Ctrl) or KLF10 along with the respective TGF-β1 promoter-luciferase reporter constructs in HeLa cells. KLF10-mediated transactivation of the TGF-β1 promoter was lost by either a 5′ deletion or a 2-bp mutation of the KLF binding site. B, CD4+CD25− WT or KLF10−/− T cells were transfected with the −453-bp TGF-β1 promoter or a −453-bp TGF-β1 MUT promoter harboring a 2-bp mutation of the KLF binding site in the presence or absence of TGF-β1. In response to TGF-β1, induction of the −453-bp TGF-β1 MUT promoter was markedly impaired in WT CD4+CD25− T cells and completely abolished in KLF10−/− CD4+CD25− T cells. C, cells from A were subjected to ChIP assays using antibodies to IgG or KLF10 as described under “Experimental Procedures.” DNA isolated from ChIP assays were analyzed in triplicate by real-time qPCR using primers at −341 and −166 bp of the TGF-β1 promoter. Values are presented as relative to DNA input. KLF10 binding was reduced in the presence of the 453-bp TGF-β1 MUT promoter bearing a 2-bp mutation of the KLF binding site. D and E, adenoviral overexpression of the TGF-β1 promoter-SEAP reporter in WT and KLF10−/− CD4+CD25− T cells (D) and CD4+CD25+ Tregs (E) demonstrate impaired TGF-β1 promoter activity in both KLF10−/− T cells. Cells were adenovirally transduced with 100 multiplicity of infection followed by treatment with TGF-β1 (1 ng/ml) or vehicle (Ctrl). Activation of the TGF-β1 promoter was quantified by determining the amount of the SEAP released to the culture medium. Error bars indicate S.D.

KLF10−/− CD4+CD25+ Tregs had up to ~67% reduced suppressor function of co-cultured CD4+CD25− T cells grown in the presence of APCs and anti-CD3 mAbs compared with WT CD4+CD25+ Tregs (Fig. 4A and supplemental Fig. S5B). Indeed, a gene dosage effect in suppressor function occurred (Fig. 4B) across WT, KLF10−/−, and KLF10−/− CD4+CD25+ Tregs, an effect suggesting that tight control of KLF10 expression may be important for T regulatory cell suppressor function. Emerging studies implicate TGF-β1 as a critical mediator of T regulatory cell suppressor function (17, 40, 41). To assess...
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FIGURE 4. Effect of KLF10 deficiency on CD4+CD25+ T regulatory cell function. A, KLF10−/− CD4+CD25+ T regs were co-cultured with WT CD4+CD25+ T cell effectors (5 × 10^5), α-CD3 Ab (1 µg/ml), and APCs for 72 h and proliferation was assessed by thymidine incorporation during the last 18 h. B, gene dosage effect of KLF10 deficiency on T reg suppressor function. Equal numbers of WT, KLF10−/−, or KLF10−/− CD4+CD25+ Tregs were subjected to suppression assays as described in A. C, reduced levels of TGF-β1 elaboration in KLF10−/− CD4+CD25+ T regs. D, rescue of KLF10−/− T regulatory cell suppressor function by exogenous TGF-β1. E, KLF10−/− T regs were stimulated with anti-CD3 Abs and TGF-β1 (1 ng/ml) for 1 h and Western blot analyses was performed for the indicated proteins. KLF10−/− T regs have markedly reduced levels of phosphorylated Smad2. Data are representative of three independent experiments and similar results were obtained as quantitated by densitometry of the bands (right). Error bars indicate S.D.

