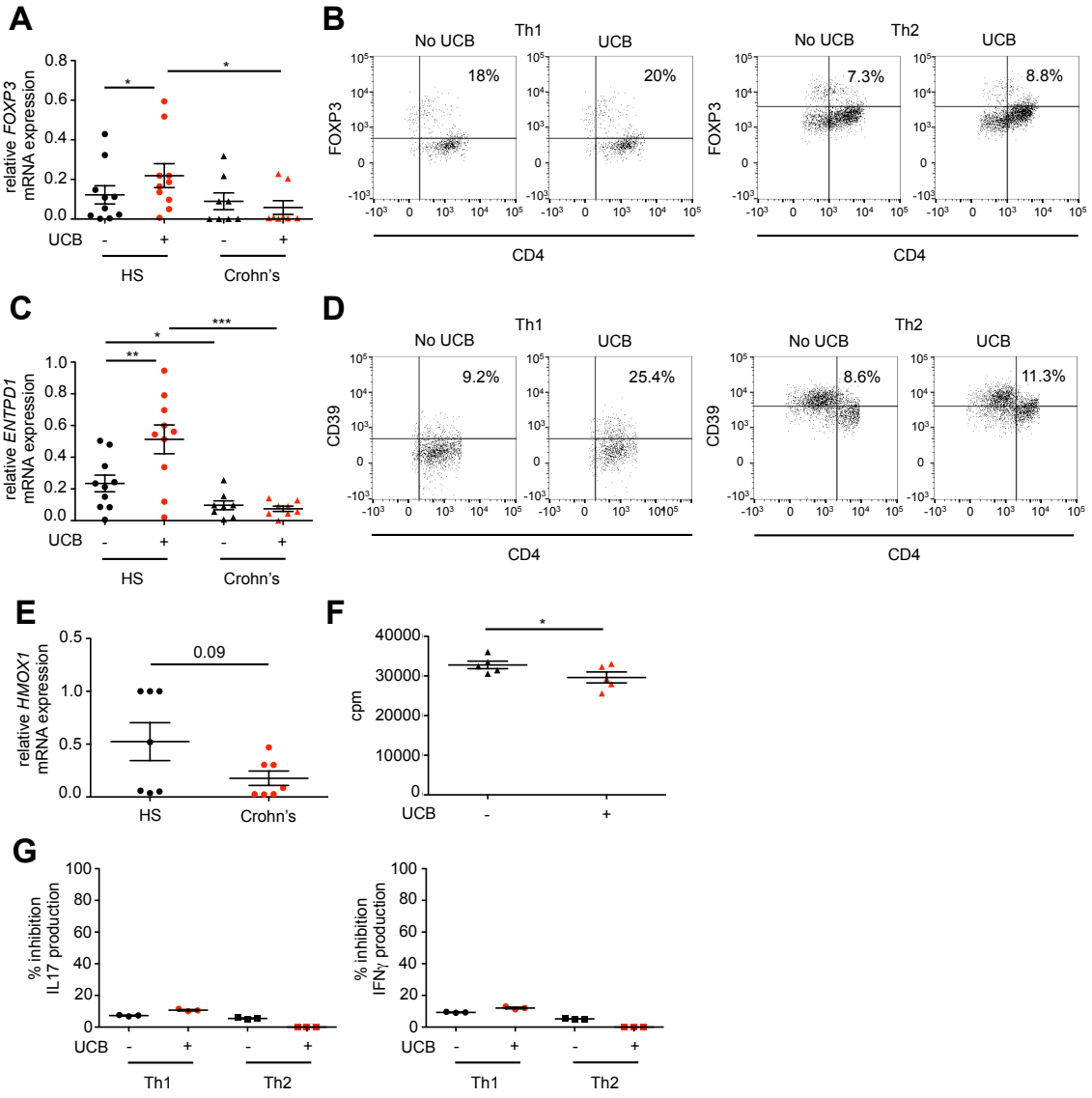
Longhi et al.

