Krüppel-like factor KLF10 deficiency predisposes to colitis through colonic macrophage dysregulation

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The abbreviations used are: KLF; krüppel-like factor, TGF-βRII; TGF-β receptor II
ABSTRACT

Krüppel-like factor (KLF)-10 is an important transcriptional regulator of TGF-β1 signaling in both CD8⁺ and CD4⁺ T lymphocytes. In the current study, we demonstrate a novel role for KLF10 in the regulation of TGFβRII expression with functional relevance in macrophage differentiation and activation. We first show that transfer of KLF10-/- BMDM into wt mice leads to exacerbation of experimental colitis. At the cell biological level, using two phenotypic strategies, we show that KLF10-deficient mice have an altered colonic macrophage phenotype with higher frequency of pro-inflammatory LyC6⁺MHCII⁺ cells and a reciprocal decrease of the anti-inflammatory LyC6⁻MCHII⁺ subset. Additionally, the anti-inflammatory CD11b⁺CX3CR1⁺ subset of colonic macrophages is significantly decreased in KLF10-/- compared to wt mice under inflammatory conditions. Molecularly, CD11b⁺ colonic macrophages from KLF10-/- mice exhibit a pro-inflammatory cytokine profile with increased production of TNF-α and lower production of IL-10 in response to LPS stimulation. Because KLF10 is a transcription factor, we explored how this protein may regulate macrophage function. Consequently, we analyzed the expression of TGFβRII in colonic macrophages and found that in the absence of KLF10, macrophages express lower levels of TGFβRII and display an attenuated Smad-2 phosphorylation following TGF-β1 stimulation. We further show that KLF10 directly binds to the TGFβRII promoter in macrophages leading to enhanced gene expression through histone H3 acetylation. Collectively, our data reveal a critical role for KLF10 in the epigenetic regulation of TGFβRII expression in macrophages and the acquisition of a “regulatory” phenotype that contributes to intestinal mucosal homeostasis.
Introduction

Macrophages (Mϕ) are the most abundant mononuclear phagocytes in the healthy intestinal lamina propria (LP) and are important regulators of immune responses during intestinal inflammation (26, 29, 42). In the murine system, intestinal macrophages rely on constant replenishment by Ly6C⁺ blood monocytes conditioned to acquire a non-inflammatory profile and differentiate into chemokine receptor CX3CR1⁺ “regulatory” macrophages (1, 2, 20, 33, 42). During experimental colitis, however, the conditioning of Ly6C⁺ blood monocytes is impaired giving rise to pro-inflammatory macrophages and migratory dendritic cells that perpetuate intestinal inflammation. IL-10/IL10R (27, 41) and TGFβ/TGFβR signaling in the mononuclear phagocyte system (MPS) have emerged as important regulators of inflammation “anergy” characteristic of LP macrophages in mice and human (24, 25, 28). However, the molecular cues that imprint this inflammatory “anergy” downstream of either TGFβ or IL-10 signaling are poorly defined (28).

The significance of TGF-β signaling, in particular, in dendritic cells (DC) and macrophages has been recently highlighted by the observation that mice with a dendritic cell-specific deletion of TGFβ receptor II (TβRII) develop a multi-organ autoimmune inflammation, including colitis and gastritis (24). Moreover, CD68TGFβDNRII mice with Mϕ-specific expression of a truncated TGF-βRII, shows enhanced colitis susceptibility and reduced recovery following intestinal injury with DSS (25). These data reveal an important contribution of TGFβ signaling in the suppression of intestinal inflammation, at least in part, through direct effects on Mϕ function (25, 28). In fact, these considerations lead us in this study to search for cellular and molecular mechanisms that acting downstream of TGFβ signaling may contribute to colitis. In this regard, recent studies have identified a role for TGFβ-inducible early gene 1 or KLF10, which belongs to the family of Sp1/Krüppel-like zinc finger transcription factors (31), in T lymphocytes and innate immune cells (5, 23, 34, 39).

