Published in final edited form as:

Science. 2015 May 29; 348(6238): 1031–1035. doi:10.1126/science.aaa4812.

Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific CD4+ T cells

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Abstract

Inflammatory CD4+ T cell responses to self or commensal bacteria underlie the pathogenesis of autoimmunity and inflammatory bowel disease (IBD), respectively. While selection of self-specific T cells in the thymus limits responses to tissue antigens, the mechanisms that control selection of commensal bacteria-specific T cells remain poorly understood. Here we demonstrate that group 3 innate lymphoid cell (ILC3)-intrinsic expression of major histocompatibility complex class II (MHCII) is regulated similarly to thymic epithelial cells, and that MHCII+ ILC3s directly induce cell death of activated commensal bacteria-specific T cells. Further, MHCII on human colonic ILC3s was reduced in pediatric IBD patients. Collectively, these results define a selection pathway for commensal bacteria-specific CD4+ T cells in the intestine, and suggest that this process is dysregulated in human IBD.

Keywords

Commensal bacteria; T cell selection; innate lymphoid cell; inflammatory bowel disease

Pathologic CD4+ T cell responses to self are limited by presentation of self-antigens in the thymus on MHCII+ thymic epithelial cells (TECs) and dendritic cells (DCs), resulting in

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clonal deletion (1-6). In contrast, commensal bacteria-specific $CD4^+$ T cells, which have been implicated in the pathogenesis of inflammatory bowel disease (IBD) (7-11), do not encounter cognate antigen in the thymus and therefore are not subject to negative selection prior to entering the periphery (12, 13). While physical and biochemical barriers separate the immune system from intestinal commensal bacteria (7, 13-17), antigens derived from commensal bacteria are continuously sampled from the intestinal lumen and presented by DCs in the draining lymph nodes (7, 10, 15, 18, 19). Regulatory T cells (T_{reg}) can in part limit dysregulated $CD4^+$ T cell responses to commensal bacteria (20, 21). However, whether other mechanisms control commensal bacteria-specific $CD4^+$ T cells in lieu of thymic negative selection is poorly defined. In recent studies, ILC3s were found to express MHCII and genetic deletion of ILC3-intrinsic MHCII resulted in spontaneous $CD4^+$ T cell-dependent intestinal inflammation (22), suggesting additional antigen-presentation pathways control commensal bacteria-specific $CD4^+$ T cell populations.

CCR6-expressing lymphoid tissue inducer (LTi)-like ILC3s ($CCR6^+$ ILC3s), are a major ILC subset present in the mesenteric lymph node (mLN) (Fig. 1A) and colon lamina propria (cLPL) (fig. S1A) of healthy mice, and constitutively express retinoic acid-related orphan receptor gamma t (RORγt) and MHCII, relative to $ST2^+$ group 2 ILCs (ILC2) (fig. S1B-C). ILC3s are regulated by various cytokine, environmental and microbial factors (23, 24). However, interleukin (IL)-23p19, the aryl hydrocarbon receptor (Ahr) or the intestinal microbiota were not required for $CCR6^+$ ILC3 expression of MHCII (fig. S1D-F), although ILC3 frequencies were reduced in the intestine of *Ahr*$^{-/-}$ mice, as previously described (fig. S1E) (25). Further, in contrast to a recent report (26), expression of MHCII, CD80 and CD86 on $CCR6^+$ ILC3s were unaffected by *ex vivo* stimulation with microbial or inflammatory stimuli, or by the absence of MyD88 or Caspase 1/11 *in vivo* (fig. S2).

Next we examined whether expression of ILC3-intrinsic MHCII was dependent on the class II transactivator (CIITA), a master transcriptional regulator of MHCII expression (27). MHCII expression was absent on $CCR6^+$ ILC3s from *Ciita*$^{-/-}$ mice, relative to *Ciita*$^{+/+}$ control mice (Fig. 1B and fig. S3A).