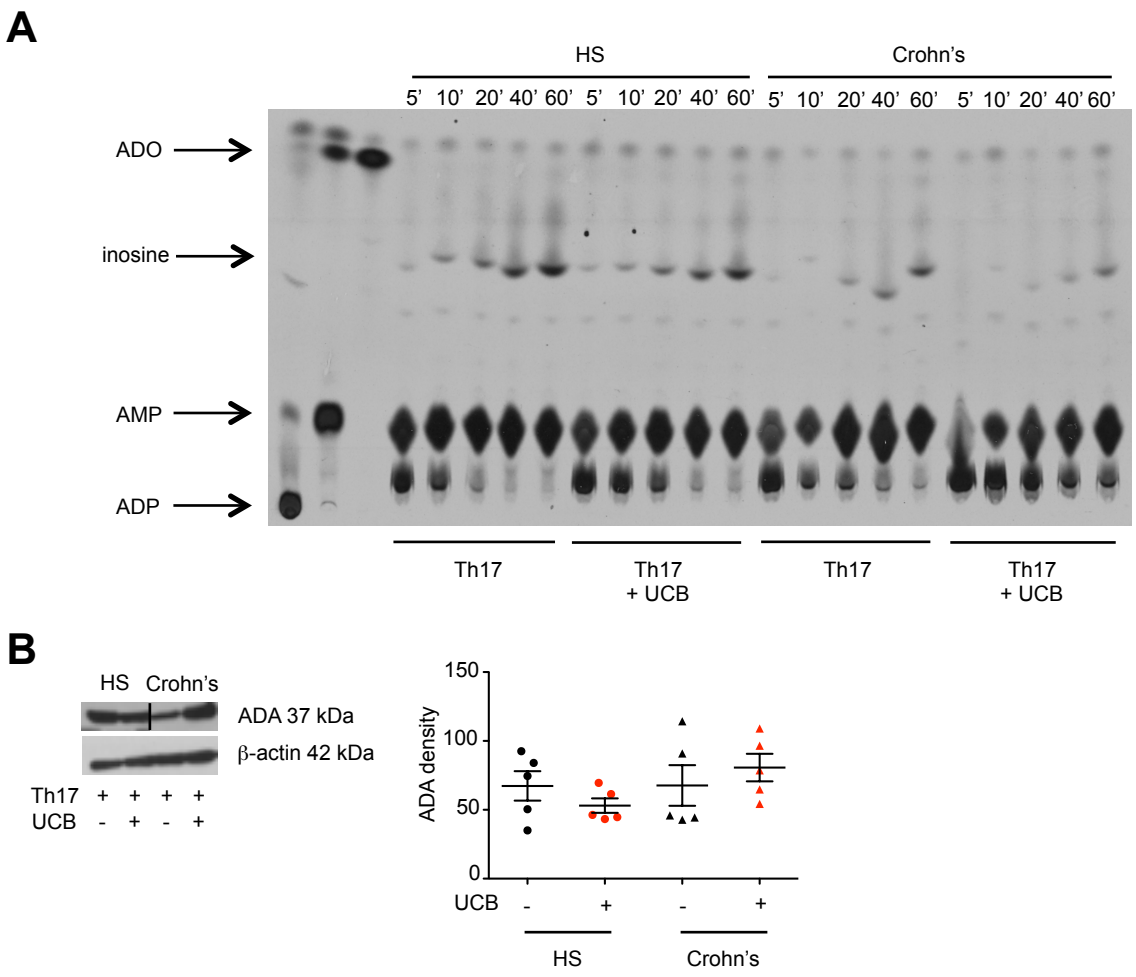
Bilirubin suppresses Th17 immunity in colitis by upregulating CD39

Supplemental material

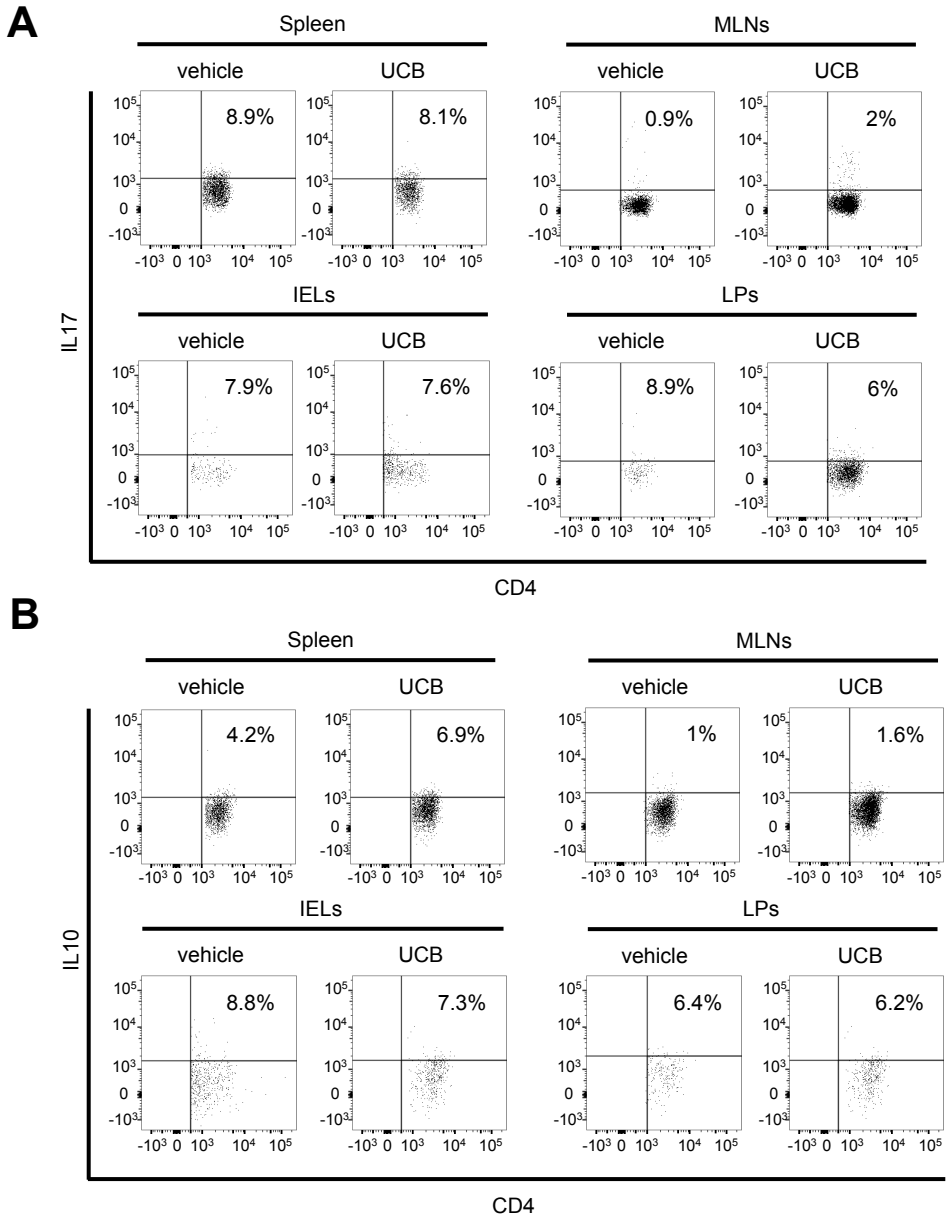


Supplemental Figure 1. Effects of UCB on Th17, Th1 and Th2 lymphocytes in health and Crohn's disease. (A) Mean±SEM relative *FOXP3* mRNA expression of untreated and UCB-treated Th17 cells obtained from peripheral blood CD4 cells in the presence of IL6, IL1β and TGFβ (n=10 HS and n=8 Crohn's disease patients). **(B)** The effects of UCB were also determined in Th1 and Th2 lymphocytes, derived from CD4 cells upon exposure to Th1 (i.e. IL12+anti-IL4) or Th2 (i.e. IL4+anti-IFNγ) skewing conditions. After 5 days skewing, Th1 and Th2 cells were exposed to UCB for the last 6 hours of culture. Frequencies of $FOXP3^+$ cells within