Supplementary Figure 1:
R161H lymphocytes are activated in the intestine

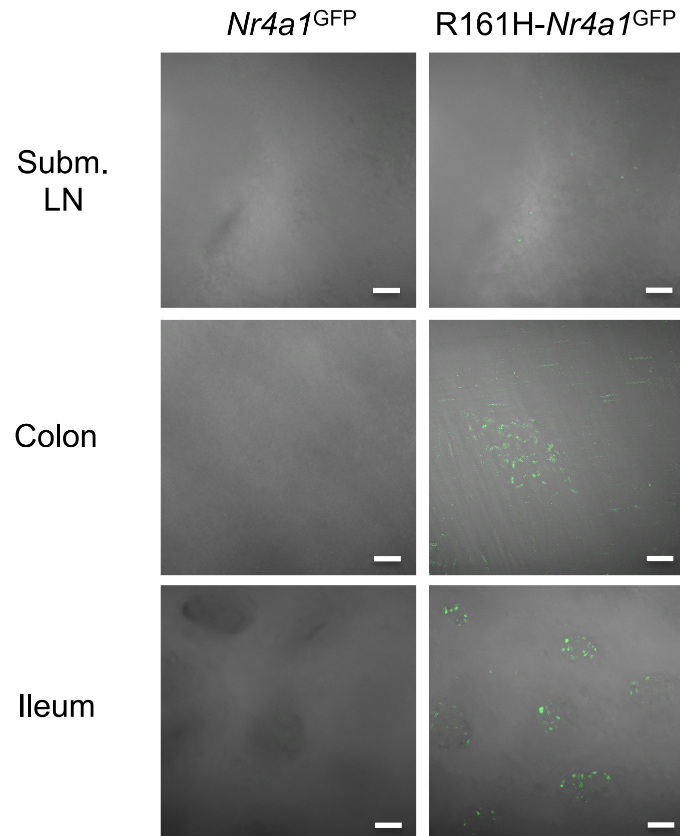
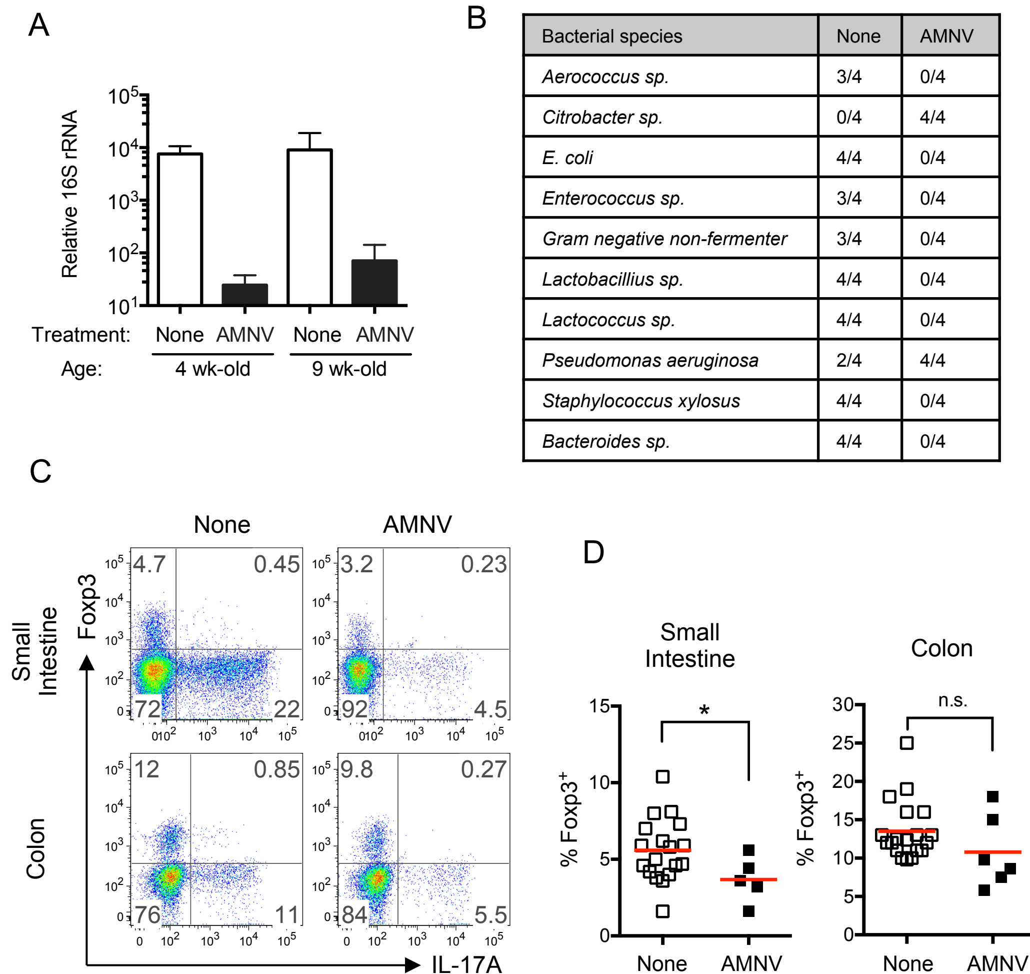