Transcription of CIITA in mice is driven via distinct promoter elements, termed pI, pIII and pIV, that are indicative of the upstream signaling events that induce MHCII expression (27). In contrast to B cells and DCs, $CCR6^+$ ILC3-intrinsic MHCII expression was absent in the mLN of both pIII/pIV$^{-/-}$ and pIV$^{-/-}$ mice (Fig 1C, fig. S3B-C), indicating that the pIV promoter of *Ciita* is required for MHCII expression on $CCR6^+$ ILC3s. The pIV promoter of *Ciita* is utilized by multiple cell types, such as epithelial cells, in response to interferon (IFN)-γ signaling (27). However, expression of MHCII on $CCR6^+$ ILC3s was not impaired in the absence of IFN-γ, IFN-γR1 or STAT-1 (Fig. 1D, fig. S3D-E). MHCII expression in TECs is also dependent upon the pIV promoter of *Ciita* (fig. S3A-B) and pIV-dependent, IFN-γ-independent CIITA expression has previously only been described in TECs (27-29), suggesting a previously unappreciated link between these cell types.

These data provoked the hypothesis that TECs and $MHCII^+$ ILC3s share similar functional roles in the selection of $CD4^+$ T cells. To test this, we examined $CD4^+$ T cells in the intestine of mice with an ILC3-intrinsic deletion in MHCII ($MHCII^{\Delta ILC3}$ mice). As we

previously reported (22), frequencies and cell numbers of $CD44^{hi}$ $CD4^+$ T cells (T_{eff}) in the cLPL of $MHCII^{\Delta ILC3}$ mice were increased relative to *H2-Ab1*$^{fl/fl}$ controls (fig. S4A-B). In contrast, the total numbers of $CD44^{lo}$ naïve T cells and $FoxP3^+$ T_{reg} were unchanged (fig. S4B). T_{eff} cell populations utilized a broad range of T cell receptor (TCR) Vβ chains and T_{eff} expansion was detected in the cLPL but not in the thymus (fig. S4C). Further, sort-purified $CD4^+$ T cells from $MHCII^{\Delta ILC3}$ mice responded to fecal-derived antigen, but not mammalian tissue-derived antigens (fig. S4D).

These data, along with previous studies (20, 30, 31), suggest the majority of $CD4^+$ T cells in the steady-state intestine are specific for commensal bacteria and that ILC3-intrinsic MHCII controls commensal bacteria-specific $CD4^+$ T cell responses through direct presentation of microbiota-derived antigens. To test this, we crossed $MHCII^{\Delta ILC3}$ mice with either TCR transgenic mice specific for CBir1, an antigen expressed by *Clostridia* species constitutively present in the murine and human microbiota (13, 32), or TCR transgenic mice specific for ovalbumin (OT-II). Loss of ILC3-intrinsic MHCII had no effect on the frequencies or cell numbers of OT-II T cells or CBir1 T cells in the thymus (Fig. 2A). In contrast, numbers of CBir1, but not OT-II, T cells were increased in the cLPL and mLN (Fig. 2B and fig. S5A), and $CBir1^{MHCII\Delta ILC3}$ mice exhibited increased frequencies of antigen-specific IFN-γ^+ and tumor necrosis factor (TNF)-α^+ colonic $CD4^+$ T cells, colonic inflammation and neutrophil infiltration, which could be prevented by administration of antibiotics (ABX), and was not observed in *Rag1*$^{-/-}$ $MHCII^{\Delta ILC3}$ mice (Fig. 2B-D and fig. S5A-D). Finally, similar to polyclonal $MHCII^{\Delta ILC3}$ mice, the population expansion and increased cytokine production of CBir1 $CD4^+$ T cells was associated with a selective increase in $CD44^+CD62L^{lo}$ T_{eff}, whereas numbers of $FoxP3^+$ T_{reg} remained unchanged (fig. S5E). These data suggest that $CCR6^+$ ILC3s selectively limit the expansion of commensal bacteria-specific $CD4^+$ effector T cells through presentation of antigen derived from endogenous commensal bacteria. In support of this hypothesis and consistent with recent findings (33), $MHCII^+$ ILC3s localized in distinct clusters in the mLN at the interface between the B- and T- cell zones in the marginal and subcapsular sinus (Fig. 2E), a site through which antigen-experienced T cells traffic.