whether KLF10−/− T regulatory cells have impaired release of TGF-β1, we isolated supernatants of WT or KLF10−/− CD4+CD25+ T regulatory cells and found a ~32% reduction in TGF-β1 secretion (Fig. 4C). Furthermore, KLF10−/− CD4+CD25+ T regulatory cells have reduced TGF-β1 mRNA levels (~40%), protein levels (up to ~50%), and cell surface expression by FACS (~40%) compared with WT T regs (supplemental Fig. S4). These observations raise the possibility that exogenous administration of TGF-β1 may, in part, rescue the defect in TGF-β1 release observed in KLF10−/− T regs and allow for more complete suppression of co-cultured T cell effectors. KLF10−/− CD4+CD25+ T regs co-cultured WT CD4+CD25+ T cell effectors to a markedly lesser extent (~58% reduced) in comparison to WT CD4+CD25+ T regs (Fig. 4D). Remarkably, exogenous administration of TGF-β1 (1 ng/ml) rescued the defect in suppressor function in KLF10−/− CD4+CD25+ T regs to levels near those achieved by WT CD4+CD25+ T regs. Finally, because KLF10−/− T regs have reduced elaboration of TGF-β1, we hypothesized that KLF10−/− T regs may have impaired TGF-β signaling. Cellular signaling through the TGF-β superfamily occurs via intra-cellular mediators, termed Smads, which translocate to the nucleus, where they direct transcriptional responses (42). Three classes of Smads (pathway-restricted, common, and inhibitory) are responsible for propagating the downstream signaling effects. TGF-β/activin receptors phosphorylate the pathway-restricted Smads, Smad2 and Smad3, whereas bone morphogenic protein receptors activate Smad1, Smad5, and Smad8. Pathway-restricted Smads may hetero-oligomerize with the only common Smad, Smad4, before translocating to the nucleus. The inhibitory Smads, Smad6 and Smad7, are structurally divergent from other Smads and block TGF-β signaling by preventing ligand-induced receptor phosphorylation of pathway-restricted Smads. Indeed, KLF10−/− T regs stimulated with anti-CD3 Abs and TGF-β1 for 1 h had profoundly reduced levels of phosphorylated Smad2, whereas no differences emerged in the protein levels of Smad2, Smad7, the TGF-β type I and II receptors, and Foxp3 (Fig. 4E). Thus, KLF10−/− T regs have altered T regulatory suppressive function independent of Foxp3 expression, largely as a consequence of reduced elaboration of TGF-β1.

KLF10−/− CD4+CD25− T Cells Are Hyperactivated and Promote Atherosclerosis—Although KLF10−/− T regs have impaired suppressor function of wild-type T cell effectors, the converse question is equally important. Can WT T regs equally suppress KLF10−/− CD4+CD25− T cell effectors compared with WT CD4+CD25− T cell effectors? To examine this, suppression assays were performed using WT CD4+CD25+ Tregs co-cultured with WT or KLF10−/− CD4+CD25+ T cell effectors. WT T regs suppressed KLF10−/− CD4+CD25− T cell effectors less potently (~41% lower) compared with WT CD4+CD25− T cell effectors (Fig. 5A), an effect suggesting that KLF10−/− CD4+CD25− T cells may be “hyperactivated.” Because such a state may be associated with altered elaboration...
of T effector cytokines, we examined mRNAs for Th1, Th2, and Th17 markers. Indeed, in response to anti-CD3 Abs, KLF10−/− CD4+ CD25+ T cells robustly increased mRNAs for IFN-γ and IL-2 (Th1), IL-4 (Th2), and IL-17 (Th17) (Fig. 5B). Consistently, upon TCR activation, we found enhanced elaboration of several cytokines in KLF10−/− CD4+ CD25+ T cells including Th1 (i.e. IFN-γ, IL-2), Th2 (i.e. IL-4, IL-5), and Th17 (i.e. IL-17) (Fig. 5C). In particular, KLF10−/− CD4+ CD25+ T cells had a number of potently induced IFN-γ-responsive proteins including MIP-1α, MIP-1β, RANTES, SDF-1β, eotaxin, MCP-1, and MMP-9. Interestingly, increased levels of several of these chemokines/factors associate with the development or progression of chronic inflammatory diseases such as atherosclerosis (43). To assess the functional contribution of CD4+ CD25+ T cells in the development of atherosclerotic lesions, we adoptively transferred WT or KLF10−/− CD4+ CD25+ T cells into ApoE−/−/scid/scid mice, which lack functional T and B cells. Previous studies demonstrate markedly reduced atherosclerotic lesion formation in ApoE−/−/scid/scid mice compared with ApoE−/− mice, an effect that adaptive transfer with CD4+ T cells can rescue (36, 44). ApoE−/−/scid/scid mice that received KLF10−/− CD4+ CD25+ T cells accelerated atherosclerotic lesion formation by ~2-fold in comparison to WT CD4+ CD25+ T cells and associated with ~3-fold increased infiltration of CD4+ T cells and Mac-3-positive macrophages after 4 weeks of a high-fat diet (Fig. 6A). Consistently, recipient ApoE−/−/scid/scid mice adoptively transferred with KLF10−/− CD4+ CD25+ T cells had higher plasma levels of pro-inflammatory mediators MCP-1, SDF-1β, and MMP-9, whereas the TGF-β1 plasma level was lower (Fig. 6B). Importantly, no differences appeared in plasma lipid profiles in recipient ApoE−/−/scid/scid mice (supplemental Table S2). Finally, to assess whether KLF10−/− CD4+ CD25+ T cells also possessed impaired TGF-β signaling as a consequence of reduced TGF-β1 elaboration, cells were stimulated with anti-CD3 Abs and TGF-β1 for 1 h, and Western blot analyses were performed. KLF10−/− CD4+ CD25+ T cells possessed significantly reduced levels of phosphorylated Smad2, whereas no differences occurred in the expression of Smad2, Smad7, or the TGF-β type I and II receptors (Fig. 6C). These findings correspond with a loss of cell intrinsic control of KLF10−/− CD4+ CD25+ T cell activation. Taken together, these findings indicate that KLF10−/− CD4+ CD25+ T cells are hyperactivated in response to TCR stimulation. No differences in proliferation were observed between WT and KLF10−/− CD4+ CD25+ T cells alone (p = NS), but KLF10−/− CD4+ CD25+ T cells are hyperactivated in response to TCR stimulation. WT or KLF10−/− CD4+ CD25+ T cells were stimulated with anti-CD3 Abs for 24 h and subjected to qPCR analyses for the indicated cytokines. Each sample was evaluated in triplicate and represents two independent experiments. Error bars indicate S.D.