KLF proteins constitute a family of transcription factors that regulate the expression of a large number of genes with established relevance to cell proliferation, apoptosis, differentiation, and transformation (6, 19, 36, 40). Given the established importance of KLF10 in TGF-β signaling in both T and innate immune cells and of TGFβ signaling in the suppression of intestinal inflammation, we hypothesized that KLF10 may contribute to intestinal mucosal homeostasis through regulation of colonic macrophage phenotype and function. Congruent with this hypothesis, we here report that indeed KLF10 plays an important role in the differentiation of intestinal macrophages towards an anti-inflammatory phenotype which contributes to the regulation of colitis in vivo (4). More specifically, we demonstrate that, through its effect on chromatin remodeling, KLF10 regulates TGFβRII expression in murine macrophages via histone H3 modification. In the absence of KLF10, colonic macrophages display a lower frequency of the P3 plus P4 anti-inflammatory subset (defined as Ly6C⁺MHCII⁺) and produce less IL-10 in response to LPS stimulation. Consistent with the anti-inflammatory role of KLF10 in macrophage regulation, transfer of KLF10⁻⁻ bone marrow derived macrophages
(BMDM) to wt mice leads to worsening experimental colitis following DSS administration.

Combined, these results advance our understanding on the KLF10-dependent molecular mechanisms underlying the function of TGF-β signaling in macrophages, while at the same time, shedding light into the innate immune contributions to colitis. Therefore, this new knowledge has both cell biological and biomedical relevance and should be taken into consideration as a potential pathophysiological mechanism in inflammatory processes.

Materials and Methods

Mouse Strains

C57BL/6 mice were purchased from the Jackson Laboratory. KLF10−/− mice were kindly provided by Thomas C. Spelsberg (Mayo Clinic, Rochester, MN) (30). The CAR transgenic mouse was obtained through the NIAID Exchange Program of the National Institutes of Health: Balb/cJ[Tg]CARdelta1-TgDO11.10 mouse line 4285 (35). All of the mice used in experiments were of 6–8 weeks in age and have been maintained under SPF conditions. The mice were age-matched in experiments comparing wild type with KLF10−/−. All animal experiments were performed per the recommendations outlined in the Guide for Care and Use of Laboratory Animals from the National Institutes of Health as required by Mayo Clinic. These guidelines were incorporated into the current study protocol (IACUC No. A13313), which was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), at Mayo Clinic, Rochester, MN.

Cell isolation

LPMC from mouse colons were isolated as previously described (20). LPMCs were further separated into CD11b+ cells using anti-CD11b Micro-Beads (Miltenyi Biotec, Auburn, CA). BMDM were obtained as previously described (27).

Flow Cytometry

Macrophage subsets in the lamina propria (LP) of wt and KLF10−/− mice were analyzed by flow cytometry using the following surface markers; CD45, CD11b, CD64, CD103, Ly6C, MHCII and CX3CR1 (eBioscience) (27, 33). Pro-inflammatory population was defined as Ly6C+MHCII+/- and anti-inflammatory as Ly6C+MHCII+/− (2). The percentage of CX3CR1hi or CX3CR1int macrophage subset between wt and KLF10−/− mice was also analyzed as previously described (20).

Western blot

Colonic CD11b+ mononuclear cells were incubated with TGFβ1 for different time points. The cells were lysed in RIPA buffer (1X lysis buffer-150 with protease inhibitors) for p-SMAD2 measurement or Laemmli buffer for total SMAD2 measurements. Proteins were separated by 10% SDS/PAGE, transferred to membrane, blocked with 5% milk and
probed overnight with primary antibodies at the following dilutions: p-SMAD2 (1:5000; Cell Signaling, Danvers, MA), SMAD2 (1:1000; Cell signaling), and β-actin (1:1000). Blots were washed and incubated with HRP-conjugated secondary antibodies (1:5000; Santa Cruz Biotechnology Inc., Dallas, TX) and developed with chemiluminescence.

Transfection and Luciferase Assays

Two million THP-1 cells were transfected using the Amaxa® Cell Line Nucleofector® Kit V from Lonza (Köln Germany) according to the optimized protocol provided with the kit. Two ug of plasmid DNA for TβRII luc promoter, KLF10, or empty vector (EV) were added to the cells. The nucleofection was done using a Nucleofector®II device. Cells were divided into triplicate and allowed to sit for 24 hours. Luciferase assays were done following the manufacturer's recommendations (Promega, Madison, WI).