To investigate the *in vivo* mechanisms through which $MHCII^+$ ILC3s control commensal bacteria-specific $CD4^+$ T cells, mice were generated in which MHCII expression was restricted to only ILC3s. This was accomplished by utilizing mice with a floxed-STOP sequence cassette inserted between the first and second $IA\beta^b$ exons ($IA\beta^bSTOP^{fl/fl}$ mice) (34). $IA\beta^bSTOP^{fl/fl}$ mice lack MHCII on antigen-presenting cells in the absence of Cre recombinase ($MHCII^{neg}$) (Fig. 2G). In contrast, $IA\beta^bSTOP^{fl/fl}$ mice crossed with *Rorc*^{Cre} mice demonstrated a partial restoration of MHCII on $CCR6^+$ ILC3s ($MHCII^{ILC3+}$ mice) (Fig. 2F and G), but not B cells, DCs and macrophages (Fig. 2G). As $MHCII^{neg}$ and $MHCII^{ILC3+}$ mice lack MHCII on TECs and have disrupted endogenous T cell selection (34), we first employed an adoptive transfer approach with naïve CFSE-labeled $CD45.1^+$ CBir1 T cells. In mice with normal MHCII expression ($MHCII^{pos}$) administration of CBir1 peptide resulted in dilution of CFSE in transferred CBir1 T cells, expansion of $CD44^{hi}CD62L^{lo}$ CBir1 T_{eff} cells and an increase in $FoxP3^+$ T_{reg} in the mLN (fig. S6A). In contrast, naïve CBir1 $CD4^+$ T cells transferred into control $MHCII^{neg}$ and $MHCII^{ILC3+}$ mice

failed to proliferate or differentiate into T_{eff} or T_{reg} following peptide administration (fig. S6A), suggesting that ILC3-intrinsic MHCII does not influence naïve $CD4^+$ T cells. As $MHCII^+$ ILC3s localize at lymphoid sites through which antigen-experienced $CD4^+$ T cells traffic (Fig. 2E) (33), CBir1 $CD4^+$ T cells were pre-activated overnight prior to transfer into $MHCII^{neg}$ or $MHCII^{ILC3+}$ mice. Following CBir1 peptide administration, $MHCII^{ILC3+}$ mice exhibited reduced frequencies and numbers of pre-activated CBir1 $CD4^+$ T cells in the mLN (Fig. 2H-I), which was due to a selective decrease in effector, but not regulatory CBir1 T cells (Fig. 2I). $MHCII^+$ ILC3-mediated effects were antigen-specific, could be driven by endogenous microbiota-derived antigen alone and were specific to ILC3s (fig. S6B-D and fig. S7). Further, in complementary loss-of-function studies, CBir1 $CD4^+$ T_{eff} expanded at higher frequencies and numbers in the mLN and cLPL of mice lacking ILC3-intrinsic MHCII ($MHCII^{\Delta ILC3}$ mice), despite exhibiting comparable proliferation (fig. S8). Finally, in line with ILC3-mediated control of antigen-experienced T cells, the frequency and number of activated CBir1 $CD4^+$ T cells reaching the small intestine lamina propria (siLPL) and cLPL of $MHCII^{ILC3+}$ mice were significantly decreased relative to $MHCII^{neg}$ controls (Fig. 2J-K).

We hypothesized that the loss of CBir1 $CD4^+$ T cells from the mLN and intestine of $MHCII^{ILC3+}$ mice could be the result of altered migration, proliferation or through induction of cell death. However, pre-activated CBir1 T cells did not accumulate in peripheral organs, such as the spleen (fig. S9A) and CBir1 T cells recovered from $MHCII^{ILC3+}$ mice exhibited comparable proliferation, relative to $MHCII^{neg}$ mice (fig. S9B). To further investigate this question, we developed an *in vitro* ILC3-$CD4^+$ T cell co-culture system. Consistent with our *in vivo* findings, co-culture of activated CBir1 T cells and sort-purified $MHCII^+$ $CCR6^+$ ILC3s resulted in a significant reduction in T cell numbers following culture in the presence of cognate antigen, which could be reversed by administration of an MHCII-blocking antibody (Fig. 3A). Reduced cell recovery was associated with an antigen and MHCII-dependent increase in Caspase-3 activation (Fig. 3B) and increased Annexin V staining (Fig. 3C) in the remaining CBir1 T cells, indicative of programmed cell death. Despite selectively regulating commensal bacteria-specific $CD4^+$ T cells in the steady state, $MHCII^+$ ILC3s were also sufficient to influence T cells with other antigen-specificities only if antigen was systemically provided (fig. S10).

