Th17 cell-derived amphiregulin promotes colitis-associated intestinal fibrosis through activation of mTOR and MEK in intestinal myo-/fibroblasts

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Supplementary Methods

$CD4^+$ T cells isolation and culture

Splenic $CD4^+$ T cells were isolated using anti-mouse CD4 magnetic particles (BD Biosciences). Splenic $CD4^+$ T cells from CBir1 Tg mice were activated with irradiated antigen presenting cells (APCs) and 1μg/ml CBir1 peptide (ThermoFisher Scientific). Splenic $CD4^+$ T cells from WT, *Areg*$^{-/-}$ and *Stat3*$^{-/-}$ mice were activated with 5 μg/ml αCD3 mAb (Bio X Cell) and 2μg/ml αCD28 mAb (Bio X Cell). $CD4^+$ T cells were cultured under neutral, Th1 (10 ng/ml IL-12), Th2 (40 ng/ml IL-4) and Th17 (2 ng/ml TGFβ, 50 ng/ml IL-6, 10 μg/ml αIFNγ, and 5 μg/ml αIL-4) polarization conditions.

T cell transfer model

$CD4^+$T cells were isolated from CBir1 TCR transgenic (CBir1 Tg) mice and cultured under Th1 and Th17 conditions for 5 days. CBir1 Th1 cells or Th17 cells (1×10^6 cells/mouse) were intravenously transferred to *Tcrβxδ*$^{-/-}$ mice. The mice were sacrificed six weeks after cell transfer.

$CD4^+$T cells were isolated from WT and *Areg*$^{-/-}$ mice and cultured under Th17 conditions for 5 days and then transferred into *Tcrβxδ*$^{-/-}$ mice (1×10^6 cells/mouse). The mice were sacrificed four weeks later.

$CD4^+$T cells were isolated from WT mice and cultured under Th1 and Th17 conditions. And then WT Th1 cells or WT Th17 cells (1×10^6 cells/mouse) were transferred into *Tcrβxδ*$^{-/-}$ mice. Mice were sacrificed six weeks post-cell transfer.

$CD4^+$T cells were isolated from CBir1 Tg mice and cultured under Th17 conditions for 5 days. CBir1 Th17 cells (1×10^6 cells/mouse) were transferred into *Tcrβxδ*$^{-/-}$ mice. Mice were injected

with or without Stat3 inhibitor (10 mg/kg, HJC0152) every other day. Mice were sacrificed four weeks post-cell transfer.

DSS-induced chronic colitis model

WT mice and Areg$^{-/-}$ mice were administrated with 3 cycles of DSS insults. In each cycle, mice were given 2.0% DSS (w/v) in drinking water for 7 days and control drinking water for 7 days.

Human peripheral blood CD4$^+$ T cell isolation

Peripheral blood CD4$^+$ T cells were isolated from healthy volunteers and CD patients with or without fibrosis using anti-human CD4 magnetic particles (BD Biosciences). EDTA anticoagulated peripheral blood samples were obtained from healthy controls or CD patients with fibrosis, and CD4$^+$T cells were then isolated using anti-human CD4 magnetic particles (BD Biosciences). Peripheral blood CD4$^+$ T cells were activated with 2 μg/ml anti-human CD3 mAb (Invitrogen) and 2μg/ml anti-human CD28 mAb (Invitrogen). CD4$^+$ T cells were cultured under Th1 (1 ng/mL IL-2, 20 ng/ml IL-12, 5 μg/mL anti-IL-4), and Th17 (25 ng/ml IL-6, 2.5 ng/ml TGF-β, 10 μg/ml anti-human IL-4, 10 μg/mL anti-human IFN-γ) polarization conditions.

Quantitative real-time PCR

RNA was extracted from cultured cells with TRIzol Reagent (Molecular Research Center) and followed by cDNA synthesis with qScript cDNA Supermix (Quanta BioSciences). qRT-PCR was performed with SYBR Green gene expression assays. Predesigned primers were ordered from

Integrated DNA Technologies and normalized against actin mRNA expression. All the primers used in this study were listed in Supplementary Table 2.

