miR-106a deficiency attenuates inflammation in murine IBD models

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Pro-inflammatory cytokine TNFα antagonizes regulatory T cell (Treg) suppressive function with a measurable reduction of IL-10 protein secretion. Tregs are critical to suppress excessive immune activation, particularly within the intestine where high antigenic loads elicit chronic subclinical immune activation. Employing a TNFα-driven murine inflammatory bowel disease (IBD) model (TNFαARE/ΔARE), which mirrors the Treg expansion and transmural ileitis seen in Crohn’s disease, we demonstrate that the TNFα-mediated loss of Treg suppressive function coincides with induction of a specific miRNA, miR-106a in both humans and mice, via NFκB promoter binding to suppress post-transcriptional regulation of IL-10 release. Elevation of miR-106a and impaired Treg function in this model recapitulate clinical data from IBD patients. MiR-106a deficiency promotes Treg induction, suppressive function and IL-10 production in vitro. MiR-106a knockout attenuated chronic murine ileitis, whereas T cell restricted deficiency of miR-106a attenuated adoptive transfer colitis. In both models, attenuated inflammation coincided with suppression of both Th1 and Th17 cell subset expansion within the intestinal lamina propria. Collectively, our data demonstrate impaired Treg suppressive function in a murine IBD model consistent with human disease and support the potential for inhibition of miR-106a as a future therapeutic approach to treat chronic inflammatory conditions including IBD.

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INTRODUCTION
Inflammatory bowel disease (IBD) currently affects ~2.5 million Europeans, though the incidence continues to increase.1 The current standard treatment, anti-tumor necrosis factor (TNFα) antibody therapy reduces remission in less than 50% of IBD patients2,3 leaving an unmet need for newer therapeutics. Clinical and experimental data indicate that IBD persistence is primarily due to the attenuation of immunosuppressive T cells.4 Regulatory T cells (Treg), defined by forkhead box P3 (FoxP3) expression represent a subset of immunosuppressive T cells.5 While Tregs utilize a variety of suppressive mechanisms including cAMP and adenosine, secretion of the anti-inflammatory cytokine IL-10 is considered one of the primary mechanisms for limiting T cell proliferation (for review ref. 6). The importance of IL-10 in preventing IBD is highlighted by spontaneous enterocolitis occurring in mice lacking IL-107 and IL-10 receptor polymorphisms correlating with early onset IBD.8 Paradoxically, numerous studies have demonstrated increased CD4+ FoxP3+ T cells in the intestinal lamina propria of IBD patients which fail to attenuate disease, potentially due to impaired Treg suppressive function.9 Interestingly, the impairment in Treg function can be reversed by anti-TNFα treatment, however, it is unclear if this is a direct consequence of TNFα blockade or due to the attenuation of intestinal inflammation.10 Nevertheless, impaired Treg function is believed to contribute to the pathogenesis of IBD.

To assess Treg suppressive function in a murine IBD model, we have employed the TNFαARE/ΔARE mouse model of Crohn’s disease (CD), a model in which CD4+ effector T cells play a central role.11 These mice express a single copy of a TNFα mutation which lacks the AU-rich element (ΔARE) controlling mRNA stability, leading to over-stabilized TNFα mRNA and systemic elevation of TNFα protein.12 This results in the spontaneous development of chronic transmural ileitis reminiscent of human CD in its histological features and highlights the pivotal role played by TNFα in IBD pathogenesis.11,12 We hypothesize that the ability of TNFα to transiently impair Treg function, as has been described in both Crohn’s disease and ulcerative colitis (UC),13 may be mediated through induction of selective microRNA (miRNA). Rather than on/off regulation of genes, miRNA mediate subtle up and down-regulation allowing for fine control of key biologic functions including IL-10 release.14 Despite limited clinical success,15 miRNA remain attractive therapeutic targets to modulate immune function in a more specific manner than current broad spectrum immunosuppressive therapies.

Here we examine the role of a select miRNA in regulating Treg suppressive function in the TNFαARE/ΔARE model. We hypothesize that disease induction and maintenance is promoted by impaired Treg function, given their role in preventing overactive inflammation. We first assessed Treg frequency at the peak of intestinal inflammation (between 8 and 12 weeks) along with their capacity.
to produce IL-10. We identified a candidate miRNA, miR-106a, that is elevated in IBD in response to TNFα, which targeted the IL-10 3′ untranslated region. MiR-106a has recently been found to be elevated in serum of IBD patients correlating with disease severity, suggesting it may also serve as a useful biomarker for both CD and UC. We confirmed that miR-106a was elevated in our murine model and that selective inhibition of miR-106a enhanced Treg function in vitro. Finally, we assessed the impact of miR-106a deficiency on inflammation in multiple IBD models using a miR-106a-363 knockout mouse strain.