Negative selection in the thymus has been shown to be associated with induction of Nur77 and subsequent upregulation of the pro-apoptotic molecule Bim (35). Antigen-dependent interactions between CBir1 T cells by $MHCII^+$ ILC3s also resulted in the upregulation of Nur77 as well as Bim, which was required for ILC3-mediated induction of cell death (Fig. 3D-G). Antigen-presentation by ILC3s *in vitro* selectively led to T_{eff} death, but did not affect T_{reg} numbers (fig. S11A). We next analyzed mLN-derived $CCR6^+$ ILC3 for expression of surface molecules that directly influence antigen-specific $CD4^+$ T cell responses (Fig. 3H). ILC3s demonstrated high levels of MHCII-associated transcripts, but had negligible expression of transcripts for canonical co-stimulatory molecules and inhibitory or death receptors (Fig. 3H). Indeed, $CCR6^+$ ILC3s lacked expression of FasL by flow cytometry and antibody-mediated neutralization of FasL did not influence ILC3-induced CBir1 T cell death (fig. S11B-D). Moreover, in contrast to *Bim*$^{-/-}$ mice, $FasL^{gld/gld}$

mice did not exhibit increased frequencies of endogenous commensal bacteria-specific $CD4^+$ T cells in gut-associated lymphoid tissues (fig. S11E).

Bim-dependent apoptotic cell death may also be induced via cytokine or growth factor starvation (36, 37). As $CCR6^+$ ILC3s constitutively express high levels of the common gamma chain cytokine receptors CD25 (IL-2R) and CD127 (IL-7Rα) (Fig. 1, fig. S1 and Fig. 3H), we hypothesized that $MHCII^+$ ILC3s may induce cell death of commensal bacteria-specific $CD4^+$ T cells synergistically through TCR-induction of an apoptotic program in concert with cytokine withdrawal. Consistent with this, cell death could be reduced upon addition of exogenous recombinant (r)IL-2, but not rIL-7, to *in vitro* co-cultures (Fig. 3I). $MHCII^+$ ILC3s exhibited over twofold higher capacity to bind IL-2 as compared to activated CBir1 $CD4^+$ T cells (fig. S11F-G), suggesting $MHCII^+$ ILC3s out-compete activated T cells for pro-survival cytokines. The requirement for IL-2 was T cell-intrinsic as activated CBir1 $CD4^+$ cells expressing a constitutively active STAT-5 molecule (S5CA), were resistant to Bim upregulation and ILC3-induced cell death *in vitro* and *in vivo* (Fig. 3J-L and fig. S11H-I). Taken together these data indicate that $MHCII^+$ ILC3s mediate a negative selection process through antigen-presentation and withdrawal of IL-2 from the local milieu, resulting in deletion of activated commensal bacteria-specific T cells.

Inflammatory $CD4^+$ T cell responses against commensal bacteria are causally associated with the pathogenesis of IBD (7-11). Furthermore, inflammatory T cells derived from Crohn's disease patients also exhibit reduced Bim-mediated cell death and cytokine-withdrawal-mediated apoptosis (38, 39). Therefore, we next examined whether ILC3-intrinsic MHCII may be dysregulated in the context of human IBD. Using a previously defined gating strategy (40), all ILC subsets could be identified in intestinal biopsies of pediatric Crohn's disease patients (Fig. 4A), including $CD127^+$ $c\text{-}kit^+$ $ST2L^-$ ILC3s, that expressed NKp44 and RORγt (fig. S12A-B). Human ILC3s expressed MHCII (HLA-DR) in intestinal biopsies from non-IBD controls, whereas MHCII expression was largely absent on other ILC subsets (Fig. 4B). Although no alterations in the total frequency of ILC3s were observed between patient cohorts (fig. S12C), MHCII expression was significantly reduced on ILC3s (Fig. 4C-E), but not $CD4^+$ T cells or professional antigen-presenting cells (fig. S12D-E), from pediatric Crohn's disease patients in comparison to non-IBD controls. Moreover, we observed an inverse correlation between MHCII levels on ILC3s and frequencies of effector T helper 17 (Th17) cells (Fig. 4F), and circulating commensal bacteria-specific immunoglobulin (Ig)G titers (Fig. 4G), in pediatric Crohn's disease patients. Taken together, these data indicate alterations in MHCII on human ILC3s are associated with elevated commensal bacteria-specific inflammatory responses.