Enzyme-linked immunosorbent assay (ELISA)

Cytokine productions were measured using ELISA kits according to the manufacturer's instruction. For *ex vivo* study, two pieces of colonic punch biopsies were obtained from colitis mice and cultured in a complete RPMI 1640 medium for 24 hours. Supernatants were collected for analysis of cytokine secretion (TNF-α and IL-6). In vitro study, splenic $CD4^+$T cells of CBir1 Tg mice were cultured with irradiated APCs and CBir1 peptide under neutral, Th1, Th2, and Th17 polarization conditions for 5 days. Supernatants were collected for analysis of Areg. 96-well ELISA plates were coated with indicated capture antibodies overnight at 4 °C. The plates were blocked with 1% BSA after washing. Supernatants were further added to the 96-well ELISA plates and incubated for 2 hours at room temperature. Then, detection antibodies were added and incubated for another 1 hour at room temperature. HRP-labeled streptavidin was added and further incubated in the plates for 1 hour. After adding the substrate, the absorbance of each well was measured at 450 nm by BioTek Gene5.

T cell proliferation assay

$CD4^+$ T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, 5mM) in 96-well plates. 5×10^4 $CD4^+$ T cells per well were cultured with 2×10^5 irradiated splenic APCs in the presence or absence of a series of Areg (0, 20, 50, and 100ng/ml). On day 3, cells were harvested and analyzed by flow cytometry.

Colitis and fibrosis histopathology

At necropsy, tissues were fixed in 10% neutral buffered formalin for 24 hours, and then embedded in paraffin. Paraffin-embedded colon was cut into 5 μm sections. The sections were stained with Hematoxylin/ Eosin (H&E) (ab245880, Abcam) and Sirius Red (IW3012, IHCWORLD). HE and Sirius red sections were imaged by Cytation 5 (BioTek). Histological changes were blindly assessed from Swiss-rolled colons. Histological score for fibrosis were assessed by Sirius red staining and collagen layer thickness were measured.

Immunofluorescence staining and analysis

Mouse colonic immunofluorescence staining was performed. After deparaffinization, antigen retrieval, and blocking with 10% goat serum in PBST, the slides were incubated with primary antibodies (Collagen Ⅰ and Alpha-Smooth Muscle Actin (αSMA)) overnight at 4℃. After washing, the slides were incubated with the secondary antibody in 10% goat serum for 2 h at room temperature in the dark. Nuclei were stained with DAPI. Imaging was recorded with Biotek Cytation 5. The thickness of αSMA layers was measured with ImageJ. The percentages of red staining for type I collagen in the whole area of each image were calculated with ImageJ. Multiple areas were selected in the same section, and the average value was calculated for each group.

Immunofluorescence staining of human intestinal tissue was performed. After deparaffinization, antigen retrieval, and blocking with 10% goat serum in PBST, the slides were incubated with primary antibodies (CD4 and Areg) overnight at 4℃. After washing, the slides were incubated with the secondary antibody in 10% goat serum for 2 h at room temperature in the dark. Nuclei were stained with DAPI. Imaging was recorded with Axio Examiner Z1 LSM 5100 confocal microscope.

Ki67 Staining

Human intestinal MF monolayers were first fixed with pre-chilled methanol at -20℃ for 10 min and were then permeabilized for 15 min at room temperature using 0.2% Triton X-100 in PBS. After blocking with 10% goat serum for 1h at room temperature, MFs were incubated at 4°C overnight with primary antibodies in blocking buffer (Ki67, 1:300, Abcam, Cat# ab15580). The cells were then washed three times with PBST before incubation with secondary antibody (1:1000) and Hoechst (1:1000) in blocking buffer at room temperature for 1 h. Cells were washed three times in PBST. Whole well images were acquired and analyzed with a Biotek Cytation 5.

