Immunity

Supplemental Information

Interleukin-17B Antagonizes Interleukin-25-Mediated Mucosal Inflammation

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Supplemental Material

Supplemental Experimental Procedures

Mice

C57BL/6 mice were purchased from NCI (01C55). *Nod2*^{tm1Flv}/J (005763), and *Vil1-cre* (018963) mice on the C57BL/6 background were purchased from The Jackson Laboratory. *Il25*$^{flox/flox}$ and IL-25-deficient mice have been previously described (Angkasekwinai et al., 2010) and were backcrossed for >8 generations onto the C57BL/6 background. To generate mice with an intestine-specific deletion of *Il25* (*Il25*$^{fl/fl}$x*Vil1-cre*), *Il25*$^{fl/fl}$ mice were crossed with *Vil1* promoter–driven *Cre* (*Vil1-c*re) mice. IL-17B-deficient mice were generated by a UPA vector gene trapping strategy targeting the *Il17b* gene in the R1 ES cell line (clone 371C12; provided by Toronto Centre for Phenogenomics, Centre for Modeling Human Disease). ES clones were microinjected into C57BL/6 blastocytes to generate chimeras. Male pups exhibiting a high level of chimerism were used to generate mice with a homozygous genetrap in the *Il17b* gene. Homozygous mice were then backcrossed for >8 generations on the C57BL/6 background. The genetrap vector was mapped to the promoter region ~1.2 kb upstream the transcriptional start site of the *Il17b* gene and resulted in substantially reduced gene expression. The genotyping primers for IL-17B-deficient mice were as follows: forward 1, 5'-TGAAGCTGTGGCCTGATTT -3'; forward 2, AGAAGCGAGAAGCGAACTG; and reverse 1, CCTAGCCAACCGTTACAGC. Forward 1 and reverse 1 amplify a wild-type 5' UTR sequence and forward 2 and reverse 1 amplify the product representing the gene trap insertion. *Il17rb*$^{-/-}$ mice have previously been described (Watarai et al., 2012). Act1-deficient mice have been described previously (Chang et al., 2011). For *in* vivo disease models, female mice from different groups were co-housed and

male mice were placed on a cage rotation system to negate potential effects of differential microbiota composition between strains. All experiments were performed on 6-12 wk-old animals housed under specific pathogen-free conditions at the MD Anderson Cancer Center and Rosalind Franklin University of Medicine and Science. All animal procedures were conducted under protocols approved by the MD Anderson Cancer Center and Rosalind Franklin University Institutional Animal Care and Use Committees.

DSS-induced colitis

All experiments were performed using 6-12wk old mice on the C57BL/6 background. C57BL/6 controls were bred in house to prevent any differential effects of microbial colonization between housing facilities. Mice were feed DSS in drinking water at a concentration of 2-3.5% (w/v) for a total period of 5 days. Starting weights were recorded and then mice were weighed and monitored daily until the experimental endpoint (d 8). After 5 days of DSS administration, mice were put back on normal water until the experimental endpoint. After euthanasia, colon samples were collected from individual mice, washed with PBS, and then measured for shortening. For experiments involving the injection of IL-17B, mice were injected with 500ng of rmIL-17B (R & D Systems) at d 0, 2, 4, and 6 following the start of DSS treatment.

Colon tissue mRNA and histology

To analyze mRNA expression in whole colon tissue, colons from healthy or DSS mice (d 8) were divided into proximal, intermediate, and distal sections followed by the harvesting of equivalent biopsies from each section. Tissue samples were then combined and homogenized in TRIzol (Invitrogen). RT-PCR was performed to generate cDNA using the MMLV system (Invitrogen) and real-time PCR was performed using IQ SYBR Green on a CFX96 instrument