ChIP Assays

ChIP assays were performed as previously described (36). Two million murine CAR-expressing BMDM were transfected with KLF10-His adenovirus or empty vector. After 48 hours cells were treated with 1% formaldehyde to cross-link histones to DNA. Fixed cells were sonicated to yield chromatin fragments of 200–1000 bp. Antibody used in the ChIP assays was Omni-probe (D-8) (catalog # sc7270) from Santa Cruz Biotechnology (Santa Cruz, CA). DNA was recovered by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation with the addition of an inert carrier. Options for critically relevant control samples include total IgG or pre-enriched chromatin (input). We chose to control with pre-enriched chromatin because nonspecific IgG frequently does not control adequately for nonspecific cross-reactivity. Furthermore, the chromatin input generates a more accurate estimation of biases introduced through sonication of chromatin and subsequent PCR (36). The TGF-βRII ChiP primers have been previously described (38). The anti-acetyl-Histone H3 antibody (#06-599, H3K9, H3K14, H3K18, H3K23) was purchased from EMD Millipore, (Billerica, MA) and the anti-KAT2B/PCAF antibody (#12188) was purchased from Abcam (Cambridge, MA).

DSS Colitis model and BM macrophage transfer:

Induction of DSS colitis was performed as previously described (7). To analyze the effect of BM macrophage transfer to DSS colitis we transferred wt or KLF10-/- BMDM one day prior and one day after the administration of DSS and followed the mice for disease activity and histological score (20, 37).

Measurement of cytokines by ELISA. Analysis of culture supernatants of stimulated colonic macrophages and BMDM were analyzed by ELISA.

Statistical Methodology

Statistical analyses were performed using GraphPad Software version 4 (GraphPad Software, Inc. La Jolla, CA, USA). Descriptive analyses including means and standard deviations were performed in normally distributed data. t tests were used to compare means between two groups. Paired t test was used to compare means between paired samples. A p value of <0.05 was considered as statistically significant.
Results

Role of KLF10 deficient macrophages in worsening colitis in wild type mice.

We have previously shown that KLF10-/- mice show enhanced susceptibility to colitis, partially through dysregulation of FOXP3 expression in Tregs (36, 37). However, the precise cellular and molecular pathways, as it regards to the participation of other cell types in this process, are not fully characterized. We have recently shown that the susceptibility to DSS colitis in KLF10 deficient mice is transferable with bone marrow (BM)(37). Given the importance of mucosal macrophages in colitis susceptibility in mice and men, we reasoned that KLF10 deficiency in innate immune cells may contribute to colitis susceptibility through dysregulation of macrophages. To address the contribution of KLF10 deficiency in macrophages to colitis susceptibility, we transferred bone marrow derived macrophages (BMDM) from wt or KLF10-/- mice into wt mice and assessed their susceptibility to colitis. As shown in Figure 1, DSS-treated wt mice that received KLF10-/- BMDM developed more severe colitis compared to the ones that received wt BMDM as indicated by the disease activity index (DAI), colon length and histological score (20). Interestingly, the mice that received either wt or KLF10-/- BMDM developed more severe colitis compared to DSS treated mice that received no macrophages (Figure 1). The DAI and colon length in mice that received wt BMDM was 10.8 ± 0.58 and 6 ± 0.17 cm vs 12.6 ± 0.5 and 5.4 ±0.18 cm in mice that received KLF10-/- BMDM, respectively (n=5, p=0.04 for both). Similarly the histological inflammatory score was 5 ± 0.6 in mice that received wt BMDM vs. 7.75 ± 0.8 in mice that received KLF10-/- BMDM (p=0.03). The observed worsening of the colitis after transfer of either wt or KLF10-/- BMDM is likely the result of the proinflammatory differentiation of transferred monocytes/macrophages in the context of colitis(2, 26) and this appears to be more pronounced in the KLF10-/- transferred macrophages. These data indicate a novel pathophysiological role for the transferred KLF10-/- macrophages in enhancing colitis severity.

Genetic Inactivation of KLF10 in the Germline leads to an altered colonic macrophage phenotype in vivo