Western blot

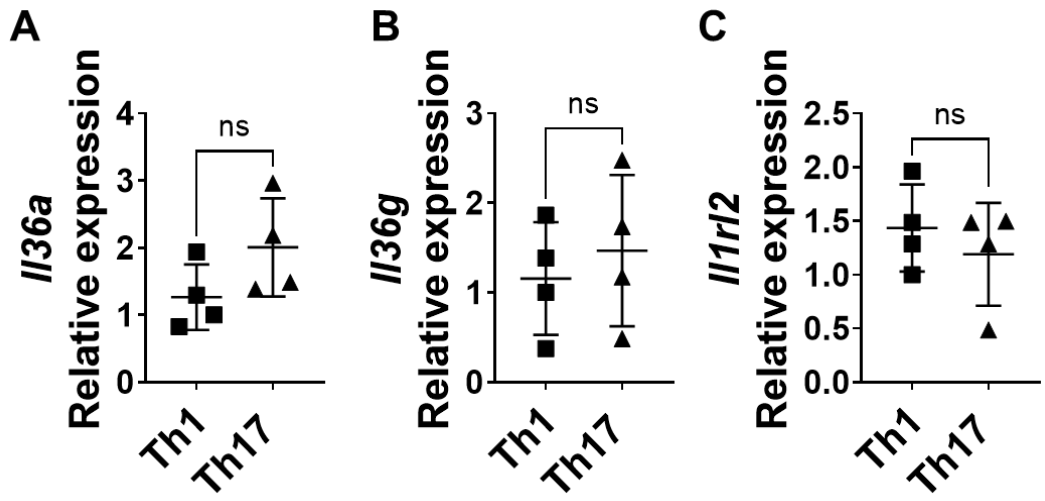
Total protein was extracted from the cells using radioimmunoprecipitation assay buffer (RIPA buffer) with protease inhibitor cocktail, phosphatase inhibitors, and phenylmethanesulphonyl (PMSF). Protein concentration was measured by BCA Protein Assay kit. Protein was loaded and separated by mini-protein TGX Stain-Free Gels. The blots were blocked with 5% skim milk in TBST for 1 hour at room temperature after electrotransfer and incubated with primary antibody overnight at 4℃ (phospho-Stat3, 1:2000, CST; total Stat3, 1:1000, CST; phospho-mTOR, 1:1000, CST; total mTOR, 1:1000, CST; phospho-MEK1/2 1:1000, CST; total MEK1/2, 1:1000, CST), followed by incubation with the corresponding secondary antibody. The blots were detected using the ChemiDoc Imaging System.

Flow cytometry

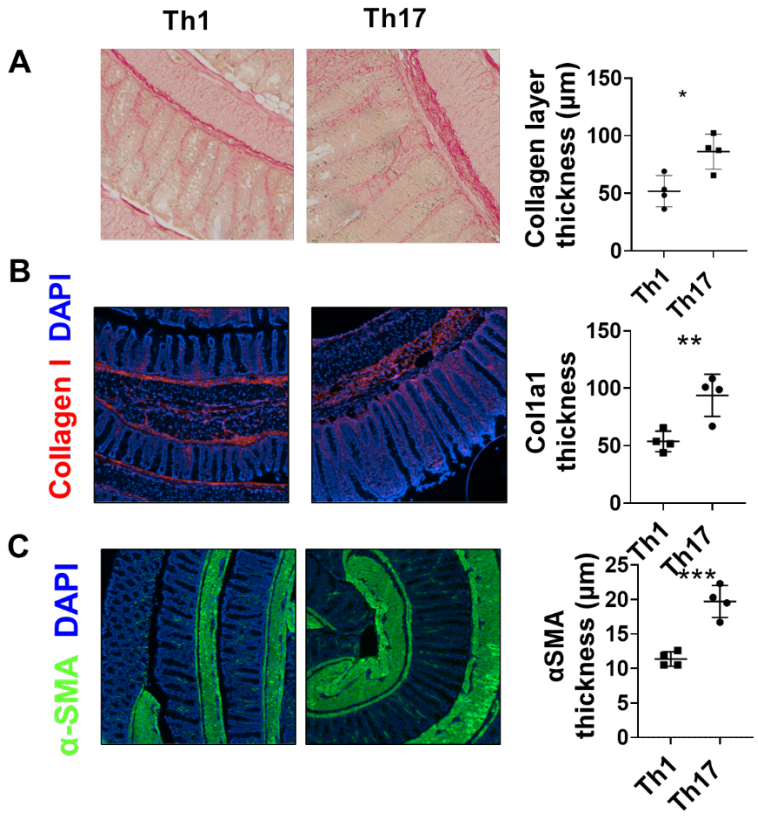
T cells were stimulated with phorbol-12-myristate 13-acetate (50 ng/ml, Sigma Aldrich) and ionomycin (750 ng/ml, Invitrogen) for 2 h, followed by 0.75 µl/ml Golgi Stop (BD GolgiStop) for an additional 3 h. And then, T cells were stained with LIVE/DEAD (Invitrogen). Anti-mouse CD4 (BioLegend) was used for surface staining. After washing, cells were fixed and permeabilized using the Fixation/Permeabilization set (Invitrogen). Intracellular staining was stained with anti-mouse IL-17 (Biolegend).