(Bio-Rad). Most primer pairs have been previously described (Angkasekwinai et al., 2007; Angkasekwinai et al., 2010; Reynolds et al., 2012). Other primers included IL-17B: forward (F), 5'- -3' and reverse (R), 5'- -3'; IL-6: (F)5'-ACCAGAGGAAATTTTCAATAGGC-3' (R)5'-TGATGCACTTGCAGAAAACA-3'; IL-17A: (F) 5'-CTCCAGAAGGCCCTCAGACTAC-3' (R) 5'-GGGTCTTCATTGCGGTGG-3'; IL-17F: (F)5'-CTGGAGGATAACACTGTGAGAGT-3' (R) 5'-TGCTGAATGGCGACGGAGTTC-3', IL-22: (F)5'-CATGCAGGAGGTGGTACCTT-3' (R)5'-CAGACGCAAGCATTTCTCAG-3' IL-25:(F)5'- CGGAGGAGTGGCTGAAGTGGAG-3' (R)5'-ATGGGTACCTTCCTCGCCATG-3',CCL2: (F)5'-CTCAGCCAGATGCAGTTAACGCCC-3' (R)5'-GGTGCTG AAGACCTTAGGGCAGAT-3'; CCL5: (F)5'-AGATCTCTGCAGCTGCCCTCA-3' (R)5'-GGAGCACTTGCTGCTGGTGTAG-3' CCL7:(F)5'-CTCATAGCCGCTGCTTTCAGCATC-3' (R)5'-GTCTAAGTATGCTATAGCCTCCTC-3'. For histological analysis, total colon tissue was prepared as described above from healthy, DSS, and *Citrobacter rodentium*-infected animals. Each section of colon tissue was then embedded in paraffin and then stained with H&E as previously described (Reynolds et al., 2012). Individual scores from each section of colon tissue isolated from individual mice were combined from each group to assess histological score (Wirtz et al., 2007).

Colon protein analysis

For the analysis of inflammatory mediator protein production in DSS-induced animals, total colon samples were first washed and then opened longitudinally. Equivalent tissue biopsies were taken from proximal, intermediate, and distal sections from individual mice and then combined in a tissue culture plate with the luminal sides positioned upward. Biopsies were then cultured with media overnight at 37^{o}C and 5 % CO_2 followed by the collection of supernatants for ELISA

determinations (BD Biosciences). For IL-17B and IL-25 determinations, we found that total colon tissue cultures lack protein expression in the supernatants, likely due to autocrine consumption of IL-25 and IL-17B. Thus, for the analysis of IL-17B and IL-25 in total colon tissues, colon samples were obtained from WT and IL-17B-deficient animals following 8 d DSS administration. Colon samples were immediately homogenized in PBS containing protease inhibitor cocktail (Sigma-Aldrich) and then assayed for the IL-17B and IL-25 production by ELISA (R & D Systems).

Cell lines and in vitro CECs

YAMC cells were a generous gift from Dr. Dingzhi Wang at the University of Texas MD Anderson Cancer Center. YAMC cells were cultured with insulin-transferrin-selenium (Gibco) and IFNγ (R & D Systems) at 33^{o}C for propagation and without supplements at 37^{o}C for experiments. The isolation of colon epithelial cells (CECs) has been described previously (Reynolds et al., 2012). Primary CECs were plated and rested in 96-well dishes prior to stimulation with LPS (Sigma-Aldrich), Pam3CSK4 (Invivogen), MDP (Invivogen), or the indicated concentration of recombinant mouse IL-17B and/or IL-25 (R & D Systems) for 6h (mRNA) or 24h (ELISA). For binding competition studies, IL-25 tagged with human Ig (IL-25-hIg) was generated for FACS analysis of binding. For binding competition studies, IL-17B and IL-25 were added at the same time. Real-time PCR was performed as described above. IL-6 protein was measured by ELISA (BD Biosciences).