RESULTS
Expansion of impaired regulatory T cells during active disease
Consistent with previously published data from 20-week-old mice, we demonstrated a significant increase in Treg frequency at peak disease (8–12 weeks) in TNF-ARE/- mice relative to WT. The increased frequency was offset by a reduction in IL-10 producing CD4+FoxP3+ Tregs in ilea of 8wk TNF-ARE/+ mice relative to WT littermates. Treg accumulation was confirmed by Taqman real-time PCR measurement of the relative FoxP3 mRNA expression in ileal whole tissue with results expressed relative to 18S or CD4. PCR analysis of isolated CD4+CD25+ Tregs from WT and TNF-ARE/+ mice indicated no significant difference in IL-10 mRNA. Results represent mean ± SEM for three mice per group from three independent studies. *P < 0.05, **P < 0.01, ***P < 0.001
support the ability of TNFα to modulate IL-10 protein secretion. We therefore assessed the impact of proteasomal inhibition on the secretion of IL-10 by Tregs from WT and TNFKnowing +/− mice. Treatment with the proteasomal inhibitor (MG132, 1 μM) significantly increased detectable levels of IL-10 in supernatants from WT (435.0 ± 20.1 pg/ml vs. 374.5 ± 16.5 pg/ml; P < 0.05; Fig. 3d) but not TNFKnowing +/− Tregs. Thus, the TNFα mediated decrease in IL-10 secretion was not altered by inhibition of the proteasome suggesting an alternative mechanism for the regulation of IL-10 expression relative to control; P < 0.001. 

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sugesting that the reduced IL-10 production was regulated at a post-transcriptional level.

Decreased suppressive function of Tregs from inflamed mice

To assess the impact of reduced IL-10 output from Tregs on their suppressive function, we performed an in vitro Treg suppression assay. Magnetically isolated CD4Knowing +/− T cells from inflamed mice exhibit impaired suppressive function. Increasing ratios of isolated CD4 Knowing +/−CD25Knowing + Tregs from both WT and TNFKnowing +/− mice were co-cultured with irradiated CD90Knowing +/− antigen-presenting cells and fluorescent CellTrace-labeled effector cells stimulated with soluble anti-CD3 (1 μg/ml) for 72 h. Proliferation of effector cells was quantified by flow cytometric analysis of the percentage of proliferating labeled lymphocytes as a surrogate marker of suppressive function. Tregs from TNFKnowing +/− displayed significantly weaker suppression of proliferation compared to WT littermates. Results represent mean ± SEM for four technical replicates from three independent studies. **P < 0.001

TNFα suppresses IL-10 production through a post-transcriptional mechanism

To examine the impact of recombinant TNFα on Treg IL-10 production, we employed the IL-10 IRES-GFP reporter strain to allow real-time monitoring of IL-10 transcription with stoichiometric GFP production. This demonstrated no significant difference when recombinant TNFα was added to culture media (Fig. 3a, b). This was further validated by harvesting RNA from isolated CD4Knowing +/−CD25Knowing + Treg following 24 h incubation with TNFα and assessment of IL-10 by Taqman real-time PCR which also showed no significant difference (not shown). In contrast, ELISA of supernatants from these cultures were consistent with previous intracellular cytokine staining, showing that adding TNFα to WT Treg cultures resulted in reduced IL-10 secretion (301.8 ± 35.7 pg/ml vs. 857.4 ± 74.3 pg/ml; P < 0.005; Fig. 3c). Similarly, Tregs isolated from TNFKnowing +/− mice produced significantly less IL-10 relative to WT controls (292.6 ± 22.3 pg/ml vs. 857.4 ± 74.3 pg/ml; P < 0.001; Fig. 3c) yielding a similar output to that of WT Tregs challenged with TNFα in vitro. Interestingly, TNFα antibody blockade partially restored IL-10 output in TNFKnowing +/− Tregs, (518.1 ± 26.3 pg/ml vs. 292.6 ± 22.3 pg/ml; P < 0.001; Fig. 3c). Taken together, these data support the ability of TNFα to modulate IL-10 protein secretion.