Scratch wound-healing assay

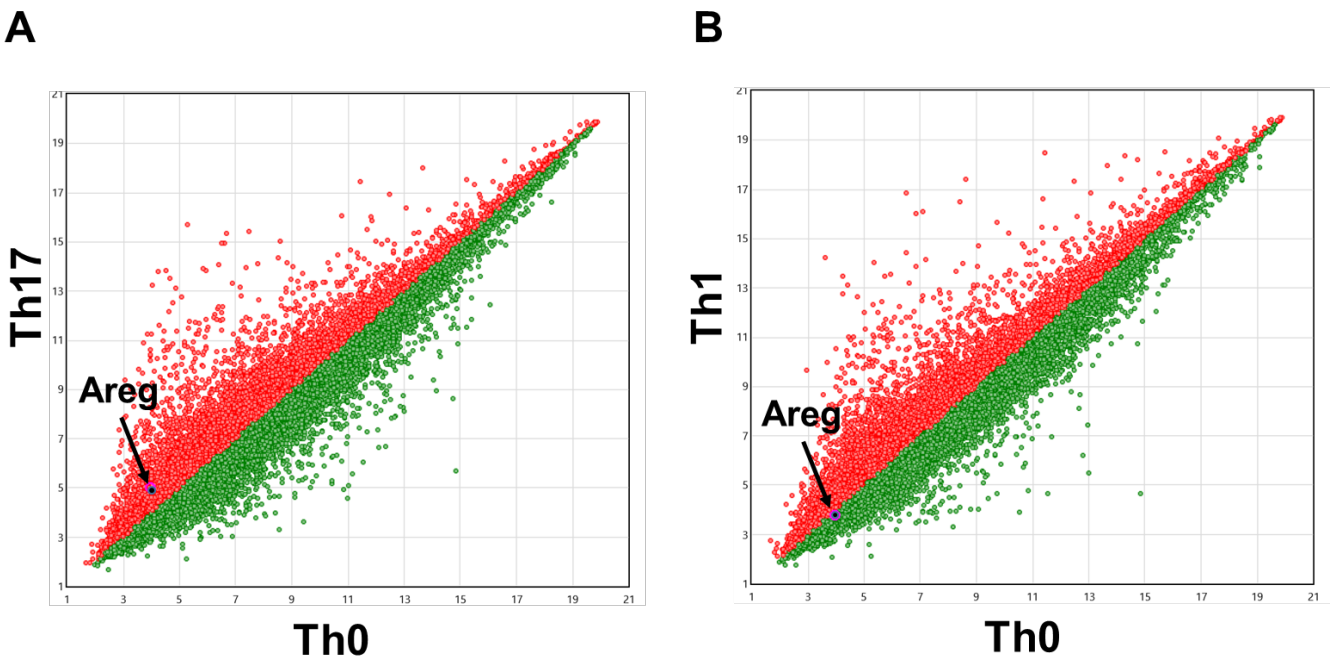
Primary human intestinal MFs were plated in a 24 or 96-well plate. Twenty-four hours later, cells were starved by placing them into serum starve media overnight. Human intestinal MF monolayers were wounded using a 200 µL pipette tip. Areg (100 ng/ml), mTOR inhibitor (1µM, Merck), and MEK inhibitor (1µM, Merck) were added to the plates. The plates were then placed onto the microscope stage located inside an Okolab cage incubator on a Nikon Eclipse TI and imaged at 4× magnification, 37°C, 5% CO_2. The migration areas were determined by an MRI Wound Healing Tool macro for FIJI software (National Institutes of Health, Bethesda, MD; http://dev.mri.cnrs.fr/attachments/download/1992/MRI_Wound_Healing_Tool.ijm). For the video, images were recorded every 2h over a 24h period.