IL-17B and IL-25 binding analysis

The IL-25-hIg (human Ig) expression vector was transfected into *Drosophila* S2 cells, and the secreted IL-25-hIg fusion protein was purified with a protein A column (Angkasekwinai et al.,

2007). Binding assays were then performed as described previously (Chang et al., 2011). Briefly, HEK 293T cells were retrovirally transfected with full length murine IL-17RB or both IL-17RA and IL-17RB vectors expressed in bicistronic RVKM plasmids. After selection, a fixed concentration of IL-25-hIg fusion protein (0.5ug/ml) was added along with increasing doses of IL-17B (0-2 μg/ml) or IL-17 for 30 min. Treated cells were then washed and preblocked with human IgG1 (Sigma-Aldrich) for 20 min. Cells were then washed and stained with anti-hIg conjugated to APC for 15 min and the APC bound to the cell surface was measured by flow cytometry.

Citrobacter rodentium infection

C. rodentium (ATCC 51459) infection was performed using 2 x 10^9 CFU as previously described (Hu et al., 2013). Briefly, the indicated mouse strains were infected with 2 x 10^9 CFU of bacteria by oral gavage. Mice were then monitored and weighed daily until experimental endpoint (d 14). Colon tissue was analyzed for mRNA and inflammation (H&E staining) as described above. For the determination of CFU, feces, liver, and spleen from infected mice were harvested, weighed, and homogenized in sterile PBS. Homogenates were then plated on MacConkey agar for 24 h at 37^{o}C and *C. rodentium* colonies were counted for CFU determination by normalizing for volume and weight of the starting tissue.

Supplemental Material References

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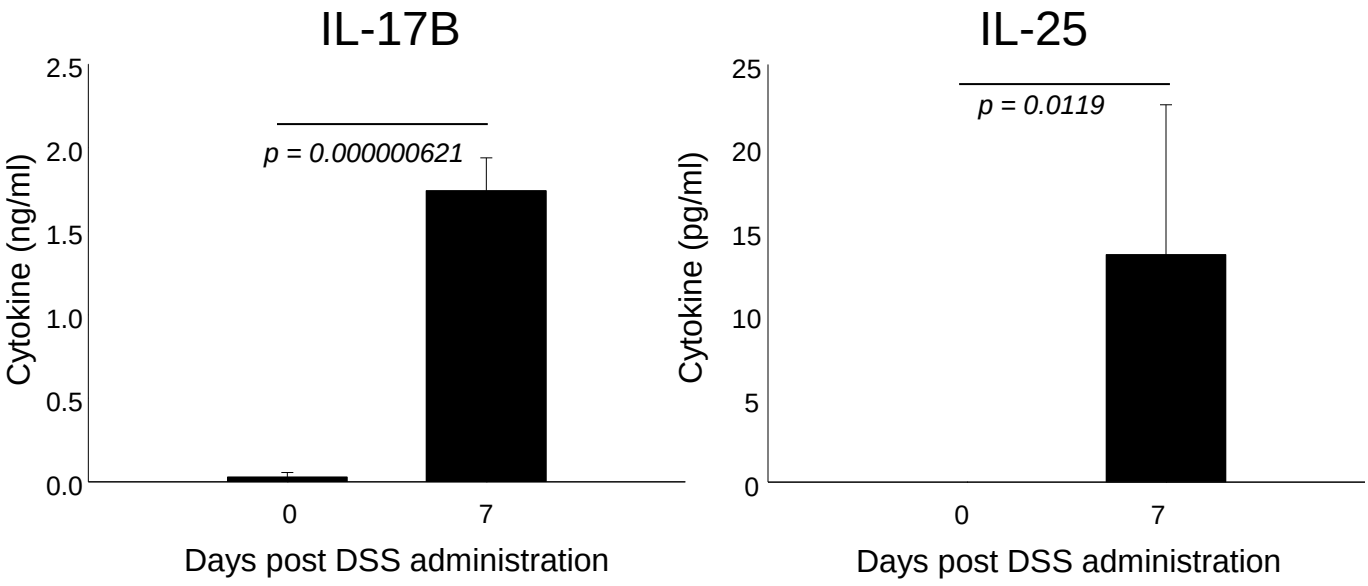