miR-106a promoter activity depends on NFκB binding

To confirm TNFα was capable of regulating miR-106a expression in human T cells, Jurkat cells were treated with recombinant TNFα. We observed a concentration-dependent increase in miR-106a expression with a significant increase observed with 10 ng/ml TNFα (3.0 ± 0.2 fold induction; P < 0.01; Fig. 4a), consistent with murine T cell data. Interestingly, TGFβ significantly suppressed miR-106a expression in a concentration-dependent manner with the greatest suppression seen at 10 ng/ml (0.18 ± 0.01 fold expression relative to control; P < 0.001; Fig. 4a). This suggests that miR-106a is critical for pro-inflammatory signaling to transiently impair immunosuppressive signaling and may also
have an important physiological role in Treg induction by TGFβ. To assess if TNFa and TGFβ are directly targeting miR-106a transcription we used a dual luciferase reporter assay with the human miR-106a promoter in Jurkat cells. Previous studies have identified a putative promoter region on human chromosome X for miR-106a23 and so we aimed to confirm the ability of TNFα and TGFβ to target this miR-106a promoter. TNFα caused a concentration-dependent induction of miR-106a promoter luciferase activity relative to Renilla luciferase (4.4 ± 0.01 RLU with 10 ng/ml TNFα treatment; P < 0.001; Fig. 4b). In contrast, TGFβ suppressed miR-106a promoter luciferase activity in a concentration-dependent manner which was greatest at 10 ng/ml of TGFβ (0.7 ± 0.01 fold induction relative to vehicle control; P < 0.01; Fig. 4b). To identify potential sites on the miR-106a promoter that are responsive to TNFα signaling, we performed luciferase assays with the full-length miR-106a construct in the presence of a NFKB inhibitor, Bay 11–7082 (10 μM; Sigma-Aldrich) and an AP-1 false substrate, c-JUN peptide (100 μM; Tocris Bioscience) using 10 ng/ml of TNFα unless otherwise stated (Fig. 4e). While inhibiting AP-1 activity failed to impact TNFα-induced luciferase activity, NFKB inhibition abolished all TNFα-induced luciferase activity (0.03 ± 0.006; P < 0.001; Fig. 4e) supporting a role for NFKB binding to the miR-106a promoter as a result of TNFα signaling. This effect appears to be selective for miR-106a as expression of other miR-106 family members (Fig. 4f) including miR-17, located on a paralogous cluster miR-17-92 cluster, miR-106b found on the miR-106a-363 polycistronic unit, were all downregulated with TNFα treatment. However, miR-18 failed to reach statistical significance, which is consistent with some shared regulation. This phenomenon was recapitulated in isolated healthy human peripheral blood CD4+ T cells in which paired analysis demonstrated a significant increase in miR-106a in response to TNFα treatment (1.8 ± 0.2 fold increase; P < 0.05; Fig. 4g). Thus, under physiological conditions, TGFβ appears to suppress miR-106a to facilitate Treg induction. In contrast, under inflammatory conditions, TNFα appears to drive upregulation of miR-106a through NFKB-dependent induction of the miR-106a promoter leading to temporary suppression of normal immune regulation.
MiR-106a deficiency enhances Treg induction and function.

Germline deletion of the miR-106a-363 cluster significantly reduced the induction of IFNγ+ (Fig. 5a) cells and IL-17+ T cell (Fig. 5b) from CD4+CD25− splenocytes under Th0 conditions relative to WT cells. In contrast, the frequency of CD4+FoxP3+ T cells induced under both Th0 and Tr1 conditions was significantly increased in cells from miR-106a−/− mice relative to WT controls (Fig. 5c). Increased Treg induction coincided with an
increased expression of IL-10 (Fig. 5d). However, fold change analysis demonstrated that under Th0 conditions, the increased frequency of IL-10 producers present in miR-106aΔ−/− cells, while preferentially occurring in Tregs, was also present in non-Tregs (Fig. 5e). Given the impaired Treg function seen in TNFΔARE/Δ mice, we next assessed in vitro suppressive function of Tregs from miR-106aΔ−/− mice relative to TNFΔARE/Δ (Fig. 5f). miR-106aΔ−/TNFΔARE/Δ Tregs displayed significantly greater suppressive function. However, this enhanced suppressive function was unaffected by IL-10R blockade, suggesting that even in the absence of IL-10 signaling miR-106a deficiency improves suppression (Fig. 5g). Because the miR-106a-363 knockout affects multiple miRNA, we validated the role of miR-106a in Treg suppressive function using a lentiviral approach. Lentiviral suppression of miR-106a reduces IL-10 secretion into cell culture supernatants measured by ELISA (192.7 ± 21.0 pg/ml vs. 130.8 ± 9.8 pg/ml; P < 0.05; Supplemental Fig. 2A,B) and corresponded to an increase in IL-10 secretion from miRZIP106a-transfected CD4+ cells (10.5 ± 0.4% vs. 6.5 ± 0.1%; P < 0.001; Supplemental Fig. 2C) measured by intracellular cytokine staining. While converted Tregs were less suppressive than their freshly isolated counterparts (not shown), miRZIP106a transduced Tregs demonstrated significantly improved Treg suppressive function relative to Tregs transduced with the control vector (Supplemental Fig. 2E) at 1:1 Treg:Teffector ratio (24.8 ± 2.4% vs. 36.1 ± 3.8%; P < 0.05) and 1:4 Treg ratio (52.9 ± 0.6% vs. 55.3 ± 0.7%; P < 0.05). Taken together, miR-106a induction appears to be at least one mechanism by which TNFα reduces IL-10 production and impairs Treg suppressive function.