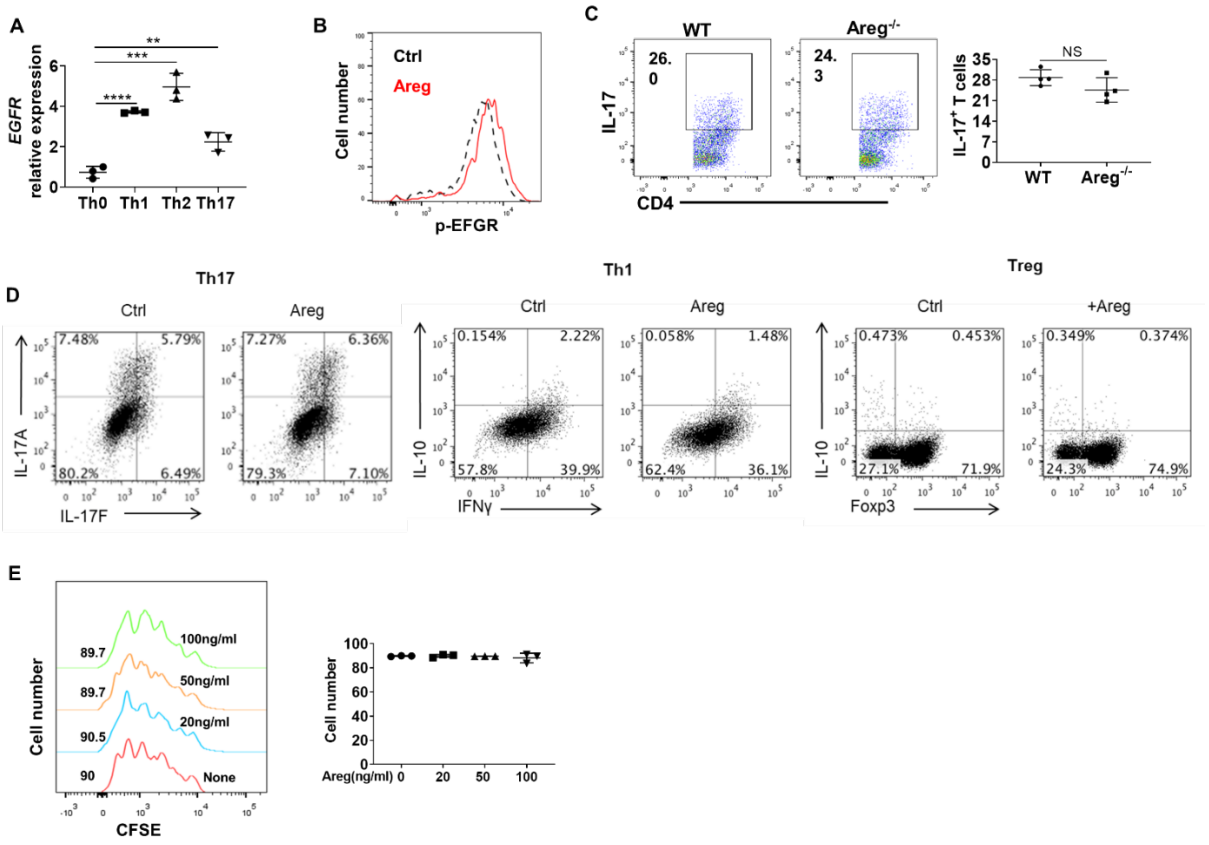
Supplementary Figure 1. Il-36-IL-36R pathway in mouse intestine in T cell-induced colitis model. $CD4^{+}$T cells were isolated from CBir1 TCR transgenic (CBir1 Tg) mice and cultured under Th1 and Th17 conditions, and then transferred into *Tcrβxδ*$^{-/-}$ mice (n=4/group). The mice were sacrificed 6 weeks after cell transfer. *Il36a, Il36g,* and *Il1rl2* levels in colonic tissues were measured by RT-PCR. Unpaired Student's t-test.



Supplementary Figure 2. WT Th17 cells induce more severe intestinal fibrosis. $CD4^{+}$T cells were isolated from WT mice and cultured under Th1 and Th17 conditions and then transferred into *Tcrβxδ*$^{-/-}$ mice (n=4/group). (A) Colon tissues were stained with Sirius Red, and collagen layer thickness was measured. (B-C) Colon tissues were stained with immunofluorescence. Collagen I thickness and αSMA layer thickness were analyzed. Unpaired Student's t-test. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$.