Figure S1. Related to Figure 1. ***R161H lymphocytes are activated in the intestine.*** Expression of the *Nr4a1* gene (Nur77) was detected by GFP (green) in the fresh tissue explants of *Nr4a1*^{GFP} and R161H-*Nr4a1*^{GFP} reporter mice. Intestines of R161H mice showed brighter and more frequent GFP signals. Subm. LN; submandibular lymph node. Scale represents 25 µm.

Supplementary Figure 2:

AMNV treatment of R161H mice reduces their intestinal bacterial mass and alters its composition, but does not expand Foxp3$^+$ cells in gut LP



Bacterial species	None	AMNV
Aerococcus sp.	3/4	0/4
Citrobacter sp.	0/4	4/4
E. coli	4/4	0/4
Enterococcus sp.	3/4	0/4
Gram negative non-fermenter	3/4	0/4
Lactobacillius sp.	4/4	0/4
Lactococcus sp.	4/4	0/4
Pseudomonas aeruginosa	2/4	4/4
Staphylococcus xylosus	4/4	0/4
Bacteroides sp.	4/4	0/4

Figure S2. Related to Figure 2. ***AMNV treatment of R161H mice reduces their intestinal bacterial mass and alters its composition, but does not expand Foxp3$^+$ cells in gut LP.***

(A) Real-time PCR detection of 16S rRNA gene in AMNV treated and untreated mice. Shown are relative 16S rRNA gene copy numbers (total eubacteria) per gram feces. N=4.

(B) Presence of culturable bacteria in the feces of untreated and AMNV-treated mice. Four individual R161H mice were tested. Shown is number of positive mice for the indicated bacteria out of total mice examined.

(C) LP lymphocytes from small intestine and colon were collected from R161H mice treated, or not, with AMNV and were intracellularly stained after a PMA and ionomycin pulse. Shown are LP data from small intestine of 4-wk old mice, gated on CD4$^+$ cells. Results in older mice were similar.

(D) Compiled data for (C) from at least 5 experiments (4-16 wk-old). *p <0.05, n.s., not significant.

Supplementary Figure 3:

Attenuation of disease by depletion of commensal micro-flora is not due to altered frequency or unresponsiveness of retina-specific T cells.