miR-106a deficiency suppresses T cell colitogenic potential

Employing the same miR-106a−/− strain, we demonstrated attenuated adoptive transfer colitis when naive CD4+CD45RBlow T cells from miR-106aΔ− mice were transferred to a lymphopenic RAG1−/− host relative to WT controls (Fig. 6a) as evidenced by a decrease in weight loss (Fig. 6b) and a reduction in the frequency of IFNγ+ (Fig. 6c) and IL-17+ CD4 T cell (Fig. 6d) infiltration into the colonic lamina propria. Interestingly, there was a small reduction in IL-10 producing T cells within the lamina propria also though this may reflect a decreased inflammatory state (Fig. 6e). Taken together, these data suggest that miR-106aΔ−/− CD4+ T cells have reduced colitogenic potential compared with WT.

miR-106a deficiency attenuates TNFα-driven intestinal inflammation in vivo

To validate our colitis findings in a second preclinical IBD model, we crossed miR-106aΔ−/− mice to the TNFΔARE/Δ−/− chronic murine ileitis model. We demonstrate a significant attenuation of histological indices of ileitis (Fig. 7a, b) including active inflammatory index (indicative of granulocytic infiltration), chronic inflammatory index (indicative of lymphocytic infiltration), villus distortion (inflammation-associated villus blunting), and total inflammatory indices (P < 0.05; n = 9–14 mice/genotype) in 8–12-week-old miR-106aΔ−/TNFΔARE/Δ−/− mice relative to TNFΔARE/Δ−/− controls. Consistent with colitis experiments, the decreased ileitis in miR-106aΔ−/−TNFΔARE/Δ−/− mice coincided with a reduction in the frequency of IFNγ+ (Fig. 7c) cells and IL-17+ T cell (Fig. 7d) infiltration into the ileal lamina propria. Once again, we observed a reduction in IL-10-producing T cells within the lamina propria likely reflecting a decreased inflammatory state (Fig. 7e). This effect is unlikely to be the result of impaired gut homing of miR-106aΔ−/− CD4+ T cells to the inflamed intestine as competitive homing assays indicated no significant difference in homing between WT and miR-106aΔ−/− cells (Supplemental Fig. 3). Taken together, these data suggest that miR-106a deficiency decreases susceptibility to spontaneous ileitis.

DISCUSSION

We have previously reported an increase in Treg frequency in the ileal lamina propria of chronically inflamed 20-week-old TNFΔARE/Δ−/− mice in the face of exacerbated inflammation.17 We now extend this finding to demonstrate that a similar induction is observed during the early stage of peak disease onset in this model at 8 weeks-of-age, which is consistent with data from patients with Crohn’s disease who display a similar increase in Tregs within the inflamed ileum.9 This was countereuitive, however, given an increase in an anti-inflammatory population would be expected to attenuate inflammation rather than facilitate it. We postulate the reason for this apparent paradox comes from understanding the impact of inflammation on Treg suppressive function. Under inflammatory conditions, pro-inflammatory cytokines acting via multiple mechanisms, including through TNFα receptors, can induce a transient resistance to suppression in effector T cells allowing rapid expansion of the effector T cell population to deal with the immediate insult before undergoing activation-induced apoptosis. Thus, it is not surprising that TNFα would be able to further amplify this effect by blunting Treg suppressive function. Consistent with that hypothesis, data from human studies of Treg function in IBD patients using anti-TNFα therapy demonstrate a concomitant improvement in Treg function.10,25,26