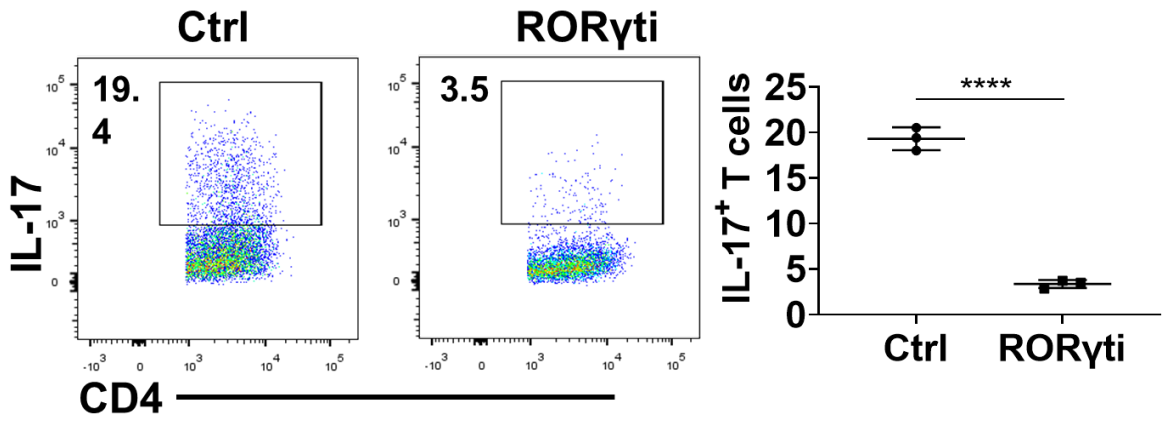


Supplementary Figure 3. Areg expression in T cells. (A-B) Spleen $CD4^+$ T cells were activated with α-CD3 and α-CD28 mAb under neutral (Th0), Th1, and Th17-polarization conditions for 24 h for analysis of gene differences by microarray analysis.

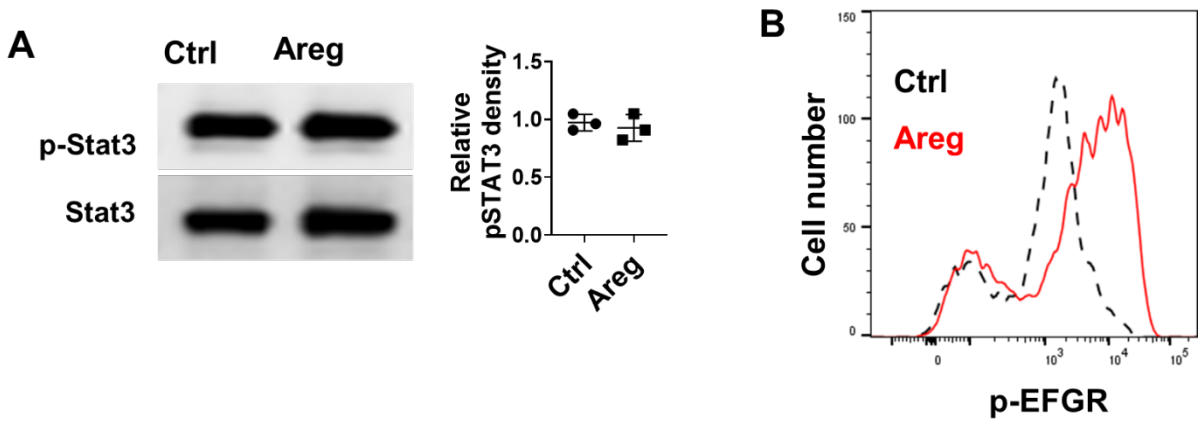


Supplementary Figure 4. Areg does not affect T cell differentiation and proliferation. (A) $CD4^+$ T cells were activated and cultured under various T cell polarization conditions for 5 days. Epidermal growth factor receptor (EGFR) expression was determined by qRT-PCR. (B) $CD4^+$ T cells were activated and cultured under various T cell polarization conditions for 5 days. Cells were treated with or without 100ng/ml Areg for 2h, and p-EFGR was determined by flow cytometry. (C) $CD4^+$ T cells were isolated from WT and *Areg*$^{-/-}$ mice and cultured under Th17 condition for 5 days. The percentage of Th17 cells was determined by FACS. (D) CBir1 Tg $CD4^+$ T cells were cultured with or without 100ng/ml Areg under Th17, Th1, and Treg polarization conditions for 5 days. T cell differentiation was determined by flow cytometry. (E) CBir1 Tg $CD4^+$ T cells were cultured for 3 days. After washing, the T cells were labeled with CFSE, and cultured in the presence or absence of 0, 20, 50, 100ng/ml Areg for another 3 days. Cell proliferation was