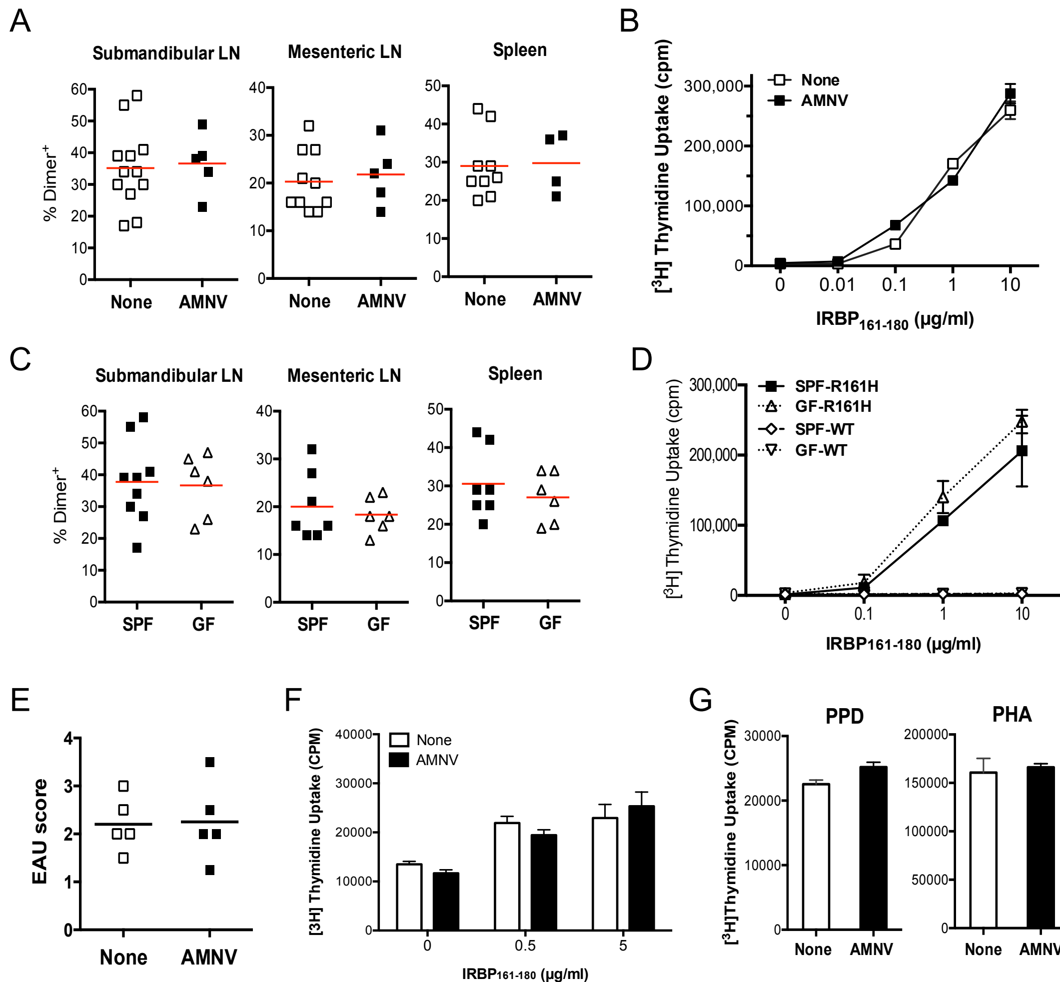


Figure S3, related to Figure 2. ***Attenuation of disease by depletion of commensal microflora is not due to altered frequency or unresponsiveness of retina-specific T cells.***

(A-D) Immunological profile of AMNV-treated *vs.* untreated R161H mice:

(A) Frequencies of IRBP-specific T cells (% Dimer^{+}) in untreated (None) and AMNV-treated R161H mice (4-12 wk-old). Compiled data from 5 independent experiments.

(B) Antigen-specific proliferation to $IRBP_{161\text{-}180}$ of peripheral lymphocytes (5x10^{5}/well) from untreated (None) and AMNV treated R161H mice of 8 wk-old mice.

(C) Frequencies of p161 dimer-positive cells in the CD4^{+} population in SPF R161H and GF R161H mice from 2 experiments.

(D) Antigen-specific proliferation was also comparable between GF R161H (dotted line) and SPF R161H (solid line) at 7 wks of age. Similar results were obtained from another experiment using 11 wk-old mice , and no response was observed in their WT littermates.