The mammalian gastrointestinal tract is colonized with trillions of beneficial commensal bacteria that regulate host nutrient metabolism, immune cell homeostasis and protect from pathogen infection (7, 11, 15). As such, commensal bacteria are an essential component of the mammalian “superorganism” required for the host to thrive (41). It is well characterized that self-specific $CD4^+$ T cells with the potential to cause pathologic inflammation in mammalian tissues are controlled through antigen-dependent thymic selection (1-5). Here, MHCII-expressing $CCR6^+$ ILC3s were found to control intestinal homeostasis through induction of apoptotic cell death and deletion of activated commensal bacteria-specific T

cells, a process with multiple similarities to negative selection in the thymus, which we propose to call “intestinal selection” (fig. S13). Thus, intestinal selection controls the peripheral commensal bacteria-specific $CD4^+$ T cell pool in concert with other previously described tolerogenic pathways, including T_{reg}, production of IgA and active maintenance of intestinal barrier function (7, 15, 17, 23). Dysregulated ILC3-intrinsic MHCII in pediatric Crohn's disease patients suggests a possible role for alterations in this pathway in the onset and/or progression of human IBD. Thus, $MHCII^+$ ILC3 may represent a novel therapeutic target to control pathologic $CD4^+$ T cell responses in chronic human inflammatory disorders associated with dysregulated host-commensal bacteria relationships (7, 10, 15).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Members of the Sonnenberg laboratory are thanked for discussions and critical reading of the manuscript. The authors thank C Hunter and S Wagage (University of Pennsylvania) for the Ahr-deficient mice, I Brodsky (University of Pennsylvania) for the Caspase 1/11-deficient mice, and M Jenkins, J Walter and T Dileepan (University of Minnesota) for tetramer reagents and protocols. Data presented in this manuscript are tabulated in the main paper and in the supplementary materials. Microarray data is accessible at GEO (http://www.ncbi.nlm.nih.gov/geo/) via accession number GSE67076. We thank the University of Alabama, Institut Pasteur, University of Minnesota and Janssen Research and Development LLC for sharing mouse strains by material transfer agreement. Research in the Sonnenberg laboratory is supported by the National Institutes of Health (DP5OD012116), the NIAID Mucosal Immunology Studies Team (MIST) Scholar Award in Mucosal Immunity and the Institute for Translational Medicine and Therapeutics Transdisciplinary Program in Translational Medicine and Therapeutics (UL1-RR024134 from the US National Center for Research Resources). MRH is supported by a research fellowship from the Crohn's and Colitis Foundation of America (CCFA, #297365). TCF is supported by a Cancer Research Institute Student Training and Research in Tumor immunology (STaRT) grant. DRW is supported by a Wellcome Trust Research Career Development Fellowship. COE is supported by the National Institutes of Health (DK071176).

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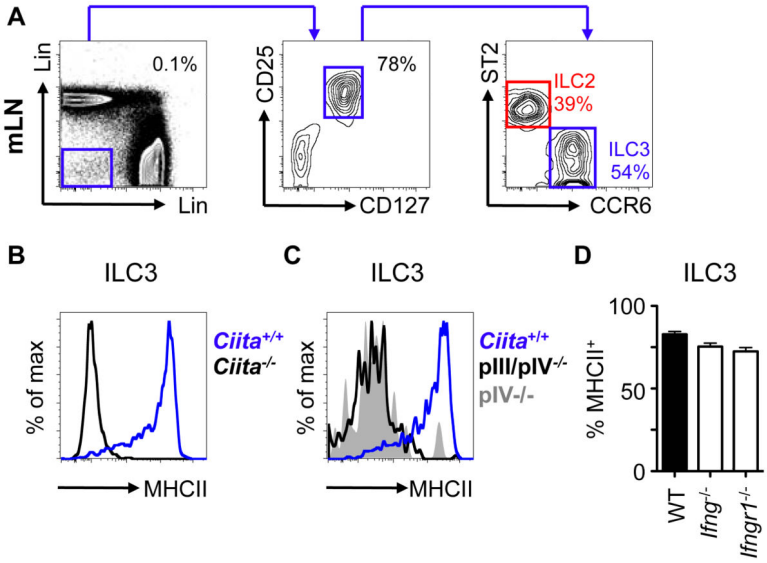