To start elucidating the mechanisms by which KLF10 deficiency in macrophages enhances colitis susceptibility, we analyzed the effect of KLF10 deficiency on colonic macrophage physiology, by first analyzing the phenotype of colonic macrophages isolated from KLF10-/- or wt mice. Given the complexity of the phenotype of the macrophage subsets that have been previously characterized from the small intestine and colon (1, 2, 26, 33, 42), we focused our attention on the phenotypic characterization
of colonic macrophages defined as CD45+CD11b+CD64+CD103- cells (27, 42). Gating on these cells, we then analyzed the different macrophage subsets based on the expression of Ly6C and MHCII as proposed by Tamoutounour at al (33). Using this gating strategy, colonic macrophages can be characterized as proinflammatory P2 stage (Ly6C+MHCII+) or antiinflammatory P3 plus P4 stage (Ly6C+MHCII+)(27, 33). We found that in the absence of KLF10, the frequency of proinflammatory P2 stage subset was significantly increased compared to wt colons (Figure 2A). The percentage of the P2 subset was 29.6 ± 1.2 % in wt mice compared to 36.4 ± 1.2% in KLF10-/- mice (n=4, p=0.01). Similarly there was a reciprocal decrease in the frequency of anti-inflammatory P3 plus P4 stage in the colons of KLF10-/- mice (Figure 2A). The percentage of the P3 plus P4 subset was 38 ± 2% in wt mice compared to 27.7 ± 1 % (n=4, p=0.009).

Recruited Ly6C+ blood monocytes to the healthy murine colon acquire a noninflammatory gene-expression profile and differentiate into resident chemokine receptor CX3CR1hi anti-inflammatory macrophages(20, 43). Moreover, during DSS colitis there is a colonic recruitment of CX3CR1int cells that are pro-inflammatory (2, 43). To further define the effect of KLF10 deficiency in the expression of CX3CR1 by resident and newly recruited colonic macrophages, we also analyzed the expression of CX3CR1 among CD11b+ mononuclear cells between KLF10 and wt mice. We observed no significant differences in the proportion of CD11b+CX3CR1hi subset between wt and KLF10-/- mice isolated from the colon (28.6% ± 2.93 vs. 29 ± 2.6, n=5, p=NS). However, we observed a significant increase of the colonic CX3CR1int subset in KLF10-/- compared to wt mice. The frequency of CD11b+CX3CR1int subset was 16.3 % ± 1.8 in wt vs 21.5% ± 2 in KLF10-/- mice (n=5, p=0.03). More importantly after the induction of DSS colitis, the colonic CD11b+CX3CR1hi subset increased significantly more in wt mice compared to KLF10-/- mice. For example by day 8 of the experiment 41.5% ± 5.6 of wt CD11b+ cells acquired high levels of CX3CR1 vs. 28.4% ± 4.5 of KLF10-/- mice (n=4, p=0.03)(Figure 2B). Although the diffence was not as pronounced for the CD11b+CX3CR1int subset, we observed a higher frequency of CD11b+CX3CR1int cells in KLF10-/- compared to wt mice following DSS colitis (20.8 % ± 0.45 vs. 16.5 % ± 1.4, n=3; p=0.05) (Figure 2B). Collectively, these results implicate an important role for KLF10 in the differentiation of intestinal macrophages in health and during acute colitis.

**KLF10 deficient macrophages display a dysregulation in the profile of pro-inflammatory cytokines**

Since the CD11b+CX3CRint macrophage subset is being characterized as pro-inflammatory with the ability to produce high levels of TNF-α and the CD11b+CX3CRhi subset as anti-inflammatory through the production of IL-10 (8, 41), we next analyzed the cytokine profile of colonic macrophages isolated from the colons of KLF10-/- or wt mice following LPS stimulation in vitro. As shown in Figure 3A, colonic macrophages from KLF10-/- mice produce significantly higher levels of TNF-α and lower levels of IL-
10 compared to macrophages isolated from wt mice following LPS stimulation. The amount of IL-10 released by LPS-stimulated wt colonic macrophages was 0.55 ± 0.03 ng/ml compared to 0.04 ± 0.009 (n=3; p<0.0001) of similarly stimulated KLF10-/- colonic macrophages. The amount of TNF-α released by LPS-stimulated wt colonic macrophages was 0.042 ± 0.004 ng/ml compared to 0.1 ± 0.016 ng/ml released by KLF10-/- colonic macrophages (Figure 3A). We found no significant differences between wt and KLF10-/- colonic macrophages in the release of IL-12 p70 following LPS stimulation (Figure 3A). Similarly stimulation of KLF10-/- BMDM with IFN-γ and LPS led to higher release of TNF-α and IL-6 compared to wt BMDM. Stimulated wt BMDM released 0.29 ± 0.05 ng/ml of TNF-α compared to 0.76 ± 0.08 ng/ml of KLF10-/- stimulated cells (n=3, p=0.004). Similarly the amount of IL-6 released by wt BMDM was 0.5 ± 0.07 ng/ml vs 0.98 ± 0.15 ng/ml of stimulated KLF10-/- cells (n=3, p=0.02) (Figure 3B), indicating that the absence of KLF10 directly affects the proinflammatory activation of macrophages irrespective of their location or state of differentiation.