(E-G) EAU induced by immunization is similar in AMNV treated *vs.* untreated WT mice (d15 after immunization):

(E) Histology scores after immunization with 7 µg of $IRBP_{161\text{-}180}$ in CFA.

(F) Proliferation in response to IRBP peptide and (G) proliferation to PPD and PHA of draining lymph node cells. One representative experiment of two is shown.

Supplementary Figure 4.

AMNV treatment reduces IL-17A-producing cells in the gut of R161H *Rbp3*$^{-/-}$ mice

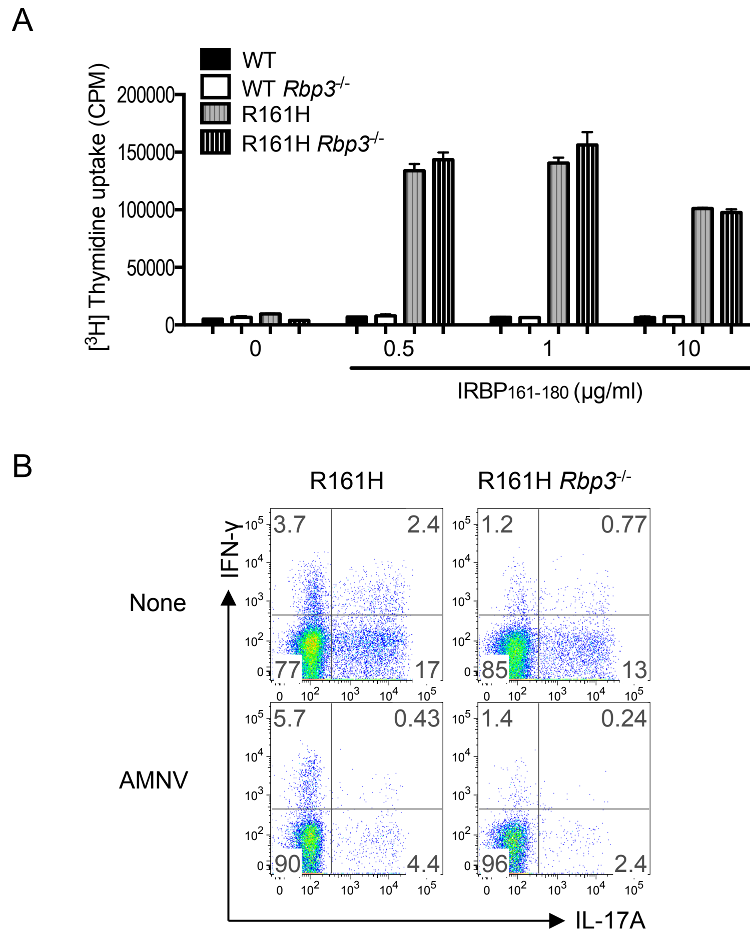


Figure S4. Related to Figure 4. ***AMNV treatment reduces IL-17A-producing cells in the gut of R161H Rbp3*$^{-/-}$ *mice.***

(A) Peripheral lymphocytes of R161H *Rbp3*$^{-/-}$ mice respond similarly to lymphocytes of R161H *Rbp3*$^{+/+}$ mice to the cognate antigen.

(B) LP lymphocytes were collected from 8 wk-old R161H and R161H *Rbp3*$^{-/-}$ mice untreated (None) or treated with AMNV, and were stained for the indicated cytokines after a PMA and ionomycin pulse. Shown are LP from small intestine gated on CD4^{+} cells. One representative experiment of two is shown (N=3 pooled in each experiment).

Supplementary Figure 5.