Figure S1 (related to Figure 1). IL-17B and IL-25 cytokines are induced in the colon following the induction of colitis. Healthy (d 0) or DSS-induced (d 7) colons were homogenized in PBS containing protease inhibitors and then supernatants were directly assayed for IL-17B (left) or IL-25 (right) expression by ELISA. n = 4-10 mice per group. Data are presented as mean + SD error bars. Data are representative of 3 independent experiments. * *p* = Students t test.

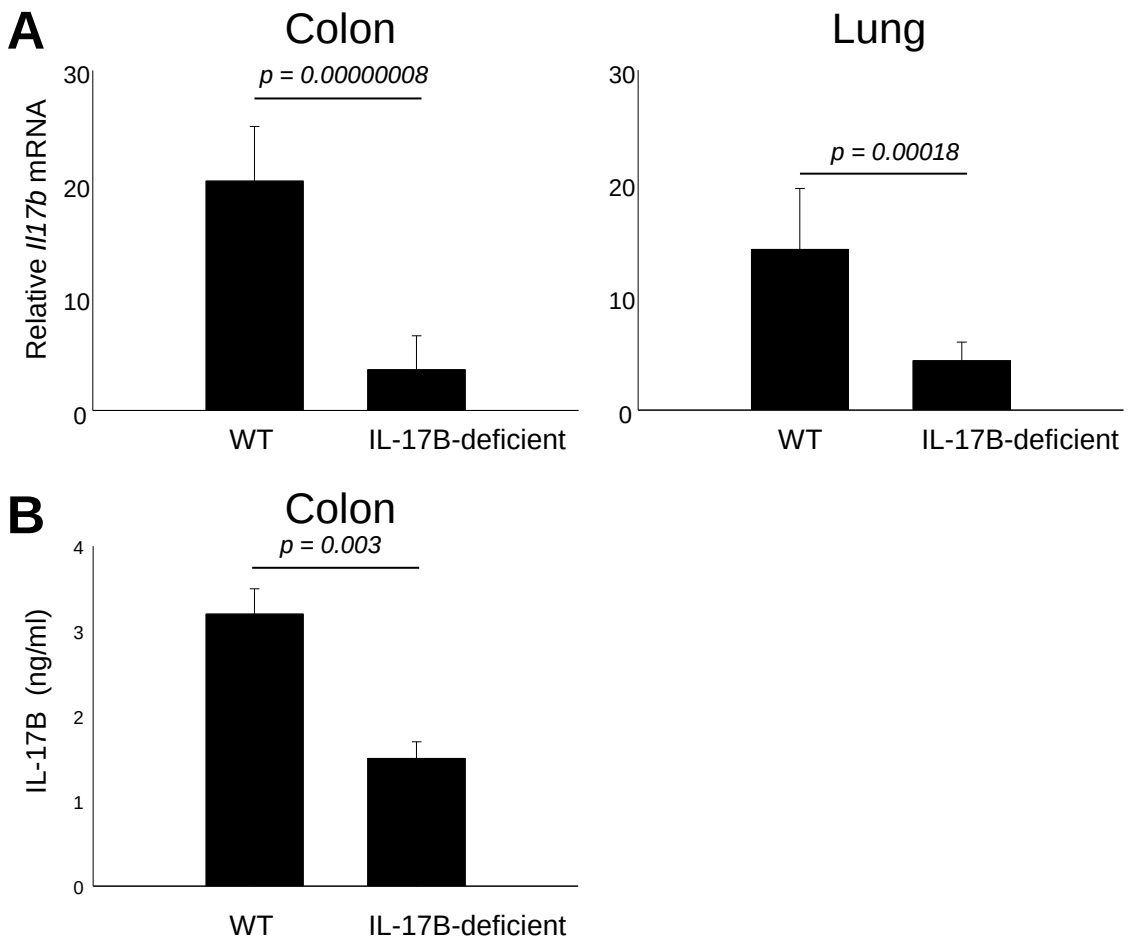

Figure S2 (related to Figure 3). Characterization of IL-17B expression in IL-17B-deficient animals. (A) *Il17b* mRNA expression was compared between C57BL/6 (WT) and IL-17B-deficient colons or lungs following 8 d DSS administration or 28 d OVA + alum administration, respectively. Relative mRNA values were obtained by assessing the expression of beta actin and using healthy tissues as the baseline for expression. n = 8-14 mice per tissue. (B) IL-17B protein in the colons of WT and IL-17B-deficient animals following 5 d DSS administration was examined by ELISA following homogenization in PBS containing protease inhibitors. n = 2 mice per tissue. Data are presented as mean + SD error bars. Data are representative of 2-3 independent experiments. *p = Students t test.

