Supplemental Experimental Procedures

Reagents and Antibodies

Unless otherwise stated all chemicals were purchased from Sigma Aldrich. The following antibodies were used in neutralization, flow cytometry and cell sorting: Galectin-9 (108A2, BioLegend), CD4 (H129.19, BD Pharmingen), CD25 (7D4, BD pharmingen), IL-17A (TC11-18H10.1, BD Pharmingen), CD45.2 (104, eBioscience), Nrp1 (R&D System), ICOS (7E.17G9, eBioscience), GITR (YGITR 765, BioLegend), CD69 (H1.2F3, BioLegend), IFN-γ (XMG1.2, BD Pharmingen), CD44 (IM7, BioLegend), CD62L (MEL-14, BioLegend), Foxp3 (FJK-16s, eBioscience), IL-10 (JES5-16E3, BioLegend), Tim-3 (8B.2C12, eBioscience). Recombinant human Galectin-9 was purchased from R&D.

In vitro T cell differentiation

Naïve ($CD44^{lo}CD62L^{+}CD25^{-}$) $CD4^{+}$ T cells were sorted from spleens and lymph nodes of indicated mice by flow cytometry. The purity of isolated T cell populations routinely exceeded 98%. Naïve T cells were stimulated with plate-bound anti-CD3 (145-2C11, 1μg/ml) and anti-CD28 (PV-1, 1μg/ml) and appropriate cytokines. All recombinant cytokines were purchased from Miltenyi Biotec.

Measurement of cytokines

Secreted cytokines were measured by ELISA at the indicated times. For intracellular cytokine staining, cells were cultured as described above and stimulated for 4 h at 37˚C in culture medium containing PMA (50ng/ml; Sigma), ionomycin (1μg/ml; Sigma) and

monensin (GolgiStop; 1µl/ml; BD Biosciences). After staining for surface markers, cells were fixed and permeabilized with the Foxp3 Staining Buffer Set, according to the manufacturer's instructions (eBiosciences). All cytokines antibodies were purchased from Biolegend. Flow cytometric analysis was performed using a FACS Calibur (Becton Dickinson).

Quantitative RT-PCR

RNA was extracted with RNAeasy minikits (Qiagen) and RNA expression were analyzed by RT-PCR according to the manufacturer's instructions using the GeneAmp 7500 Sequence Detection System (Applied Biosystems). Expression was normalized to the expression of GAPDH. Primers-probe mixtures were purchased from Applied Biosystems: *Lgals9* (Mm00495295), *Foxp3* (Mm00475162), *Cd25* (Mm01340213), *Tnfrsf18* (Mm00437136), *Ctla4* (Mm00486849), *Il10* (Mm00439614), *Icos* (Mm00497600), *GAPDH* (4352339E).

Western blot analysis

2-5 × 10^6 cells were lysed in whole cell extract buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Igepal CA-630, 0.2 mM EDTA, 10 mM Na_2VO_4, 10% glycerol, protease inhibitors). Proteins were separated by SDS-PAGE gel electrophoresis using 4-12% NuPAGE Bis-Tris gels (Invitrogen) followed by transfer to nitrocellulose membrane. To block unspecific binding, membranes were incubated with 5% milk in TBST (0.5 M NaCl, Tris-HCl, pH 7.5, 0.1% (v/v) Tween-20) for 60 min and washed once with TBST. Proteins of interest were detected by incubating membranes over night at 4°C in 5% BSA

and BST with anti-p-Smad3 (Cell Signaling, D12E11) or anti-β-Actin (Santa Cruz, sc-47778), washing with TBST three times 10 min and incubating with horseradish peroxidase–conjugated anti-rabbit or anti-mouse antibody (Cell Signaling, 7074 and 7076). Bound antibody was detected by using Immobilon Western chemiluminescent HRP substrate (Millipore).

Immunoprecipitation

Cell lysates were prepared as described above and proteins were immunoprecipitated by incubation of lysates with 3 μg antibody (listed below) over night at 4°C and pull-down of antibody-protein precipitates with Dynabeads Protein G (Invitrogen). Beads were washed extensively and proteins eluted with NuPAGE LDS sample buffer (10% β-mercaptoethanol). The presence of immuncomplexed proteins was determined by Western blot analysis with the antibodies listed. Anti-TGF-βRI (ab31013, Abcam), anti-CD44 (8E2, Cell signaling), anti-galectin-9 (M20, Santa Cruz) for precipitation, and anti-TGF-βRI (ab121024, Abcam), anti-CD44 (KM114, BD Pharmingen), anti-galectin-9 (C-20, Santa Cruz) for western blotting.

Retroviral transduction

Retroviral particles were produced by transiently transfecting HEK 293T cells with retroviral packaging constructs eco and gag and pol and expression plasmids MSCV-IRES-GFP or MSCV-IRES-GFP-Smad3 (kind gift of Dr. Bing Su) using Fugene HD (Roche). 72 hours after transfection, viral culture supernatants were harvested, supplemented with polybrene (8 mg/ml) and added to previously stimulated T cells (5

$\times 10^5$/well, plate-bound anti-CD3 and anti-CD28 and different combination of cytokines for 24 h). Cultures were centrifuged at 800 G for 45 min at 25°C.