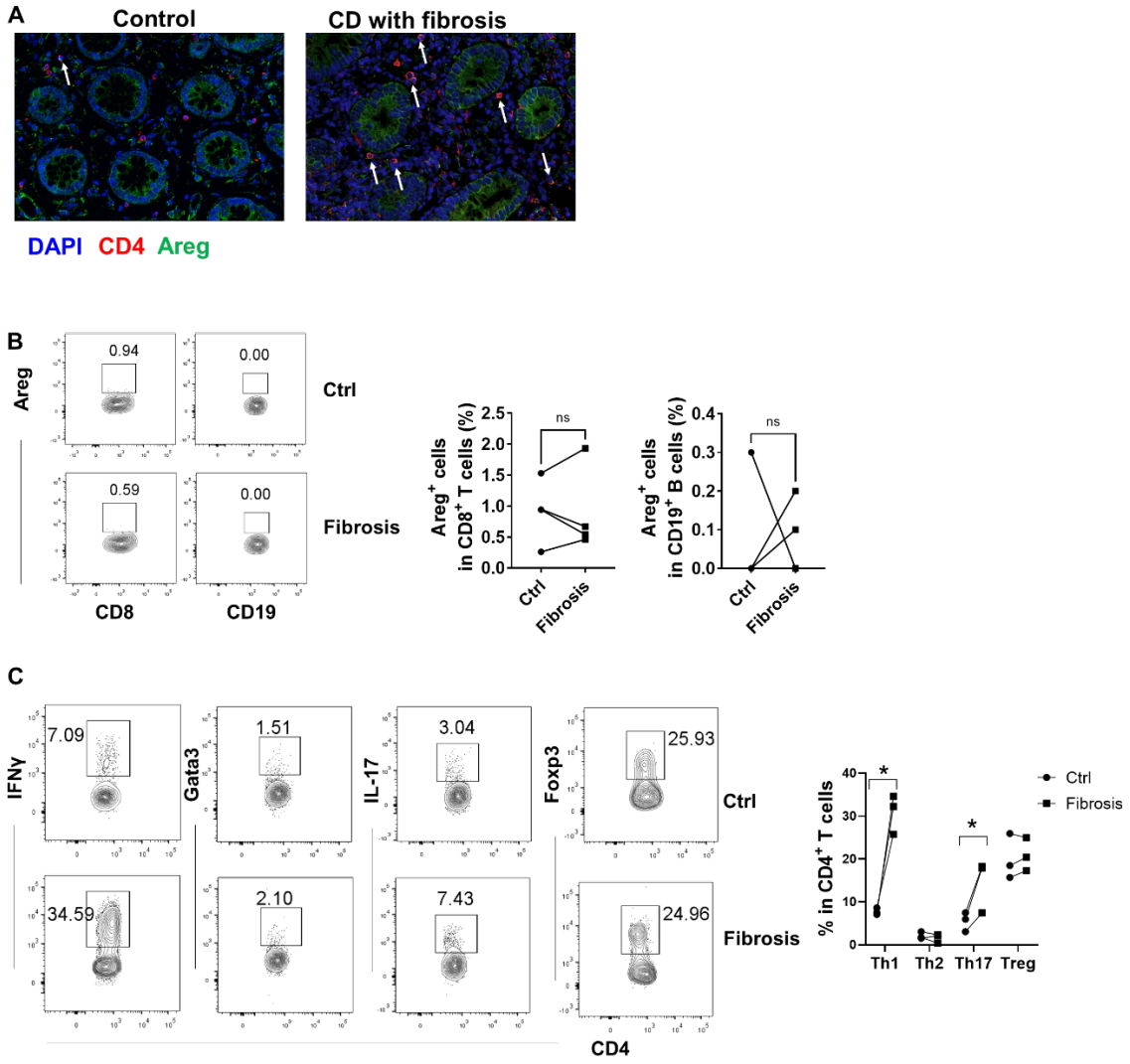
measured by CFSE dilution. Representative data from 2-3 independent experiments with similar results. (A and D) one-way ANOVA; (C) unpaired Student's t-test. $^{*}p < .05$; $^{***}p < 0.001$.



Supplementary Figure 5. The efficiency of RORγt inhibitor in inhibition of Th17 differentiation. (A) $CD4^+$ T cells were cultured under Th17-polarization conditions in the presence or absence of the RORγt inhibitor (GSK805). Areg expression was detected by RT-PCR. Representative data from 3 independent experiments with similar results.



Supplementary Figure 6. Stat3 and EFGR pathway in intestinal MFs treated with Areg. (A) Intestinal MFs were treated with Areg for 48h, and phosphorylation of Stat3 was determined by western blot. (B) Intestinal MFs were treated with or without 100ng/ml Areg for 2h, and p-EFGR was determined by flow cytometry.