**Genetic inactivation of KLF10 directly impairs TGF-βRII expression and TGF-β signaling in macrophages**

Given the critical role of KLF10 in TGF-β signaling and the importance of TGF-β/TGF-βR pathway in mucosal immune homeostasis (5, 23), we reasoned that KLF10 may regulate TGF-β1 signaling in innate immune cells, including macrophages. To this end we have analyzed colonic macrophages and showed that TGF-βRII expression is lower in KLF10-/- CD11b⁺ colonic macrophages compared to wt mice (Fig. 4A). Moreover, TGF-β1 signaling in KLF10-/- macrophages led to an early attenuation of Smad-2 phosphorylation compared to wt mice (Fig. 4B), indicating that in the absence of KLF10, TGF-β signaling in macrophages is impaired. Thus, to further explore the molecular pathways by which KLF10 regulates TGFβRII expression in macrophages we addressed the notion that KLF10 binds to and transcriptionally activates the TGF-βRII gene promoter in macrophages. To this end we used chromatin immunoprecipitation (ChIP) assays to confirm binding of the KLF10 transcription factor to the core TGFβRII promoter in macrophages. BMDM from KLF10-/- and wt mice were analyzed for KLF10 binding to the TGF-βRII promoter as described in Materials and Methods. As shown in Figure 5A, KLF10 binds to the TGF-βRII core promoter region between -500 to +500 relative to the transcriptional start site (TSS).

We next transfected the macrophage cell line THP-1 with KLF10 and TGF-βRII reporter constructs and analyzed luciferase activity. As shown in Figure 5B, co-transfection of KLF10 with TGFβRII led to a dose-dependent induction of luciferase activity compared
to the transfection with empty vector. Transfection with 1 μg of KLF10 and 1 μg of TGFβRII led to a four-fold enhancement of promoter activity relative to the empty vector (n=4; p=0.004) (Fig. 5B). Co-transfection of KLF10 with different TGFβRII reporter constructs that carry either a deletion or mutation of the KLF binding sites, significantly abrogates luciferase activity (0.35 ± 0.32 for the deletion and 0.057 ± 0.006 for the mutant TGF-βRII promoter, p=0.005 and p=0.007 respectively) (Fig. 5B).

Collectively, our data show that KLF10 directly binds to the core TGF-βRII promoter region in BMDM and transcriptionally activates the TGFβRII promoter in macrophages.

**KLF10 functions by recruiting the histone acetyl transferase PCAF to the TGF-βRII promoter in macrophages**

Chromatin remodeling has emerged as one of the most important aspects of gene regulation (13, 15, 16). To further address the chromatin modifying events by which KLF10 may activate the TGF-βRII promoter in macrophages, we examined histone modifications by ChIP assays using pan AcH3 as an indicator of open chromatin. We observed that in the absence of KLF10, there is significantly lower Histone H3 acetylation (Fig 6A). These data suggest that KLF10 is required for the recruitment of an H3 specific acetyltransferase (HAT) to modify chromatin required for gene activation. Our group have previously discovered a critical interaction between KLF10 and p300/CBP-associated factor (PCAF) in immune cells, in particular the regulation of FOXP3 promoter activity in T cells (37). Therefore, we explored the possibility that KLF10 interacts with PCAF to regulate TGF-βRII promoter activity in macrophages. We indeed observed a significant decrease of PCAF binding to the TGF-βRII core promoter in the absence of KLF10 (Fig. 6B). Taken together our data provide a novel pathway by which KLF10 specifically recruits PCAF to induce histone H3 acetylation to activate the TGFβRII gene transcription in macrophages.
Discussion

In the current manuscript we report several novel observations. We first demonstrate that transfer of KLF10-/- BMDM to wt mice worsens experimental colitis. Secondly, we show that, at the cellular mechanistic level, in the absence of KLF10 intestinal macrophages display an altered phenotype and a pro-inflammatory cytokine profile in response to LPS stimulation. Moreover, colonic macrophages display lower expression of TGFβRII and attenuated Smad-2 phosphorylation in response to TGFβ stimulation. Third, at the molecular level, exploring the chromatin modifying events we demonstrate that KLF10 binds to the core TGFβRII promoter and epigenetically regulates TGFβRII gene transcription in macrophages via histone H3 acetylation through recruitment of the histone acetyl transferase PCAF. Thus in light of these novel observations, we propose that KLF10 is an important transcriptional regulator of intestinal macrophage differentiation and anti-inflammatory function in vivo.