**DISCUSSION**

We have shown here that KLF10 is a regulator of CD4+ CD25+ T cell activation, T regulatory cell differentiation, and T reg cell suppressor function. By gain and loss of function experiments, we demonstrate that KLF10 targets Tgf-β1 and Foxp3 in CD4+ CD25+ T cells, whereas it targets only TGF-β1 in CD4+ CD25+ T regs. In support of this notion, KLF10 was found to be a TGF-β1-responsive gene expressed highly in T regulatory cells. It programmed CD4+ CD25+ T cell fate toward the CD4+ CD25+ T regulatory cell lineage by targeted induction of both Tgf-β1 and Foxp3 genes. Consistently, we found that KLF10−/− mice have decreased numbers of peripheral CD4+ CD25+ and CD4+ CD25+ Foxp3+ T regulatory cells, and that KLF10-deficient CD4+ CD25+ T cells have impaired TGF-β1 and Foxp3 expression in response to TGF-β1.
KLF10 Targets TGF-β1 in CD4⁺CD25⁻ T Cells and T Regs

Remarkably, KLF10-deficient T reg cells have reduced suppressor function, independent of Foxp3 expression, due to decreased expression and elaboration of TGF-β1 and, consequently, defective Smad signaling, an effect rescued by exogenous TGF-β1 administration to KLF10⁻/⁻ T reg cells. Furthermore, we provide cogent evidence for a lack of cell-intrinsic control of T cell activation by reduced TGF-β1 levels, promoter activity, and consequently, TGF-β signaling in KLF10⁻/⁻ CD4⁺CD25⁻ T cells with enhanced elaboration of Th1 and Th2 cytokines. When introduced into ApoE⁻/⁻/scid/scid mice, KLF10⁻/⁻/CD4⁺CD25⁻ T cells accelerated atherosclerosis with accumulation of T cells and macrophages into lesions and increased elaboration of a range of proinflammatory cytokines, chemokines, and metalloproteinases. A recent study by Venuprasad et al. (45) identified that the E3 ubiquitin ligase Itch regulated the expression of KLF10 to modulate in vitro generated expression of Foxp3; in addition, they found that the in vitro generated "TGF-β1 converted" T regs from CD4⁺CD25⁻ T cells of KLF10⁻/⁻ mice could not suppress airway inflammation. However, the mechanisms by which KLF10 controls both CD4⁺CD25⁻ T cell activation and T regulatory cell suppression function remained unclear. Our findings delineate unique mechanisms in which KLF10 exerts its function in CD4⁺CD25⁻ T cells and T regulatory cells by targeting Tgf-β1 and highlight a critical role for KLF10 in vascular inflammation.

KLFs in T Cells—Other Kruppel-like factors play critical roles in various aspects of T cell differentiation, activation, and function (25, 26). For example, KLF2 expression is induced upon differentiation of immature double positive T cells (CD4⁺CD8⁺) to single positive T cells (CD4⁺ or CD8⁺) that circulate in the bloodstream. Indeed, gene-targeting studies revealed a critical role for this factor in programming the quiescent phenotype of single positive T cells (29). KLF2 also regulates T cell egress from the thymus and peripheral trafficking (46). Expression cloning originally identified KLF13/RFLAT-1 (RANTES factor of late activated T lymphocytes) by its ability to bind to a site and activate the RANTES promoter (47, 48). Recently, targeted disruption of KLF13 verified an important role for its regulation of RANTES expression and T cell survival (49). Additional KLFS identified in T cells include KLF5 and KLF6, albeit their functional roles are not as well defined.