untreated and UCB-treated Th1 and Th2 lymphocytes were determined by flow cytometry. Representative flow cytometry plots of CD4 and FOXP3 from one representative healthy individual are shown. Cells are gated on $IFN\gamma^+$ lymphocytes. **(C)** Mean±SEM relative *ENTPD1* mRNA expression of untreated and UCB-treated Th17 cells (n=10 HS and n=8 Crohn's disease patients). **(D)** Representative flow cytometry plots of CD4 and CD39 fluorescence in Th1 and Th2 lymphocytes from one healthy individual are shown. Cells are gated on $IL4^+$

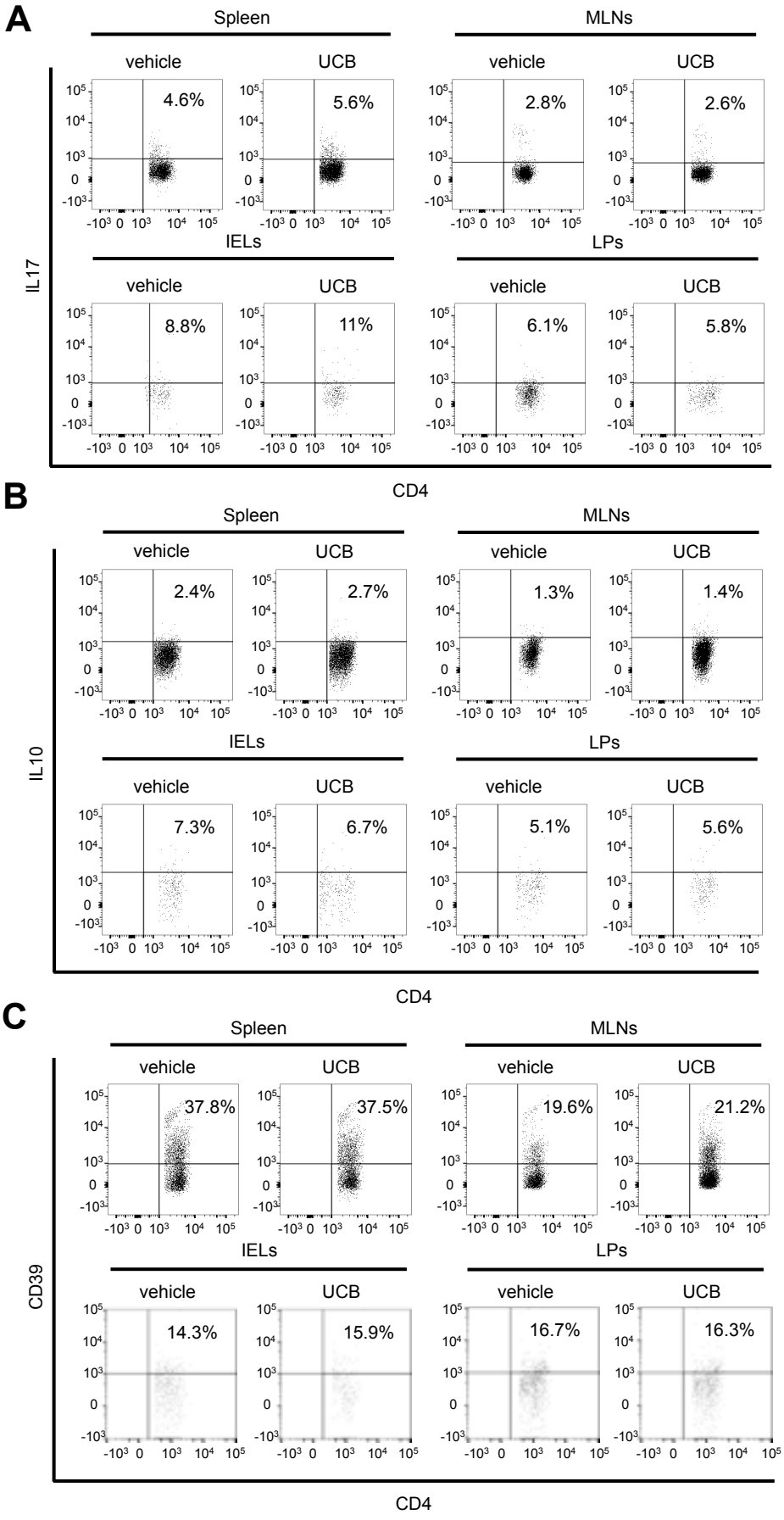
lymphocytes. **(E)** Mean±SEM relative *HMOX1* mRNA expression in peripheral blood-derived Th17 cells in n=7 HS and in n=7 patients with Crohn's disease. **(F)** Mean±SEM cpm of untreated and UCB-treated Th17 cells (n=5 Crohn's