All CD4+ T cells in R161H *Tcra*-/- mice express the retina-specific αβTCR and expanded Th17 cells are present in colonic LP

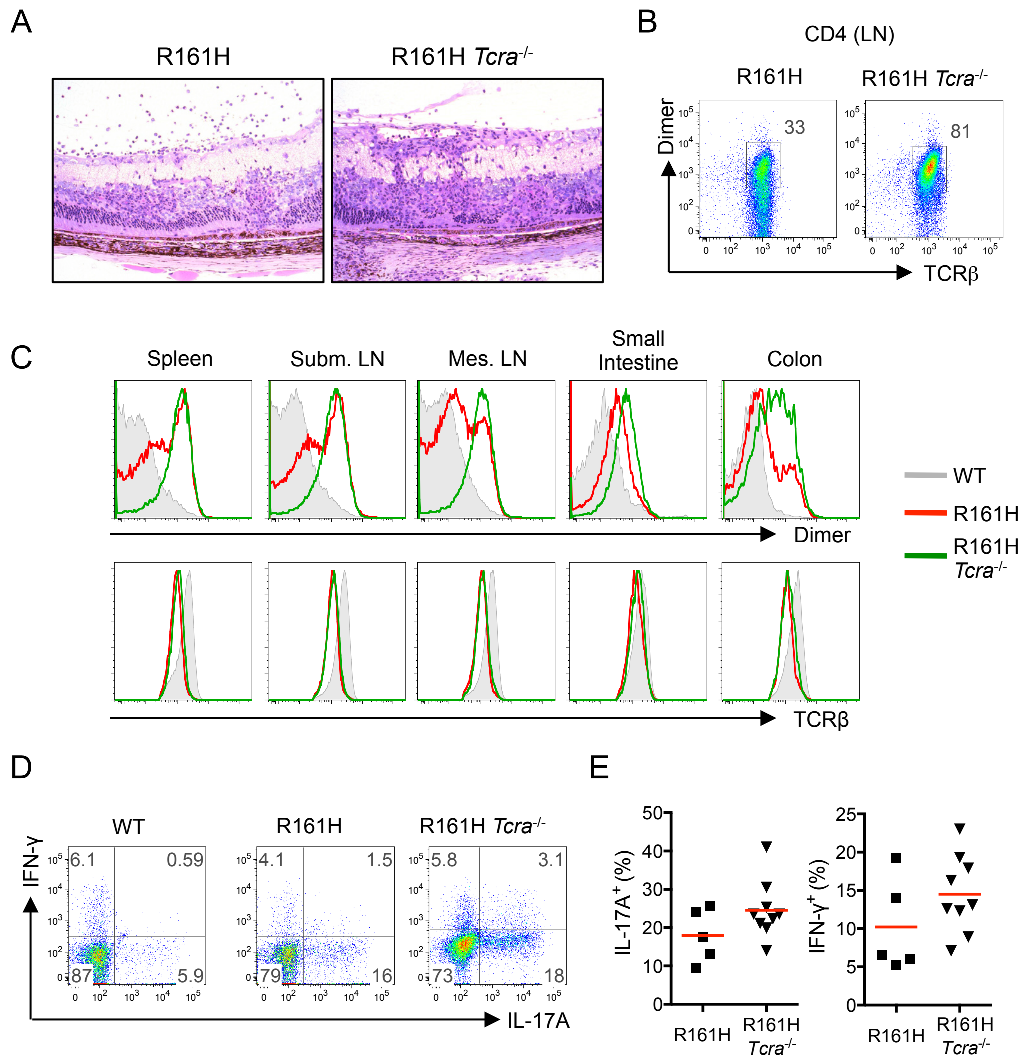


Figure S5. Related to Figure 5. ***All CD4+ T cells in R161H Tcra-/- mice express the retina-specific αβTCR and expanded Th17 cells are present in colonic LP.***

(A) R161H *Tcra*-/- mice develop spontaneous uveitis similarly to their R161H *Tcra*+/+ littermates.

(B) Dot plots showing pooled peripheral LN cells gated on CD4.

(C) Overlaid histograms of WT, R161H and R161H *Tcra*-/- CD4 cells to compare the expression levels of R161 TCR (Dimer) and the common TCRβ chain in peripheral tissues. One representative mouse of 5 is shown.

(D) Colonic LP of R161H *Tcra*-/- mice. The high proportion of IL-17A-producing CD4+ T cells compares to WT is maintained.