Assessment of intestinal inflammation

Tissues were graded semiquantitatively from 0 to 5 in a blinded fashion. Score 0: No changes observed; Score 1: Minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; Score 2: Mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with minimal to mild epithelial hyperplasia and minimal to mild mucin depletion from goblet cells; Score 3: mild to moderate inflammatory cell infiltrates that were sometimes transmural, often associated with ulceration, with moderate epithelial hyperplasia and mucin depletion; Score 4: marked inflammatory cell infiltrates that were often transmural and associated with ulceration, with marked epithelial hyperplasia and mucin depletion; Score 5: marked transmural inflammation with severe ulceration and loss of intestinal glands.

Kinase activity assays

Naïve cells from WT or *Cd44*$^{-/-}$ mice were stimulated with 5 ng/ml TGF-β1 and/or recombinant galectin-9 for 30 min before they were lysed in whole cell extract buffer. TGF-βRI was immunoprecipitated from the lysate as described above and TGF-βRI kinase activity in the precipitate was assessed with the ADP-Glo TGF-βRI kinase enzyme assay system following the manufacturer's instructions (Promega, V4093). Data

is presented as fold change over samples immunoprecipitated with isotype control antibodies.

Galectin-9 promoter luciferase reporter construction

A fragment of the galectin-9 promoter (+1 to -2478 bp relative to the transcription start site) was amplified by PCR using a BAC DNA containing the *Lgals9* locus. The primer sequences for PCR were 5'-AGATCTCACTTCTTCAAGGGGAGGAG-3' and 5'-ACGCGTGTCTGTGCCTCTCTCAGGC-3'. The fragment was directionally cloned into the pGL3 luciferase reporter vector (Promega) through MluI and BglII restriction sites and the DNA sequence of the insert was verified.

Reporter assays

EL4 LAF cells (1×10^5 cells/well, 48 well plate, kind gift of Dr. Masahide Tone) were transiently transfected with the indicated expression vectors, empty vector controls as well as the promoter firefly luciferase-reporter constructs and Renilla luciferase reporter vector (Promega) with Fugene HD (Promega). 24 h after transfection cells were stimulated with different combinations of plate-bound anti-CD3and anti-CD28, TGF-β and galectin-9. 24 h after stimulation luciferase expression was determined by measuring luminescence with the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase activity was normalized to renilla luciferase activity. Data is representative of at least two independent experiments, each data point represents duplicate values. The following vectors were used; pCMV5-Smad3 (Dr. Akihiko Yoshimura), pGL3-Lgals9 reporter.

Proximity Ligation Assay

PLA detection was performed with a Duolink II kit (Olink Bioscience).

Immunofluorescence and Confocal Microscopy

Purified $CD4^+$ T cells from WT or *Lgals9*$^{-/-}$ mice were grown on chamber slides (Nalge Nunc International, Naperville, IL) and stimulated with 1μg/ml anti-CD3 and anti-CD28 antibodies in the presence or absence of TGF-β. Cells were fixed, blocked with 5% goat serum, and then incubated with primary antibodies (TGF-βRI and CD44) from Santa Cruz Biotechnology or Abcam. Slides were washed, incubated in Alexa Fluor 488- or Alexa Fluor 568-conjugated secondary Abs (Invitrogen) containing DAPI and examined on a LSM 710 Zeiss confocal microscope.

ChIP

Experiments were carried out following manufacturer's instructions (Simple ChIP kit, Cell signaling). The following antibodies were used for ChIP: anti-Smad3 (ab28379, Abcam), antibody to acetylated H4 (06-866; Millipore). The following primer pairs were used:

Sequences of primers used in chromatin immunoprecipitation (ChIP) assay

Lgals9 promoter:	
-1700 – -1500 F	TGACTGCATGGTTGAAGGCAGAAG
-1700 – -1500 R	CACAGCATCAGTGTTGGGAAGTGT
-1450 – -1150 F	AGCCATAGTGCAATCCCAAAGTCC
-1450 – -1150 R	CCAGCCTTTCCCTGCCTTAAACAA
-1100 – -750 F	ACAACCACATGGTGGCTTACAACC
-1100 – -750 R	TCATGTAGACCAGGCTGGCTTTGA

-450 – -150 F	ATTTCGGAGTGCCCGTAAGGAGTT
-450 – -150 R	TGAGCCTTCATTTGTCCTCTGGGT
-150 – +50 F	AAAGTCAAGGCAGAGCAGGGAACA
-150 – +50 R	GGCTGAGCTAGGAAACAGAAACCA
+250 – +550 F	TGATGCAGCTCGTGTCTCATGCTA
+250 – +550 R	CCAACAAGCCCATCCACATCATCA
Foxp3 CNS region	
Foxp3 CNS1 F	CCCATGTTGGCTTCCAGTCTCCTTTATGG
Foxp3 CNS1 R	AGGTACAGAGAGGTTAAGAGCCTGGGT
Foxp3 CNS2 F	GTTGCCGATGAAGCCCAAT
Foxp3 CNS2 R	ATCTGGGCCCTGTTGTCACA