Fig. 1. ILC3 expression of MHCII is controlled by a transcriptional pathway previously associated with thymic epithelial cells

A) Mesenteric lymph node (mLN) cells from naïve mice were gated as $CD45^+$ lineage (x-axis; CD3, CD5, CD8, NK1.1, y-axis; B220, CD11c, CD11b) negative, $CD25^+$ and $CD127^+$ and further divided by expression of ST2 (ILC2; red) or CCR6 (ILC3; blue). MHCII expression was determined on ILC3s in mice deficient in **B)** CIITA, **C)** CIITA promoter regions (pIII/pIV, pIV) or **D)** IFN-γ and IFN-γR1. All data representative of at least 3 independent experiments with n=2-3 mice per group. SPF, specific pathogen free.

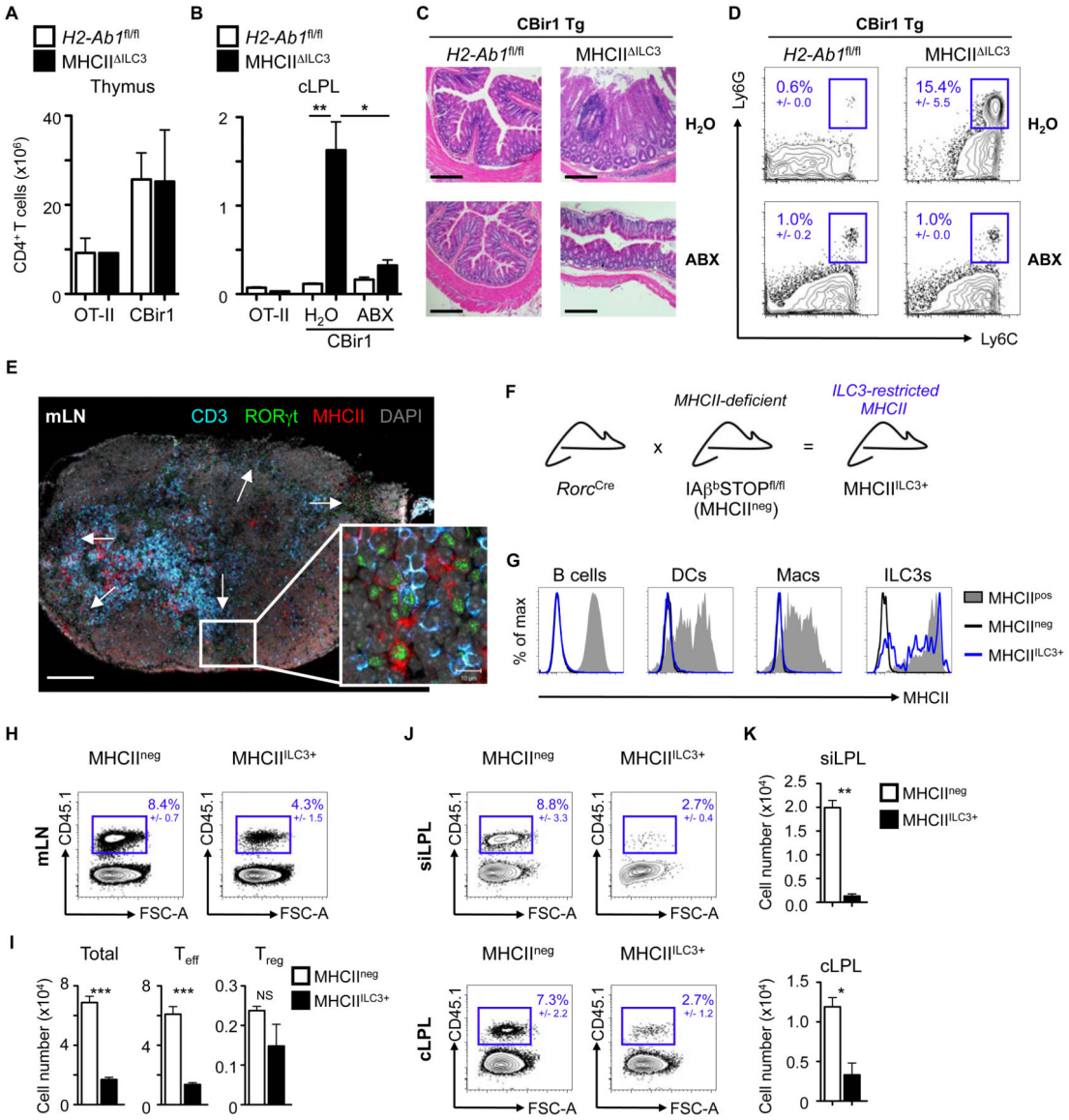

Fig. 2. MHCII^{+} ILC3s induce deletion of commensal bacteria-specific CD4^{+} T effector cells in the intestine and associated lymph nodes

Total Vβ5^{+} (OT-II) or Vβ8.3^{+} (CBir1) CD4^{+} T cell numbers determined in **A)** the thymus and **B)** cLPL of control (H_20) or antibiotic (ABX)-treated OT-II and CBir1 transgenic mice crossed with MHCII$^{\Delta ILC3}$ or *H2-Ab1*$^{fl/fl}$ littermate controls. **C)** Colon histology (scale bar 200μm) and **D)** frequencies of (CD45^{+} B220^{-} CD3^{-}) Ly6C^{+} Ly6G^{+} neutrophils in the cLPL of CBir1$^{MHCII\Delta ILC3}$ and