![Image of Figure 6](https://example.com/image.png)

**Figure 6.** KLF10⁻/⁻/CD4⁺CD25⁻ T cells promote atherosclerosis and produce enhanced pro-inflammatory profiles in vivo. A, KLF10⁻/⁻/CD4⁺CD25⁻ T cells promote atherosclerotic lesion formation in ApoE⁻/⁻/scid/scid mice. WT or KLF10⁻/⁻/CD4⁺CD25⁻ T cells were transferred intravenously into ApoE⁻/⁻/scid/scid mice (n = 5 recipient mice for WT cells; n = 7 recipient mice for KLF10⁻/⁻ cells), on a high-fat diet for 5 weeks, then aortic root lesion size (μm²) was quantitated morphometrically after Oil Red O staining (left). Quantitation of staining for CD4⁺ T cells and Mac3+ macrophages (right). Data were obtained by counting lesions at the aortic root of 5 to 7 mice from each group. B, plasma levels of pro-inflammatory mediators increased, whereas TGF-β1 levels decreased in recipient ApoE⁻/⁻/scid/scid adoptively transferred with KLF10⁻/⁻/CD4⁺CD25⁻ T cells. Plasma from ApoE⁻/⁻/scid/scid mice receiving WT or KLF10⁻/⁻/CD4⁺CD25⁻ T cells was collected and assessed by SearchLight Proteome Arrays/multiplex sandwich ELISA as described under "Experimental Procedures." Although pro-inflammatory factors such as MMP-9, MCP-1, SDF-1β, and platelet-derived growth factor-BB increased, and the anti-inflammatory factor TGF-β1 was reduced. C, KLF10⁻/⁻/CD4⁺CD25⁻ T cells have defective TGF-β signaling. WT and KLF10⁻/⁻/CD4⁺CD25⁻ T cells were stimulated with anti-CD3 Abs and TGF-β1 (1 ng/ml) for 1 h and Western blot analyses were performed for the indicated proteins. KLF10⁻/⁻/CD4⁺CD25⁻ T cells have markedly reduced levels of phosphorylated Smad2, whereas there was no effect on Smad2, Smad7, TGFβRI, and TGFβRII. Data represent three independent experiments and similar results were obtained as quantitated by densitometry of the bands (right). Error bars indicate S.D.
KLF10 Targets TGF-β1 in CD4+ CD25− T Cells and T Regs

TGF-β1 and KLF10 Control of T Regulatory Cell Differentiation and Suppressor Function—Accumulating evidence supports a critical role for TGF-β1 and TGF-β signaling in the maintenance of self-tolerance and peripheral T regulatory cell development and function. TGF-β1−/− mice die within 4 weeks of birth due to a multifocal autoimmune disease characterized by severe multior- gan infiltration of autoreactive T cells and other leukocytes (52, 53). Phenotypic similarities in TGF-β1−/− mice and Foxp3-mutant mice elicited investigation of the connection of TGF-β signaling to Foxp3 expression and generation of T regs. Indeed, TGF-β signaling induces Foxp3 and promotes peripheral conversion of naïve CD4+CD25+Foxp3+ T cells into CD4+CD25+Foxp3+ cells in vitro and in vivo (8–12, 14–16). Moreover, TGF-β1−/− mice have impaired T reg suppressor function that stems from defective TGF-β signaling (38, 40). Consistently, we found that KLF10−/− mice also have reduced peripheral T regs, T reg suppressor function, and TGF-β signaling. Because elaboration of TGF-β1 from KLF10−/− Tregs markedly decreased and exogenous TGF-β1 rescued the defect in KLF10−/− T reg suppressor function, the reduced Smad2 phosphorylation likely resulted from lower TGF-β1 levels rather than a downstream defect in Smad2 phosphorylation. In support, we found no difference in levels of TGF-β type I or II receptors, Smad2, or the inhibitory Smad, Smad7, in KLF10−/− and WT T regs. These findings support the premise that KLF10 serves as a positive autoregulator of TGF-β signaling by target- ing TGF-β1. Finally, our studies reveal persistent impairment in suppressor function in these KLF10−/− CD4+CD25+ Tregs, an effect that exogenous administration of TGF-β1 rescued. Indeed, KLF10−/− CD4+CD25+ Tregs elaborated lower TGF-β1 levels than WT CD4+CD25+ Tregs. Recent studies have determined that Foxp3 transcription factor-dependent and -independent molecules including TGF-β1, IL-10, CTLA4, granzyme B, perforin, heme oxygenase-1 (HO-1), C/EBP, CD39, galectins, or IL-35, among others, contribute to T reg suppressor function (41, 54). Additional studies are required to assess the relationship of TGF-β1, KLF10, and these other potential mediators in T reg suppressor function.