In our experiments, the increase in Treg frequency coincided with a reduction in IL-10 output in vivo and reduced in vitro suppressive function of Tregs from TNFΔARE/Δ−/− mice. Similar outcomes were seen if the proliferating cells were from inflamed TNFΔARE/Δ−/− or wildtype (not shown) suggesting a predominately defect in the TNFΔARE/Δ−/− Tregs. The reduction in IL-10 output could be directly linked to TNFα, as treatment with an anti-TNFα antibody reversed the effect. Interestingly, the decrease in IL-10 output was not mediated by downregulation of IL-10 mRNA as demonstrated using IL-10 IRES-GFP reporter mice alongside real-time PCR. IL-10 production in vivo is highly regulated with excess IL-10 increasing susceptibility to infection27 and inadequate IL-10 signaling exacerbating IBD.8 As a result, a large number of regulatory mechanisms including AU-rich elements which control mRNA stability, stress-response factors, 28 miRNA, suppressor of cytokine signaling 3 (SOCS3), nucleotide-binding oligomerization domain-containing protein two expression (NOD2) dysfunction of which is positively associated with IBD.
Fig. 5  MiR-106a deficiency promotes anti-inflammatory T cell induction. Under Th0 conditions, isolated CD4^+CD25 Neg cells from miR-106a^-/- mice displayed a significant decrease in expression of (a) IFNγ and (b) IL-17 as well as an increase in (c) FoxP3 relative to WT controls. In the presence of TGFβ, miR-106a^-/- CD4^+CD25 Neg cells underwent significantly greater Treg conversion compared to WT (c) with greater IL-10 production under both conditions (d). The fold change increase in IL-10 production by miR-106a^-/- cells under Th0 conditions was greater in FoxP3^+ but also occurred in FoxP3 Neg cells. f Isolated Tregs from miR-106a^-/-TNF^ΔARE/- mice displayed significantly greater in vitro suppressive function relative to TNF^ΔARE/- alone. g Enhanced suppressive function was maintained with antibody blockade of IL-10R signaling. Results represent mean ± SEM for four technical replicates from three independent studies. *P < 0.05, **P < 0.01, ***P < 0.001
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Fig. 6  MiR-106a deficiency attenuates colitogenic T cell potential in vivo.  

a Histology of the colonic lamina propria demonstrates significant villus distortion in WT CD45RB<sup>high</sup> T cell recipients relative to miR-106a<sup>-/-</sup> T cell recipients following adoptive transfer.  

b Adoptive transfer of CD4<sup>+</sup>CD45RB<sup>high</sup> T cells from miR-106a<sup>-/-</sup> mice resulted in significantly attenuated weight loss, a surrogate marker of intestinal inflammation, relative to WT controls.  This result coincided with a significant reduction in IFN<sub>γ</sub><sup>+</sup> CD4<sup>+</sup> T cells (c) and IL-17<sup>+</sup> CD4<sup>+</sup> T cells (d) in the colonic lamina propria as well as a reduction in IL-10-producing CD4<sup>+</sup> T cells (e). Results represent mean ± SEM for 4–7 mice per group from two or more independent studies.  *P < 0.05, **P < 0.01, ***P < 0.001

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susceptibility and IL-10 itself control IL-10 proteins levels
(reviewed in ref. 30).

In the absence of transcriptional regulation of IL-10 output, we chose to assess the potential for miRNA to provide the necessary modification of Treg function based on the established alterations in miRNA associated with IBD. Our search criteria for candidate miRNA were confined to miRNA induced by TNFα, that target and suppress IL-10 translation and are induced both in human IBD and in our murine model. Based on these restrictions, one particularly suitable candidate was identified, miR-106a. MiR-106a deficiency attenuates inflammation in murine IBD models.

Fig. 7 MiR-106a-/- mice display attenuated ileitis in vivo. Blinded histological evaluation of intestinal inflammation in the TNFΔARE/+ ileum demonstrated a significant decrease in acute and chronic inflammatory indices in addition to a reduction in villus distortion in miR-106a-/-TNFΔARE/+ relative to TNFΔARE/+ (a, b). Flow cytometry identified a concomitant reduction in c IFNγ, d IL-17, and e IL-10 producing CD4+ T the ileal lamina propria of 8–12-week-old mice. Results represent mean ± SEM for 4–7 mice per group from two or more independent studies. *P < 0.05, **P < 0.01, ***P < 0.001

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upregulated in both TNF-ARE/-/+ Tregs as well as IBD tissues and known to negatively affect IL-10 production. Excitingly, miR-106a also represents one of the few miRNAs that are actually upregulated upon T cell activation, while the majority of miRNA are downregulated.