CBir1^{H2}-*Ab1fl/fl* mice. **E)** Immunofluorescence imaging of mLN sections stained for CD3 (blue), RORγt (green), MHCII (red) or DAPI (grey). White arrows indicate RORγt^{+} cell clusters. Scale bar = 200μm. Insert demonstrates colocalization of RORγt^{+} MHCII^{+} ILCs and CD3^{+} T cells. Insert scale bar = 10μm. **F)** MHCII expression was restricted to RORγt^{+} ILC3s (MHCII^{ILC3+} mice) by crossing *Rorc*^{Cre} mice with IAβ^{b}STOP$^{fl/fl}$ (MHCII^{neg}) mice and **G)** MHCII levels were determined on B220^{+} B cells, CD11b^{+} CD11c^{hi} dendritic cells (DCs), CD11b^{+} F4/80^{+} macrophages (Macs) or Lin^{neg} CD25^{+} CD127^{+} CCR6^{+} ILC3s in the mLN of heterozygote littermates (IAβ^{b}STOP$^{+/fl}$,

MHCII^{pos}), MHCII^{neg} or MHCII^{ILC3+} mice. (H-K) MHCII^{neg} and MHCII^{ILC3+} received pre-activated CD45.1^{+} CBir1 transgenic CD4^{+} T cells and were injected with CBir1 peptide i.p. every 2 days. Frequencies and numbers of transferred T cells were analyzed in the mLN **(H,I)**, siLPL and cLPL 9 days post transfer **(J,K)**. All data representative of at least 4 independent experiments with n=2-3 mice per group. Results are shown as the mean +/- s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (two-tailed students t-test).