TGF-β1 and KLF10 Regulation of CD4+ CD25− T Cell Balancing Effector Th1 and Th2 Pathways—Accumulating studies have demonstrated that TGF-β1 inhibits both Th1 and Th2 cell differentiation pathways by inhibiting T-bet and Gata3, Th1, and Th2 master transcription factors, respectively (17, 55, 56). Although several direct and indirect mechanisms have been proposed, emerging evidence suggests that induction of Foxp3 may also be a major determinant of T reg cell development. This process leads to reduced levels of cytokines conductive for differentiation into Th1 and Th2 pathways (39, 40, 57). Consis- tently, we found that overexpression of KLF10 induced both TGF-β1 and Foxp3 expression, whereas it repressed the expression of T-bet and Gata3 (Fig. 3). Because KLF10 targets TGF-β1, we cannot rule out the possibility that retroviral overexpression of KLF10 may convert non-transduced cells into Foxp3 expressing cells by paracrine-mediated effects of TGF-β1. Indeed, our studies indicate that both mechanisms are operative in CD4+CD25− T cells (Figs. 1A and 2, A-E, and supplemental Fig. S2). Conversely, KLF10−/− CD4+CD25− T cells had enhanced mRNA and secreted proteins for Th1 (e.g. IFN-γ and IL-2) and Th2 (e.g. IL-4, IL-5, and IL-13) markers in response to TCR activation (Fig. 5). Moreover, in response to forced skewing along the Th1 and Th2 pathways, KLF10−/− CD4+ T cells had markedly increased expression for Th1 markers, T-bet and IFN-γ, and Th2 markers, Gata3 and IL-5, sup- porting the notion that these cells are hyperactivated upon differ- entiation of these pathways (Fig. 3). Consistently, KLF10−/− CD4+CD25− T cells had a markedly impaired ability to be sup- pressed by co-cultured WT T regs. In addition, in response to TGF-β1, KLF10−/− CD4+CD25− T cells had induced impor- tation of TGF-β1 and downstream Smad2 phosphorylation (Figs. 3A and 6C). Although after TCR stimulation, we found no differences in basal levels of TGF-β1 mRNA and secreted protein from KLF10−/− CD4+CD25− T cells in vitro (data not shown); secreted TGF-β1 plasma levels decreased in vivo after adoptive transfer of KLF10−/− CD4+CD25− T cells in ApoE−/−/scid/ scid mice (Fig. 5B). Collectively, these findings highlight that loss of the cell intrinsic control of T cell activation by TGF-β1 contributes, in part, to the CD4+CD25− T cell phenotype from KLF10−/− mice. Indeed, TGF-βRII-deficient mice have reduced peripheral T regs, yet adoptive transfer of WT T regs still cannot correct the phenotype of hyperactivated effector T cells observed in these mice (20). Thus, in response to TGF-β1, KLF10 maintains T cell tolerance by controlling both effector CD4+CD25− T cells and T reg cell responses.

TGF-β1/Smad Signaling in Vascular Inflammation—The composite anti-proliferative and anti-inflammatory effects of TGF-β1 on both the immune and nonimmune cellular constit- uents of the atherosclerotic lesion suggest an inhibitory role in atherogenesis. For example, blockade of the TGF-β1 ligand or TGF-β type II receptor accelerates the development of athero- sclerotic lesion formation in ApoE−/−/scid/scid prone mice (23, 58, 59). In patients, serum concentration of active TGF-β1 inversely correlates with the severity of atherosclerotic disease (60). In the context of transplant-associated arterioscle- rosis (TxAA), cardiac allografts from recipient mice heterozy- gous for TGF-β1 displayed a marked increase in TxAA compared with wild-type controls (61). We previously demon- strated that allografts from Smad3-deficient mice develop robust leukocyte infiltration and accelerated vascular arterio- pathy (62). Our studies here demonstrate that KLF10-defi- cient CD4+CD25− T cells have hyperactivated T cell cytokine profiles and accelerate atherosclerotic lesion formation in ApoE−/−/scid/scid mice, effects consistent with the premise that reduced TGF-β1 levels promote atherogenesis. To our knowledge, KLF10 constitutes the first KLF family member identified to regulate T-cell mediated atherosclerotic lesion formation in vivo. Exploration into whether KLFs anti-thetically expressed in T reg cells such as KLF4 or KLF5 (supplemental
KLF10 Targets TGF-β1 in CD4⁺CD25⁻ T Cells and T Regs

Fig. S1) may also participate in immune response and athero-
sclerotic lesion formation will hold interest.

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