Previous promoter studies have focused on a region immediately upstream of miR-106a, however, because of how single-stranded miRNA are processed, promoters of miRNA are significantly further away (as much as 90 kb) from the miRNA hairpin region. We validated the predicted miR-106a promoter region and demonstrated it was not only induced by TNFα in an NFκB-dependent manner as previously reported but that it was also inversely affected by TGFβ, consistent with a physiological role for miR-106a in Treg development. In addition, we demonstrated in vitro that miR-106a deficiency suppressed IFNγ Th1 and IL-17+ Th17 induction, while promoting FoxP3+ Treg induction, further supporting a role for TGFβ-mediated miR-106a suppression in Treg development. This increased Treg conversion coincided with increasing IL-10 production in both Tregs and non-Tregs. However, whether or not increased IL-10 output is responsible for improved Treg induction remains unclear. MiR-106a deficiency also rescued Treg suppressive functions in isolated Tregs from miR-106a-/-TNF-ARE/-/+ mice relative to TNF-ARE/-/+ mice. However, antibody-mediated IL-10 receptor blockade failed to reverse this improvement. Similar to the conversion studies, these results suggest that additional mechanisms targeted by miR-106a remain unidentified. In addition, we know that impaired IL-10 production is not the only dysfunction of Tregs impacting disease pathogenesis. Increased apoptosis and IL-17 production by Tregs are also associated with IBD. Thus, while data from these studies support using miRNA-modification to enhance Treg function as a valid therapeutic approach for the treatment of IBD, this approach may be more successful if combined with additional miRNA.

Based on our in vitro data, we hypothesized that targeting the miR-106a cluster would attenuate intestinal inflammation in vivo. Initially, we examined the impact of miR-106a deletion on isolated CD4+ T cell colitogenic potential. Using the well-established CD45RBB+ colitis model, we demonstrated for the first time that deletion of the miR-106a miRNA family in CD4+ T cells resulted in attenuation of inflammation in lymphopenic recipients. This was characterized by a decrease in Th1 and Th17 cells known to perpetuate inflammation, along with a reduction in IL-10 producing T cells, which are induced in response to inflammation to serve as a negative feedback mechanism. While the small but significant decrease in the percent of CD4+ IL-10+ T cells in the colonic lamina propria of mice receiving miR-106a-/-/+ T cells appears contrary to our hypothesis and data showing that miR-106a-deficiency promotes IL-10 production, we also recognize that IL-10 can be produced by Th1 and Th17 cells and this decrease more than likely reflects the marked decrease in the frequency of those cells. We further validated these studies with similar findings in a second preclinical mouse model of IBD, the TNF-ARE/-/+ model of Crohn’s disease. As with adoptive transfer colitis, this model is heavily CD4+ T cell dependent. Once again, miR-106a deficiency was associated with attenuation of intestinal inflammation. Based on recent evidence for a role for miR-106a-5P in renal pathogenesis, increased apoptosis and IL-17 production by Tregs are associated with IBD.

Flow cytometry and intracellular cytokine staining

For detection of intracellular cytokine producing cells, cells were incubated with phorbol-12-myristate-13-acetate (PMA, 50 μg/ml; #P8139, Sigma-Aldrich, St. Louis, MO), calcium ionomycin (1 mg/ml; #I0634, Sigma-Aldrich) and brefeldin-A (10 μg/ml; #B7651, Sigma-Aldrich) for 4 h at 37 °C. Cells were stained with antibodies against CD4 (RM4-5), CD25 (PC61.5) and IL-17 production by Tregs are also associated with IBD.

Mice

The B6.1295-Tnfm2gr/Jarn strain (TNF-ARE/-/+; Mgi:3720980) was generated by continuous backcrosses between heterozygous TNF-ARE/-/+ on a mixed background to C57BL/6/J mice and kept under specific pathogen-free conditions. Experimental animals were heterozygous for the ARE mutation (TNF-ARE/-/) or homozygous wildtype (WT), which served as controls. MiR-106a-363Δ mice on the C57BL/6/J background were obtained from Jackson Laboratories (#008461, Bar Harbor, ME). MiR-106a-363Δ mice were crossed with B6.129P-Tnfm2gr/Tnfα+/-(TNF-ARE/-+) mice to produce miR-106a-363Δ/TNF-ARE/-/+ mice. WT/FoxP3-green fluorescent protein (GFP) and TNF-ARE/-/+FoxP3-GFP reporter strains were kindly provided by Dr. de Zoeten. IL-10 internal ribosomal entry site (IRES)-GFP reporter mice were provided by Dr. Eric Clambe (Southern University of Science and Technology, Vancouver, CA) flow sorted into live CD4+ CD45RB+CD25+ CD45RBlow CD45RAlow naive T cells (BD FACSAria II, BD Biosciences, Franklin Lakes, NJ). Fetal samples were negative for Helicobacter, protozoa and helminths. All animals were handled according to procedures approved by the institutional committee for animal use.