(E) Compiled data from individual colons from 3 experiments.

Supplementary Figure 6.

Extract of intestinal contents, but not innate stimuli alone, activate R161 T cells from mice deficient in endogenous IRBP

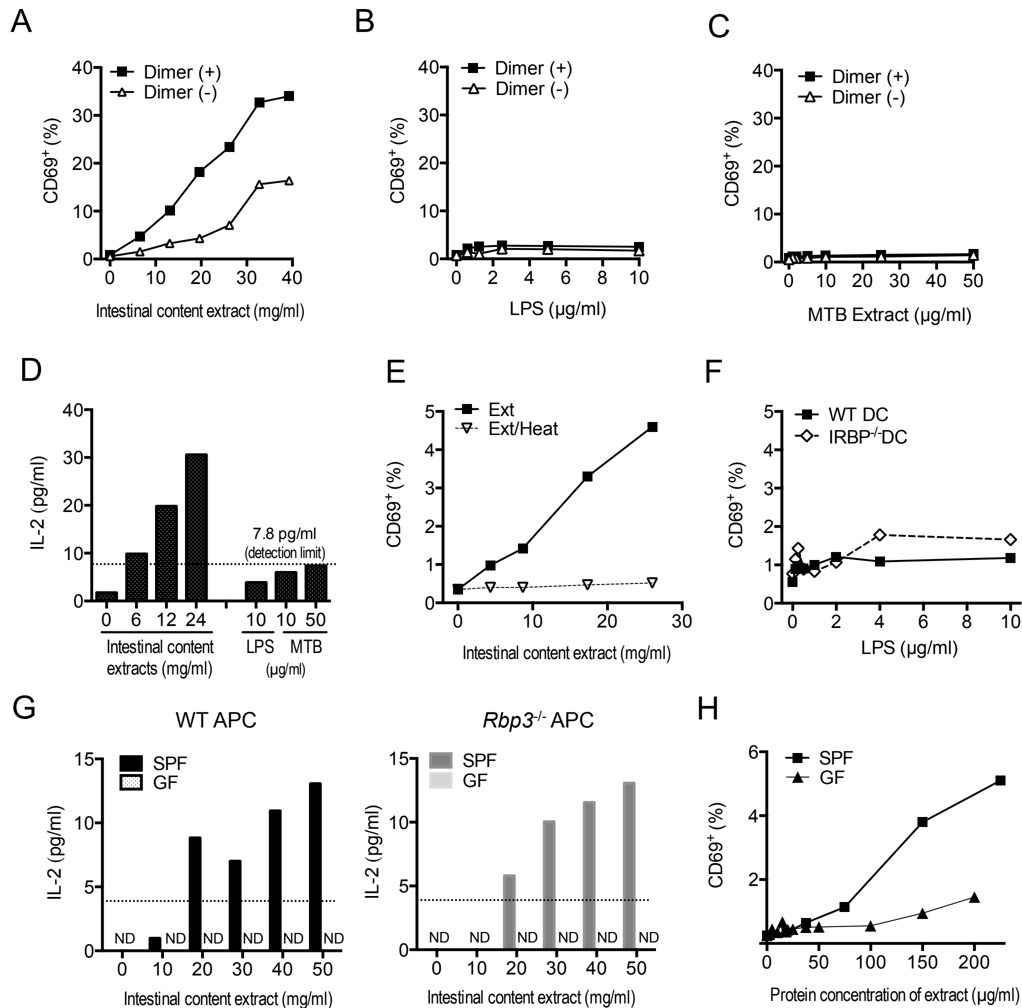


Figure S6. Related to Figure 6. ***Extract of intestinal contents, but not innate stimuli alone, activate R161 T cells from mice deficient in endogenous IRBP.***

(A-D): Responses of total LNC from R161H *Rbp3*$^{-/-}$ mice.

(A) Induction of CD69 in Dimer (+) vs. Dimer (-) T cells after 20 h of stimulation with extracts.

(B, C) CD69 induction in total LNC using innate stimuli contained in LPS (B) or MTB (C).