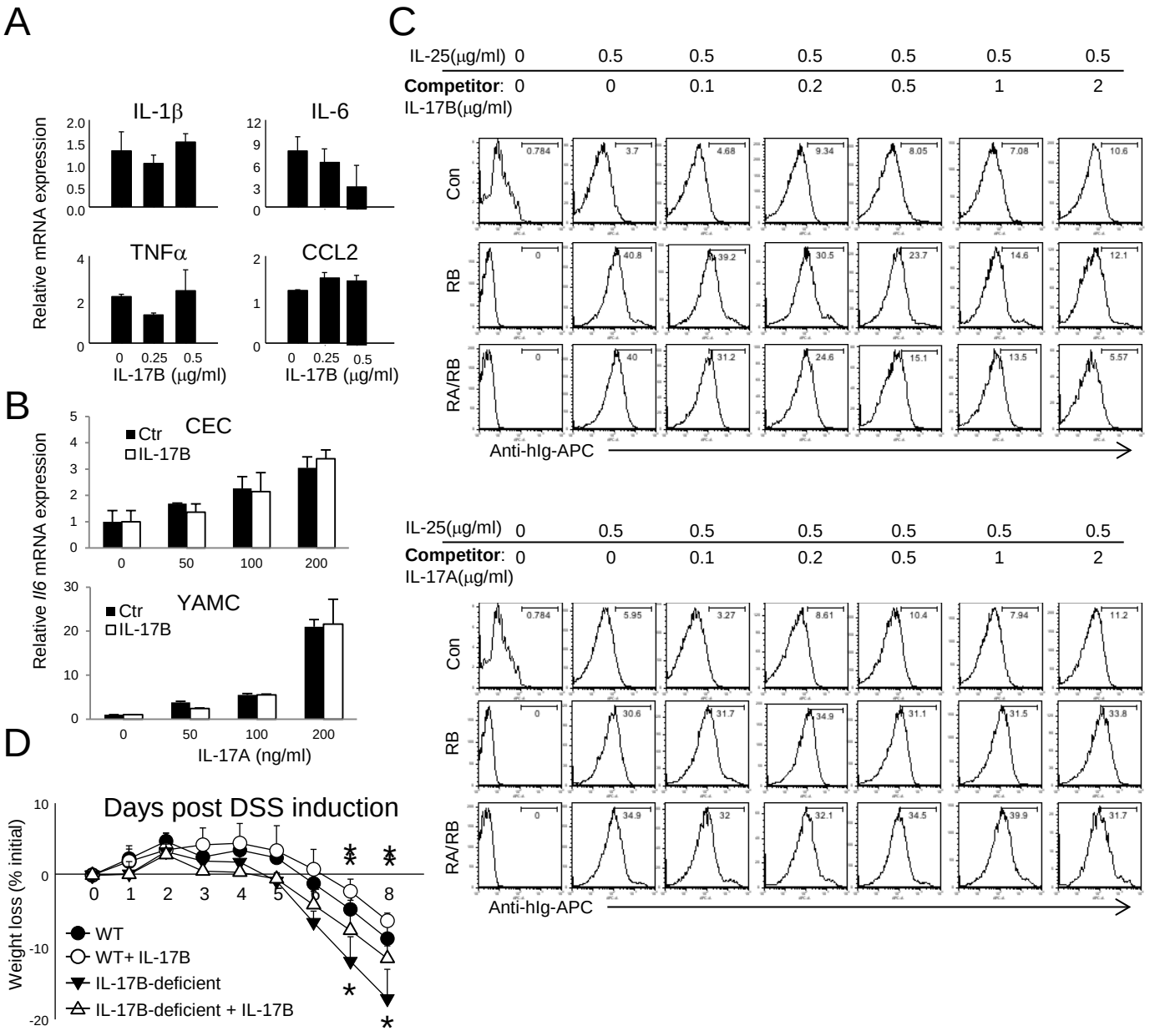

Figure S3 (related to Figure 4). IL-17B antagonizes IL-25 binding to IL-17RB. (A) Primary CECs were isolated from WT mice and stimulated for 6 h with the indicated doses of recombinant IL-17B. The expression values for inflammatory mRNAs were then obtained and normalized to the expression of the reference gene, *actb*. (B) Primary CECs and the YAMC cells were stimulated with 200 ng/ml IL-17B and increasing doses of IL-17A for 6 h. *Il-6* mRNA was then quantified by qPCR. (C) 293T cells were transfected with murine empty vector control, IL17RA, IL-17RB, or IL-17RA and IL-17RB expression vectors and then incubated with increasing concentrations of IL-17A or IL-17B along with fixed IL-25-hIg (0.5μg/ml). The binding was detected by anti-hIg conjugated with APC. (D) Combined weight loss data from WT and IL-17B-deficient DSS mice in the absence or presence of 500ng rmIL-17B injections at D0, D3, and D6. n = 5 mice per group. *Student's t test, *p< 0.05 for comparisons between IL-17B-deficient with or without IL-17B injections; **p< 0.05 for comparisons between WT mice with or without IL-17B injections. Data are presented as mean + SD error bars and are representative of 3-5 independent experiments.