Lymphocyte isolation

Cells from indicated organs were isolated as previously described. Briefly, spleens and mesenteric lymph node (MLN) cells were pressed through a 70 μm filter to release leukocytes. Intestinal tissues were rinsed with phosphate-buffered saline and agitated in 1 mM EDTA solution to disperse intestinal epithelia, followed by collagenase digestion of the remaining lamina propria cells to facilitate release of intestinal leukocytes.

RNA isolation, cDNA synthesis, and real-time PCR

Ligamentous tissues were snap frozen in liquid nitrogen prior to homogenization and RNA isolation using the RNeasy isolation kit (Qiagen, Valencia, CA). Freshly isolated CD4+ CD25+ from WT and TNF-ARE/-/+ mice were purified by magnetic selection prior to RNA isolation. Synthesis of cDNA was performed using Superscript III cDNA synthesis kit (Invitrogen). Expression analysis was performed using Taqman (ThermoFisher, Waltham, MA) assays for FoxP3 (Mm00475162_m1), CD4 (Mm00442754_m1), and IL-10 (Mm00439614_m1) multiplexed with the endogenous 18S control (Eukaryotic 18S rRNA).

Jurkat T cells were harvested in RLT buffer and RNA extracted using RNeasy columns (Qiagen) prior to cDNA synthesis using miScript II RT Kit (Qiagen). MiRNA quantification for miR-106a, RN65-2, and SNORD68 were performed using miScript Primer
Assays (Qiagen) with analysis performed on the AB7300 real-time PCR system (Applied Biosystems, Foster City, CA). For multiplex miRNA analysis, isolated Tregs from WT/FoxP3-GFP and TNFΔARE/-/FoxP3-GFP reporter mice were obtained by pre-enrichment using an EasySep CD4 negative selection kit (STEMCELL) followed by flow sorting for CD4+ FoxP3-GFP+ cells.

T cell suppression assay
Treg-mediated suppression was measured as previously described. Briefly, CD4+CD25+ Tregs were isolated by negative selection of CD4+ T cells, followed by positive selection of CD25+ cells using the EasySep Treg isolation kit (STEMCELL). Treg frequency was previously confirmed by flow cytometry with ~85% of enriched CD4+CD25+ expressing FoxP3. CD4+CD25− effector T cells were labeled with CellTrace Violet (Invitrogen) according to manufacturer's instructions prior to stimulation with anti-CD3 mAb (1 μg/ml; 145-2C11, BioLegend) in the presence of irradiated syngeneic APC to allow for fluorescent monitoring of cell proliferation. Proliferating cells were co-cultured with varying ratios of converted CD4+CD25+ Treg from WT or TNFΔARE/-/ which had been transduced with either miRZIP000 or miRZIP106a to determine the impact of selective inhibition of miR-106a on Treg suppressive function. Anti-IL-10R (10 μg/ml; 1B1.3a, BioLegend) or recombinant IL-10 (10 ng/ml; Peprotech, Rocky Hill, NJ) were added to culture conditions as indicated.

Cytokine production assays
Cytokine detection was performed on isolated cells by stimulating cells for 24 h with anti-CD3/CD28 antibodies (1 μg/ml; 37.51, BioLegend) and snap freezing cell supernatants for subsequent analysis. Anti-inflammatory cytokine IL-10 concentration was measured using the Mouse IL-10 ELISA Ready-SET-Go! Kit (eBioscience, San Diego, CA).

T cell conversion assays
Magnetically separated CD4+CD25−Tregs were cultured for 72 h in 96-well round-bottom plates stimulated with plate-bound anti-CD3 (1 μg/ml), soluble anti-CD28 (1 μg/ml), recombinant murine IL-2 (10 ng/ml; Peprotech) and without (Th0 conditions) or with (Th1 conditions) recombinant human TGFβ (10 ng/ml; R&D systems, Minneapolis, MN). Cells were then restimulated for 4 h with PMA/ionomycin/brefeldin-A prior to fixation and permeabilization using the FoxP3 staining kit (eBioscience) according to manufacturer’s instructions and staining for IFNγ, IL-17, IL-10, and FoxP3.