disease). **(G)** % inhibition of IL17 and IFNγ production by $CD4^{+}CD25^{-}$ responders in the presence of untreated and UCB-treated Th1 and Th2 cells in HS (n=3). P value derived using One-way ANOVA, followed by Tukey's multiple comparisons test (Panels A and C), or paired *t* test (Panels E and F). *P≤0.05; **P≤0.01; ***P≤0.001. UCB: unconjugated bilirubin; HS: healthy subjects; *HMOX1*: heme oxygenase 1.



Supplemental Figure 2: Effect of UCB on Th17 cell ADPase ectoenzymatic activity. **(A)** ADPase ectoenzymatic activity of untreated and UCB-treated Th17 cells was determined by TLC upon cell incubation with ^{14}C-labeled ADP. A representative of 4 independent experiments is shown. **(B)** Western Blot analysis of ADA expression of untreated and UCB-treated Th17 cells from one representative HS and one Crohn's patient. For the ADA immunoblot analysis lanes representative of samples from HS and Crohn's patient were run on the same gel but were noncontiguous. Mean±SEM ADA density levels of untreated and UCB-treated Th17 cells (n=5 HS and n=5 CD patients) are also shown. HS: healthy subjects; ADO: adenosine; UCB: unconjugated bilirubin; ADA: adenosine deaminase.



Supplemental Figure 3. UCB has limited impact on immunophenotypes of *Entpd1*$^{-/-}$ mice. Dot plots showing the frequency of **(A)** $CD4^{+}IL17^{+}$ and **(B)** $CD4^{+}IL10^{+}$ lymphocytes among mononuclear cells from the spleen, MLNs, IELs and LPs in a representative *Entpd1*$^{-/-}$ mouse. UCB: unconjugated bilirubin; MLNs: mesenteric lymph nodes; IELs: intra-epithelial lymphocytes; LPs: lamina propria lymphocytes.