Macrophages are the most abundant leukocytes in the healthy intestinal lamina propria and contribute significantly to gut homeostasis (22, 29) through several mechanisms including phagocytosis, degradation of microorganisms and dead tissue cells, and epithelial cell restitution (22). Importantly they produce large amounts of IL-10 which blocks TLR-induced inflammatory responses and enhances the survival and function of local FOXP3+ Tregs (8, 22, 41). Moreover, the response of macrophages to mucosally produced IL-10 is critical in maintaining homeostasis since disruption of IL10-receptor signaling leads to spontaneous colitis in mice (27, 41).

Small intestinal and colonic macrophages are continuously replenished by chemokine receptor CCR2–dependent influx of Ly6C^hi monocytes that differentiate locally into mature, anti-inflammatory macrophages (1, 8, 41). Recent evidence also suggests that TGFβ-signaling contributes to the conditioning of recruited blood macrophages to an anti-inflammatory phenotype (9, 11, 25). For example, mice with a Mϕ-specific transgene of TGFβDNRII(CD68TGFβDNRII) have an impaired ability to resolve colitic inflammation, produce less IL-10 but increased levels of IL-33+ Mϕ compared with control littermates, suggesting that TGFβ may promote the suppression of intestinal inflammation, at least in part, through direct effects on Mϕ function (25). A recent report also demonstrated that TGFβ, primarily the TGFβ2 isoform, suppresses Mϕ inflammatory responses in the developing intestine and protects against inflammatory mucosal injury (18). In addition TGFβ signaling plays a critical role in promoting alternative macrophage activation, thus limiting systemic inflammatory responses (9). Moreover, TGF-β produced by intestinal epithelial cells is required for the tolerogenic condition of intestinal dendritic cells and possibly Mϕ (11). Therefore, characterizing the molecular cues by which IL-10 or TGFβ contribute to the regulatory phenotype of
intestinal mononuclear phagocyte system, including macrophages, is of critical importance in understanding perturbation of these pathways that may contribute to the development of mucosal inflammation. In this study, we provide novel insight into the chromatin modifying events that control the regulation of TGF-βRII gene expression in macrophages, namely the requirement of TGF-β-induced KLF10 in recruiting PCAF to the core TGF-βRII promoter and regulating gene transcription. PCAF is one of the histone modifying enzymes that associates with KLF10 to the TGF-βRII promoter and is consistent with our previous studies showing that KLF10 can recruit PCAF in the FOXP3 promoter to induce Treg differentiation (36, 37). The critical role of KLF10 is highlighted by the significant decrease of Histone H3 acetylation in KLF10/-/- BMDM and the inability to transactivate the TGF-βRII promoter in THP-1 cells when KLF10 binding sites of the promoter were deleted or mutated. Additional protein partners may be recruited by KLF10 to the core TGF-βRII promoter in macrophages in addition to PCAF that will enable for the full gene activation and these possibilities are currently under investigation.

KLF10 has been implicated in cell differentiation, as a target gene for a variety of signaling pathways, and in serving as a potential marker for human diseases such as breast cancer, cardiac hypertrophy, and osteoporosis (32). Certain genetic variants of KFL10 have been described for cardiomyopathy (3) and minor contributions of Smad 7 and KLF10 in type 2 diabetes (10). Recent report also suggested KLF10 as a susceptibility gene for IgA nephropathy in Han Chinese (17). Higher levels of KLF10 have also been described in human COPD and liver cirrhosis (14). There is no known effect of low KLF10 that may affect innate immune response in human disease. However, the relevance of these regulatory pathways in human inflammatory bowel disease, particularly Crohn’s disease (CD), is highlighted by the fact that KLF10 is a critical regulator of Smad 7 (12), the latter being an important negative regulator of TGF-β signaling of T cells and other cell types. Indeed the lower Smad 2 phosphorylation we observed in TGF-β stimulated KLF10/-/- macrophages (Figure 4) could be partly related to increased Smad 7 expression as the result of absent suppressive role of KLF10 on Smad 7 expression. The role of KLF10 in regulating Smad 7 expression in macrophages is currently under investigation. Recently, Smad 7 has been targeted (Mongersen®, Celgene, NJ) for the treatment of Crohn’s disease with encouraging results (21). Although, in theory Mongersen® may target Smad 7 in intestinal T cells its effect on intestinal macrophages may contribute to its therapeutic effect. In this context it would be interesting to see, if indeed Smad 7 is dysregulated in the absence of KLF10, whether targeting of Smad 7 in KLF10/-/- mice may ameliorate the severity of colitis.