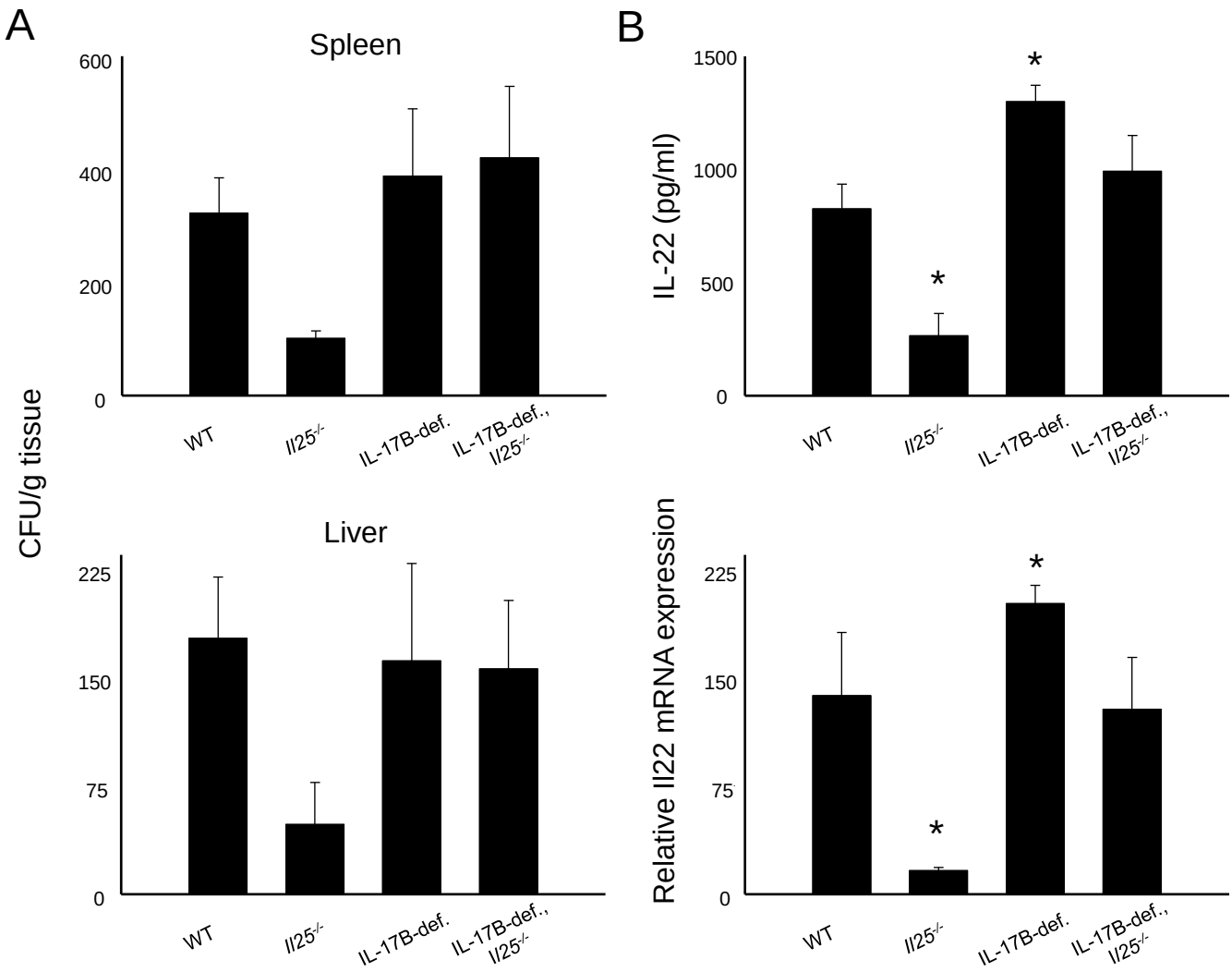

Figure S4 (related to Figure 5). Dissemination of *Citrobacter rodentium* and colonic IL-22 expression in IL-17B- and IL-25-deficient animals. (A) Spleen and liver samples were obtained from WT, *Il25*$^{-/-}$, IL-17B-deficient (IL-17B-def.), and IL-17B-deficient, *Il25*$^{-/-}$ mice following 13 d oral infection with *Citrobacter rodentium*. CFU was determined by plating homogenized tissue on MacConkey agar plates for 24 h. (B) Colon samples were obtained from the same groups of animals and analyzed for IL-22 expression by ELISA following tissue culture overnight (top) or by qPCR of total colon tissue (bottom). For mRNA analysis all expression levels were normalized to the expression of *actb* where *Il22* expression in healthy colon samples served as the baseline for expression. *Students t test, *$p<0.05$ in comparison to the WT group. Data are presented as mean + SD error bars and are representative of 3 independent experiments.