(D) IL-2 secretion from cultures in A–C (detection limit 7.8 pg/ml).

(E-G): Responses of naïve CD4 cells from R161H *Rbp3*$^{-/-}$ *Rag2*$^{-/-}$mice.

(E) CD69 in T cells stimulated with heat-treated intestinal content extracts (Ext) and WT APC.

(F) CD69 expression of T cells stimulated with LPS.

(G) IL-2 secretion was detectable after stimulation by intestinal content extracts from SPF mice but not after stimulation with intestinal content extracts from GF mice. *Rbp3*$^{-/-}$ and *Rbp3*$^{+/+}$ (WT) APC were used with similar results. Dotted lines show detection limit (3.9 pg/ml). ND. Not detected.

(H) CD69 expression of R161H *Rbp3*$^{-/-}$ *Rag2*$^{-/-}$CD4^{+} T cells stimulated with GF or SPF intestinal extracts normalized by protein concentration.

Supplemental Experimental Procedures

Isolation of lamina propria lymphocytes

The procedures of LP isolation were modified from the methods described before (Atarashi et al., 2011; Scheiffele and Fuss, 2002). Small and large intestines were collected separately and Peyer's patches were removed from small intestine. The proximal one third of small intestine and cecum were not included in the processing. Pieces of small intestine or colon from 2-3 mice were pooled, respectively, and cut open longitudinally to remove the contents. Intestines were rinsed in PBS and minced into small pieces using 2 surgical blades and were then transferred to a new tube containing calcium- and magnesium-free HBSS (Life Technologies). After 2 washes the minced tissue was treated with HBSS containing 10% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES, 5 mM EDTA and 1 mM DTT at 37ºC for 20 min under agitation (200 rpm) to disrupt epithelial and mucosal cells. After repeating this procedure, pieces of intestines were washed with HBSS and treated with RPMI containing 10% FBS, 25 mM HEPES, 200 µg/ml collagenase D and 10 µg/ml DNase I (Roche) at 37ºC for 45 min under agitation (200 rpm). The supernatant containing LP lymphocytes was collected, and the pellet was treated with collagenase D and DNase I for another 30 min under the same conditions and the supernatant collected. Both supernatants containing LP lymphocytes were combined, cells were washed with HBSS and filtered through 100 µm and 40 µm cell strainers. For individual LP analysis, after removal of epithelial cells, residual fat tissue and muscular coat in PBS following HBSS-5 mM EDTA treatment, intestinal tissues were cut in small pieces in RPMI-4%FBS containing 500 µg/ml collagenase D, 62.5 µg/ml dispase II (Roche) and 40 µg/ml DNase I and digested at 37ºC for 1 h (Atarashi et al., 2011). Digested tissues were resuspended in 40% Percoll (GE Healthcare). The cell suspension was layered over 80% Percoll and centrifuged at 2000 rpm at 20 °C for 20 min. The LP lymphocytes were collected, washed in RPMI-10% FBS and counted for further analyses.

Flow cytometric analysis

Eyes, lymph nodes and spleens were isolated to prepare the single cell suspensions. Red blood cells in spleen suspensions were lysed using ACK lysing buffer. Eyes were minced (after removal of the lens) and treated with 1 mg/ml of collagenase D for 30-45 min at 37°C. Cells were washed and resuspended in media for ex vivo stimulation, or in FACS buffer. Fc receptors were blocked using CD16/32 (2.4G2), and stained with the following anti-mouse antibodies labeled with various fluorochromes (FITC, PE, PerCP-Cy5.5, PerCP-eF710, PE-Cy7, APC,