In summary, we have identified important molecular cues by which TGFβ-induced KLF10 may further enhance TGFβ signaling through regulating the expression of its
own receptor in colonic macrophages. These molecular mechanisms include epigenetic modifications of the TGFβRII promoter locus through recruitment of chromatin modifying enzymes, such as PCAF. Dissecting further the molecular pathways by which KLF10 may regulate the acquisition of a “regulatory” phenotype in colonic macrophages will shed light on unique pathways that may be attractive therapeutic targets to treat mucosal inflammation.
References


FIGURE LEGENDS

**Figure 1.** Transfer of KLF10-/- BMDM worsens DSS colitis. BMDM obtained from wt or KLF10-/- mice were administered intraperitoneally (i.p.) at day -1 and +1 to wt mice who were subjected to 3% DSS treatment at Day 0. Disease activity index (DAI, upper left), colon length (upper right) and histological score (lower panel) were compared between the different treatment groups.

**Figure 2.** Characterization of colonic macrophages in adult (6-8 week-old) KLF10-/- mice. **A:** LPMC were isolated from wt or KLF10-/- mice and stained for CD45, CD11b, CD64, CD103, LyC6 and MCHII and analyzed by flow cytometry. Cells were gated on CD45+ live CD11b+CD64+CD103- subset and analyzed for the expression of Ly6C and MHCII. The different macrophage subsets were then characterized as pro-inflammatory (Ly6C+MHCII+) P2 or anti-inflammatory (Ly6C-MHCII+) P3 and P4. Average percentages ± SEM of the different colonic macrophage subsets P2, P3 and P4 in wt and KLF10-/- colon are shown (right panel, n=4). **B:** Representative dot-plot analysis of CX3CR1 expression among CD11b+ murine colonic mononuclear cells between wt and KLF10-/- mice at Day 8 following DSS treatment. Average percentages of CX3CR1^{int} or CX3CR1^{hi} subset in wt vs. KLF10-/- mice are shown in lower panel B (n=4 mice/group).

**Figure 3.** Cytokine dysregulation of KLF10-/- macrophages. **A.** Cytokine release by CD11b+ colonic mononuclear cells isolated from wt or KLF10-/- mice. Cells were incubated in medium with or without LPS (100 ng/ml) for 24 hours and supernatants were collected and analyzed for the expression of IL-10, TNF-α and IL-12p70. The data represent the mean ± SD of three independent experiments from 3 different wt or KLF10-/- mice. NT=not treated. **B.** Cytokine release by wt or KLF10-/- BMDM. BMDM were stimulated with LPS plus IFN-γ for 24 hours and supernatants were analyzed for the production of TNF-α and IL-6. The data represent the mean ± SD of three independent experiments from 3 different wt or KLF10-/- mice.

**Figure 4.** Colonic macrophages in KLF10-/- mice express lower levels of TGFβRII and have altered TGF-β signaling. **A.** Colonic macrophages were isolated and stained for CD45, CD11b, CD64 and CD103. Cells were gated on CD11b^+CD64^+ cells and analyzed for the expression of TGFβRII. The depicted representative histogram indicates the MFI of TGFβRII expression in wt vs. KLF10-/- colonic macrophages from three independent experiments with similar results. **B.** Attenuated early Smad-2 phosphorylation in KLF10-/- macrophages in response to TGF-β1 stimulation. For the analysis of pSmad2 cells were serum starved for 24 hours and activated for the indicated time-points with TGF-β1 (5 ng/ml) lysed and analyzed for p-Smad2 and total Smad2 by western blotting. Representative of 4 experiments with similar results is shown. The average ratio of p-Smad2/total Smad2 between wt and KLF10-/-
macrophages at the indicated time points from 4 independent experiments is shown in the lower panel (p<0.001 by 1-way ANOVA analysis).