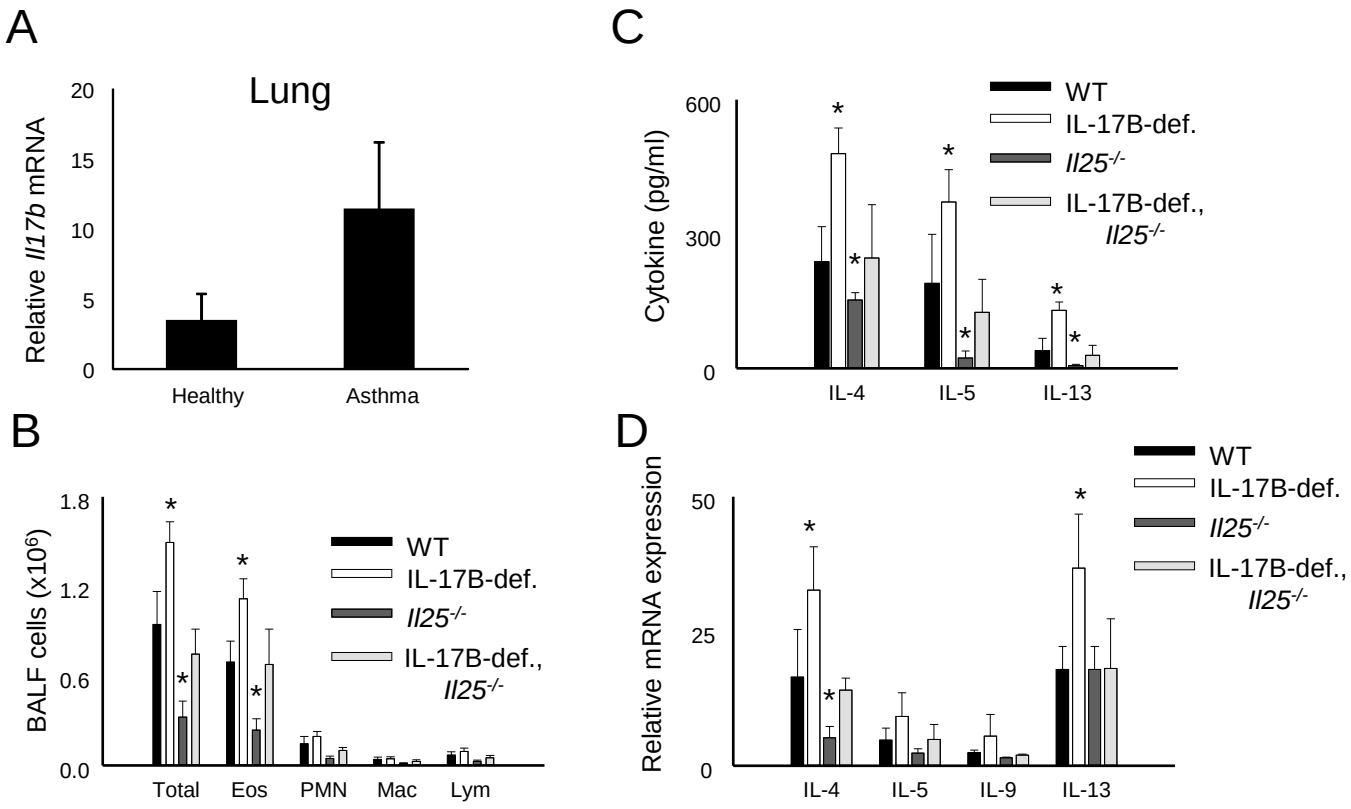

Figure S5 (related to Figure 6). IL-17B and IL-25 in experimental asthma. (A) Lung tissue obtained from WT animals was measured for the expression of IL-17B under normal (Healthy) conditions or following OVA and alum sensitization (Asthma). (B) Total cell counts in the BALF of WT, IL-17B-, IL-25-, and IL-17B- and IL-25-deficient (IL-17B-def.,*Il25*$^{-/-}$) animals sensitized with OVA in alum at day 0 and 14, followed by intranasal challenge with OVA at day 14, 25, and 26. (C) BALF derived from the animals in (A) was assayed for the presence of IL-4, IL-5, and IL-13 by ELISA. (D) Combined mRNA analysis of Th2 and Th9 cell genes from total lung samples derived from the mice in (A). Data are presented as mean of data obtained from individual mice + SD error bars and are representative of at least 3 independent experiments. *Student's t test; *$p < 0.05$ for all comparisons to WT.