APC-eF780, eFluor 450, V500, Brilliant Violet 421, 570 or 605): CD4 (RM4-5), CD8 (53-6.7), CD25 (PC61), CD44 (IM7), CD62L (Mel-14), CD69 (H1.2F3), TCRβ (H57-597) (BD Biosciences, BioLegend, or eBioscience), and CCR6-PE (R&D). In some experiments, propidium iodide (PI, Miltenyi Biotech) or Fixable Viability Dye eFluro 780 (eBioscience) was used to exclude dead cells from analysis. $IRBP_{161-180}$-specific T cells were detected by $IRBP_{161-180}$ peptide-MHC class II-mouse IgG dimer (p161 dimer)(Karabekian et al., 2005) conjugated with Alexa Fluor 647 (Life Technologies). Frequencies of CD69 expressing cells were compared within the $CD3^+TCR\beta^+CD4^+$ gate between p161 dimer-positive and -negative populations, or in $CD3^+TCR\beta^+CD4^+$ gate between R161H and WT samples. For intracellular cytokine staining, cells were stimulated in the complete RPMI-10% FBS with 10 ng/ml PMA and 500 ng/ml Ionomycin for 4 h in the presence of Brefeldin A. Cells were harvested, fixed in 4% paraformaldehyde and permeabilized with Triton buffer (0.5% Triton X-100 and 0.1% BSA in PBS). Intracellular staining was performed using anti-mouse IFN-γ (XMG1.2), IL-17A (eBio17B7, TC11-18H10.1), IL-22 (IL22JOP), and Foxp3 (FJK-16s) (eBioscience or BioLegend) with various fluorochromes as above. Stained cells were acquired using FACSCalibur, FACSAria (BD Bioscience), or MACSQuant (Miltenyi Biotech). Data were analyzed in FlowJo (Tree Star).

Depletion of intestinal commensals

R161H mice were treated with a combination of broad-spectrum antibiotics cocktail "AMNV" consisting of ampicillin (1 g/l), metronidazole (1 g/l), neomycin (1 g/l) and vancomycin, (500 mg/l) in drinking water to eliminate gut commensal microflora as described (Rakoff-Nahoum et al., 2004). The AMNV treatment was given to breeders and pups continued to receive it continuously after weaning. The cages, bedding, food and water, were autoclaved before antibiotics were added, and were changed weekly. Control animals were housed in conventional set-ups in the same rack.

DNA extraction from fecal samples and 16S rRNA gene quantitation by qPCR

Fecal samples from individual mice were obtained and used for bacterial genomic DNA isolation and 16S rRNA gene quantitative PCR analysis. The DNA was isolated using the QIAamp DNA Stool Mini kit, following the instructions of the manufacturer (Qiagen). Quantitation of total bacteria was performed as described (Barman et al., 2008) using primers 5'-ACTCCTACGGGAGGCAGCAGT-3' and 5'-ATTACCGCGGCTGCTGGC-3'.

EAU induction by immunization

WT littermates (B10.RIII) of untreated or AMNV-treated R161H mice were used for the immunization study. Mice were immunized with 7 μg $IRBP_{161-180}$ peptide emulsified in complete Freund's adjuvant (CFA). Fundoscopic evaluation of disease scores (Horai and Caspi, 2010) was performed on day 10, 14, 17 and 20. Eyes collected on day 21 were processed for histopathology and scored as described (Horai and Caspi, 2010). Draining (inguinal and iliac), submandibular and mesenteric lymph node cells were collected for antigen-specific proliferation analysis.

Supplemental References

Barman, M., Unold, D., Shifley, K., Amir, E., Hung, K., Bos, N., and Salzman, N. (2008). Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. Infect Immun *76*, 907-915.

Supplemental Movie Titles

Movie S1 (Related to Figure 1E).
GFP expression in fresh ileum of *Nr4a1*^{GFP} reporter mice at 17 days of age.

Movie S2 (Related to Figure 1E).
GFP expression in fresh ileum of R161H *Nr4a1*^{GFP} reporter mice at 17 days of age, before onset of uveitis.

Movie S3 (Related to Figure 1F).
GFP expression in fresh ileum of adult *Nr4a1*^{GFP} reporter mice.

Movie S4 (Related to Figure 1F).
GFP expression in fresh ileum of adult R161H *Nr4a1*^{GFP} reporter mice after onset of uveitis.