A
Spleen
MLNs
vehicle
UCB
vehicle
UCB
4.6%
5.6%
2.8%
2.6%
IELs
LPs
vehicle
UCB
vehicle
UCB
8.8%
11%
6.1%
5.8%
IL17
CD4
B
Spleen
MLNs
vehicle
UCB
vehicle
UCB
2.4%
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1.3%
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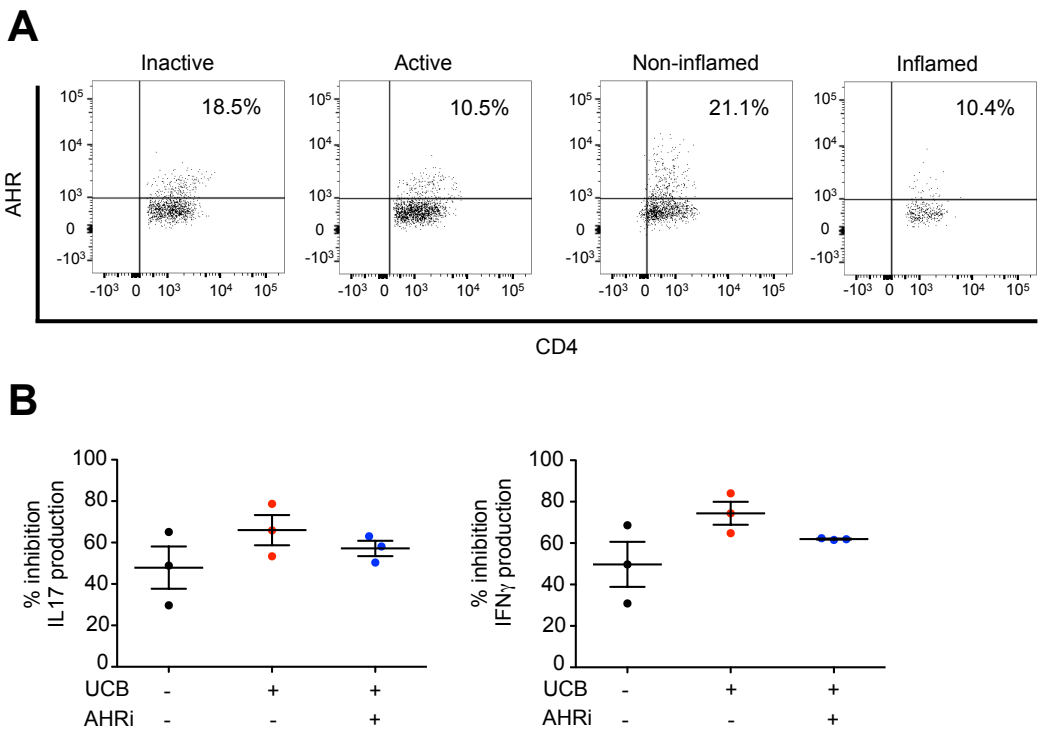
IELs
LPs
vehicle
UCB
vehicle
UCB
7.3%
6.7%
5.1%
5.6%
IL10
CD4
C
Spleen
MLNs
vehicle
UCB
vehicle
UCB
37.8%
37.5%
19.6%
21.2%
IELs
LPs
vehicle
UCB
vehicle
UCB
14.3%
15.9%
16.7%
16.3%
CD39
CD4

Supplemental Figure 4. UCB has limited impact on immunophenotypes of *Ahr*^{d} mice. Dot plots showing the frequency of **(A)** $CD4^{+}IL17^{+}$ and **(B)** $CD4^{+}IL10^{+}$ and **(C)** $CD4^{+}CD39^{+}$ lymphocytes among mononuclear cells from the spleen, MLNs, IELs and LPs in a representative *Ahr*^{d} mouse. UCB: unconjugated bilirubin; MLNs: mesenteric lymph nodes; IELs: intra-epithelial lymphocytes; LPs: lamina propria lymphocytes.



Supplemental Figure 5. Diminished AHR expression in patients with active Crohn's disease and effects of AHR inhibition on Th17 cell suppressive function. (A) Dot plots showing the frequency of $CD4^{+}AHR^{+}$ cells within LPMC-derived Th17 cells obtained from representative Crohn's patients with inactive and active disease; and from non-inflamed and inflamed bioptic colonic areas. **(B)** Mean±SEM % inhibition of IL17 and IFNγ production by untreated, UCB-treated and UCB+AHRi-treated Th17 cells (n=3 HS). AHR: aryl hydrocarbon receptor; UCB: unconjugated bilirubin; AHRi: aryl hydrocarbon receptor inhibitor.