**Figure 5.** KLF10 binds to and transactivates the promoter activity of TGFβRII in macrophages. **A:** Chromatin immunoprecipitation assay demonstrating binding of KLF10 to the TGFβRII core promoter locus in BMDM. Semi-Quantitative PCR analysis of the expression of TGFβRII post immunoprecipitation for His-tagged KLF10 in BMDM transfected with KLF10-His expression vector demonstrate significant binding of KLF10 to the core promoter. Results presented are controlled to TGF-βRII expression of pre-immunoprecipitated sample (input). Inset gel, left panel, demonstrates representative DNA gel for PCR reaction analysis of the expression of TGFβRII in BMDM post immunoprecipitation for His. The data is representative of 3 independent experiments combined in right panel shown as fold over input (p=0.006). **B:** THP-1 cells were transfected with empty vector (EV) or 1 μg of KLF10 plus TGF-βRII promoter reporter as described in Materials and Methods and analyzed by a dual-luciferase assay system. Co-transfection of a TGF-βRII promoter reporter that carries a mutation (Mut1) or a deletion (Del1) of the KLF10 binding site completely abrogates the promoter activity. The results represent the average of three transfection experiments performed in duplicate. The lower panel indicates the TGF-βRII promoter sequences with the respective deletion or mutation construct used for the transfection experiments.

**Figure 6.** Histone H3 acetylation is a KLF10-dependent event through recruitment of PCAF. **A:** ChiP assay of BMDM from KLF10-/- or wt mice for Acetylated histone H3 was performed as described in materials and methods. We observed lower Histone H3 acetylation in the absence of KLF10 in BMDM. The data is representative of 4 independent experiments combined in right panel shown as fold over input (p=0.001). **B:** P300/CBP-associated factor (PCAF) is recruited to the TGFβRII promoter. BMDM were isolated from wt or KLF10-/- mice and chromatin immunoprecipitation assays (ChiP) were performed using anti-PCAF antibody. Primers flanking the core TGFβRII promoter was used to amplify the cross-linked DNA by semi-quantitative PCR. The data is representative of 3 independent experiments combined in right panel shown as fold over input (p=0.05).
DISCLOSURES

The authors have no financial conflict of interest

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Figure 1
Figure 2

(A) Flow cytometry analysis showing the expression of Ly6C and MHCII in WT and KLF10-/- mice. The histograms on the right show the percentage of cells in each quadrant (P2, P3 + P4) with statistical significance indicated (P=0.01 for P2 and P=0.009 for P3 + P4).

(B) Flow cytometry analysis showing the expression of CD11b and CX3CR1 in WT and KLF10-/- mice. The histograms on the right show the percentage of CD11b+CX3CR1hi cells with statistical significance indicated (P=0.05 for WT and P=0.03 for KLF10-/-).
Figure 3

**A**

- IL-10
  - *p < 0.0001
  - *p = NS
  - WT NT - KLF10-/- NT - KLF10-/- 24 hours

- TNFα
  - *p = 0.02
  - *p = NS
  - WT NT - KLF10-/- NT - KLF10-/- 24 hours

- IL-12p70
  - *p = NS
  - *p = NS
  - WT NT - KLF10-/- NT - KLF10-/- 24 hours

**B**

- TNF-α
  - *p = 0.004
  - WT Media - KLF10-/- media

- IL-6
  - *p = 0.02
  - WT Media - KLF10-/- media
Figure 4

A

CD11b+CD64+CD103-

Isotype control

KLF10

wt

TGF-βRII

B

WT

KLF10−/−

pSmad2

Smad2

0 30 60 120 0 30 60 120 120 min

P<0.001

Ratio pSmad2/Smad2

wt

KLF10−/−

Figure 4
>TGFBRII promoter

GGTACCGCAGATGTCTGATCTACTAGGAACATTGACGGTTTTCTGTTGTTACTTTGGAACCTGGCTGCA

CTTATGTCATTTCGAGTAATAGCTTGGAGGAAGAACCTGCTGAGCTGTTGCTGGGCGGCTGGAGGCAA

TGAAAGTCGCGCCAAGCTCTCGAGGGGCTGGCTGAGGAACATGATGTTGCGACAGCAGAGAGCTAGGGGC

TG6ACGTGCGAGAGAAGAGAGCTCTCGGGCGAGAAGAGGTGGTCCTGCCCCGCTAGATCT

Figure 5
Figure 6

A

WT   KLF10-/-

H3Ac

Input

Fold over input

WT   KLF10

* p=0.001

B

WT   KLF10-/-

PCAF

Input

Fold over input

WT   KLF10

* p=0.05