Supplementary Figure 7. Intestinal lamina propria cell phenotypes in CD patients with fibrosis. (A) Intestinal mucosal biopsies, obtained from healthy controls and CD patients with fibrosis, were stained with anti-CD4 and anti-Areg antibodies. (B-C) Intestinal lamina propria lymphocytes were isolated from fibrotic and non-fibrotic sites of the same CD patients (n=5). (B) Areg$^+$ CD8$^+$ T cells and Areg$^+$ CD19$^+$ B cells were determined by flow cytometry. (C) IFNγ$^+$ Th1, Gata3$^+$ Th2, IL-17$^+$ Th17, and Foxp3$^+$ Treg cells were determined by flow cytometry. Paired Student's t-test. $^*p < .05$.

Supplementary table 1. The clinical characteristics of subjects.

A. Clinical characteristics of CD patients (Figure 7B)

	Biopsy samples		
	Con	**CD without fibrosis**	**CD with fibrosis**
Number of patients	10	8	10
Age(years)	42.4±13.3	38.4±14.6	35.2±11.5
Gender			
male	6	5	6
female	4	3	4
Disease duration (months)		42.8±71.6	33.1±54.9
Current therapy			
5-aminosalicylates		7	6
Immunosuppressants		1	1
Biologics		2	4
Nutritional therapy		0	6
Disease location (CD)			
L1		2	3
L2		0	0
L3		6	7
L4		0	0
CRP (mg/L)		20.3±30.2	10.3±11.5

*According to the Montreal classification system. +A/R: Active/Remission; Con, healthy controls; CD, Crohn's disease;

B. Clinical characteristics of CD patients (Figure 7C)

	Biopsy samples
	CD with fibrosis
Number of patients	11
Age(years)	33.6±11.6
Gender	
male	8
female	3
Disease duration (months)	58.5±45.2
Current therapy	
5-aminosalicylates	9
Immunosuppressants	1
Biologics	7
Nutritional therapy	1
Disease location (CD)	
L1	3
L2	1
L3	7
L4	5
CRP (mg/L)	15.9±20.2

*According to the Montreal classification system. +A/R: Active/Remission; CD, Crohn's disease;

C. Clinical characteristics of CD patients (Figure 7E)

	Blood samples	
	Con	**CD with fibrosis**
Number of patients	5	5
Age(years)	29.0±4.4	26.8±6.5
Gender		
male	3	4
female	2	1
Disease duration (months)		32.4±9.7
Current therapy		
5-aminosalicylates		3
Immunosuppressants		0
Biologics		4
Nutritional therapy		1
Disease location (CD)		
L1		0
L2		1
L3		4
L4		0
CRP (mg/L)		8.1±6.8

*According to the Montreal classification system. +A/R: Active/Remission; Con, healthy controls; CD, Crohn's disease;

Supplementary table 2. The primers for qRT-PCR.

Gene (mouse)	SYBR green primers
Areg	Forward 5' CCTCCTTCTTTCTTCTGTTTCTCC 3'
	Reverse 5' GTCACTATCTTTGTCTCTGCC 3'
Col1a1	Forward 5' GCTCCTCTTAGGGGCCACT
	Reverse 5' ATTGGGGACCCTTAGGCCAT
Col6a1	Forward 5' CTGCTGCTACAAGCCTGCT
	Reverse 5 GCACGAAGAATAGATCCACAGGG
Col6a3	Forward 5' GCTGCGGAATCACTTTGTGC
	Reverse 5'CACCTTGACACCTTTCTGGGT
Il36a	Forward 5' AGCAGCATCACCTTCGCTTAG
	Reverse 5' GTGTCCAGATATTGGCATGGG
Il36g	Forward 5' CAGGTGTGGATCTTTCGTAATCA
	Reverse 5' CATGGGAGGATAGTCACGCTG
Il1rl2	Forward 5' GCAGCAGATACGTGTGAGGAC
	Reverse 5' GCGGGTATGTGCAGTTGAAA
β-Actin	Forward 5' CCATGTCGTCCAGTTGGTAA 3'
	Reverse 5' GAATGGGTCAGAAGGACTCCT 3'

Gene (human)	SYBR green primers
Areg	Forward 5' GAGCCGACTATGACTACTCAGA 3'
	Reverse 5' TCACTTTCCGTCTTGTTTTGGG 3'
Col1a1	Forward 5' GAGGGCCAAGACGAAGACATC
	Reverse 5' CAGATCACGTCATCGCACAAC
β-Actin	Forward 5' GGACTTCGAGCAAGAGATGG 3'
	Reverse 5' AGCACTGTGTTGGCGTACAG 3'