Luciferase assays
Early passage Jurkat cells were plated at 0.5 × 10^6 cells/well in a 6-well format and transfected using Lipofectamine LTX (Thermo-Fisher) with 2.5 μg of DNA/well of mir-106a promoter luciferase/reporter construct along with 50 ng of TK Renilla control reporter vector. 24 h post transfection cells were replated to 2.5 × 10^5 cells/well in a 96-well plate and treated with increasing concentrations of recombinant human TNFα or TGFβ for 6 h. Cells were then centrifuged, supernatants removed, and cells lysed in 25 μl of passive lysis buffer (Promega, Madison, WI) prior to measuring luciferase activity on a GloMax®-Multi Microplate Multimode Reader (Promega). The full and truncated mir-106a promoter constructs incorporating the human chromosome X:133137287 to 133138287 region were synthesized by GenScript (Piscataway, NJ) based on predicted sequences described previously.

Human CD4+ T cell assays
Peripheral blood mononuclear cells were obtained from healthy donors using CPT tubes (BD Biosciences) according to manufacturer’s instructions. Frozen PBMC were rapidly thawed and rested for 2 h in serum-free X-VIVO media (Lonza, Allendale, NJ) at 37 °C prior to treatment with recombinant human TNFα (10 ng/ml, eBioscience) for 6 h. Unfractionated RNA was isolated using mirVana miRNA isolation kit (Invitrogen), followed by target-specific cDNA synthesis using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) and expression analysis performed using TaqMan miRNA probe sets relative to the endogenous control RPL21.

Lentiviral transduction
Lentiviral antagonimors to inhibit miR-106a (miRZIP106a) or scrambled control vector (miRZIP000) were generated according to manufacturer’s instructions (System Biosciences, Palo Alto, CA). Briefly, viral particles were generated in HEK293T cells transfected with miRZIP vectors and pPackH1 packaging mixing containing pVSV-G, pPackH1-GAG, and pPACK-H1-REV plasmids. Viral supernatant was concentrated using PEG-it virus precipitation solution. High titer virus was then incubated with isolated CD4+CD25−Tregs, spinoculated for 2 h at 300×g on day 0 and day 1 with polybrene (4 mg/ml) and cultured for 72 h in the presence of TGFβ (10 ng/ml), IL-2 (5 units/ml), soluble anti-CD28 antibody (1 μg/ml), and plate-bound anti-CD3 antibody (1 μg/ml) to promote Treg induction based on a previously published method. Suppressive function of transfected cells was assayed as previously described.

Competitive homing assays
Magnetically separated CD4+ splenocytes were isolated from WT and mir-106a−/− donors, and labeled with either CellTrace or Calcein Red-orange (Invitrogen) according to manufacturer’s instructions. Cells were then recounted and mixed in a 1:1 ratio prior to retro-orbital injection of 5 × 10^6 cells/mouse. Mice were killed after 18 h and expression of labeled cells identified by flow cytometry. Dye-swap experiments were performed to exclude an impact of either dye on gut homing. Results were expressed relative to injection fraction.

Statistics
Statistical analyzes were performed using the Student’s t test or 2-way ANOVA with Prism Data Analysis software (GraphPad Software, La Jolla, CA). Data were expressed as mean ± standard error of the mean (SEM). Statistical significance was set at a P < 0.05.

Study approval
Experimental animal protocols were carried out in accordance with the AAALAC-accredited Institutional Animal Care and Use Committee (IACUC) at the University of Colorado School of Medicine and in compliance with recommendations in the Guide for the Care and Use of Laboratory Animals, Animal Welfare Act and PHS Policy. De-identified healthy human patient samples were collected and used in accordance with Colorado Multiple Institutional Review Board approval COMIRB #00-510.

AUTHOR CONTRIBUTIONS
All authors provided important input to experimental design, critical review, and final approval of the manuscript. R.H.H., M.R.S., and C.B.C. co-wrote the manuscript and corrections, and performed flow and animal experiments. A.A.J. and M.R.S. performed suppression assays. M.R.S. and C.B.C. performed conversion assays. M.E.L. and C.B.C. performed PCR analysis and luciferase assays. C.M.A. assisted with luciferase assays, proofing the manuscript, and experimental design. P.J. provided blinded histological evaluation of intestinal inflammation. E.d.Z. provided guidance with experimental design, data interpretation, and manuscript editing.

ADDITIONAL INFORMATION
The online version of this article (https://doi.org/10.1038/s41385-018-0091-7) contains supplementary material, which is available to authorized users.

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