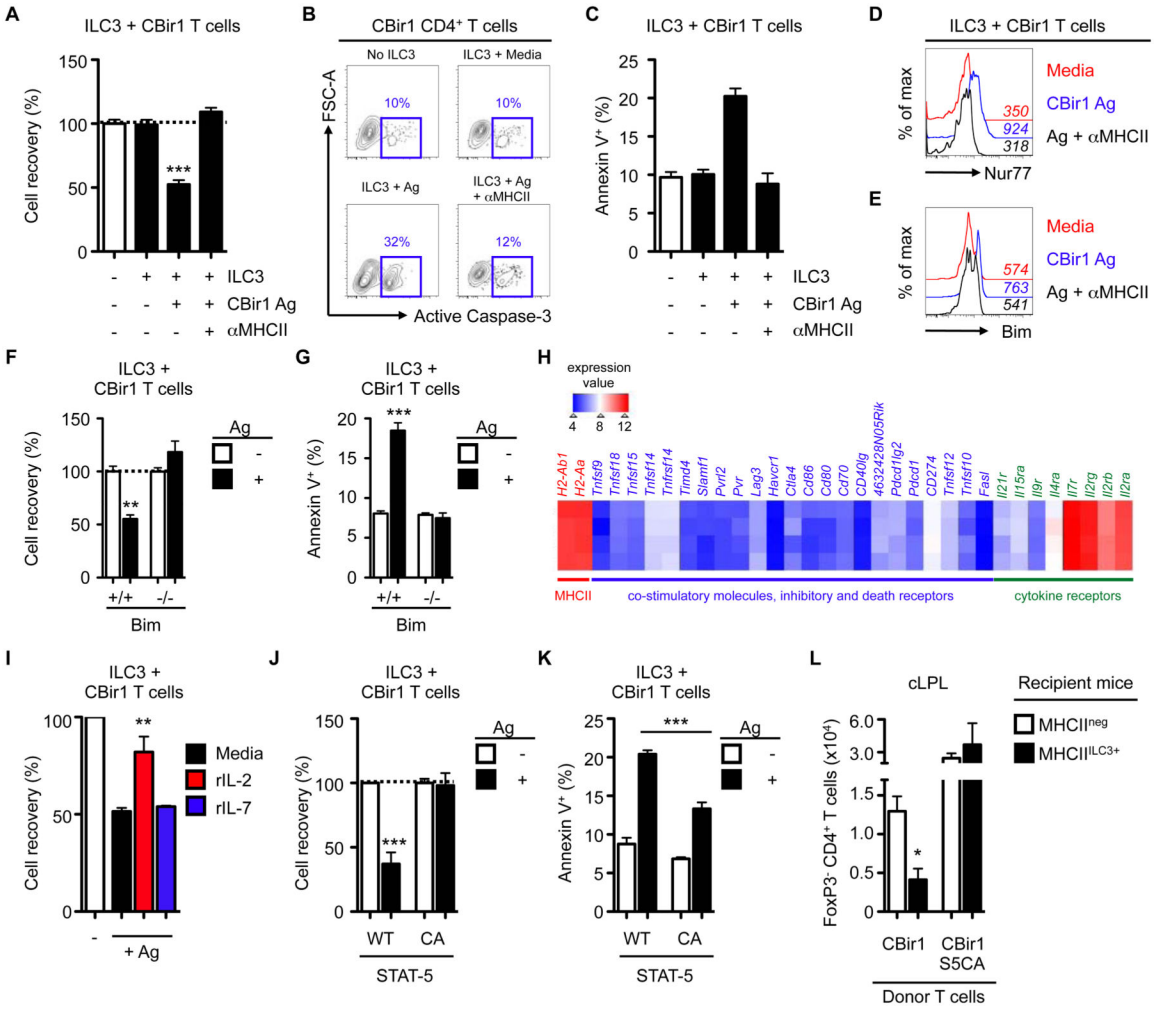

Fig. 3. MHCII$^+$ ILC3s directly induce cell death of commensal bacteria-specific CD4$^+$ T cells

(A-D) Activated CBir1 CD4$^+$ T cells were co-cultured with sort-purified CCR6$^+$ ILC3s in the presence or absence of CBir1 antigen or an anti-MHCII neutralizing antibody and **A)** relative cell recovery (%) was quantified relative to T cells cultured alone, **B)** frequency of active Caspase-3 expressing cells were assessed and **C)** frequencies of Annexin V$^+$ Dead cell exclusion dye-negative (pre-apoptotic) cells were quantified. **D)** Expression of Nur77 by CBir1 CD4$^+$ T cells and **E)** expression of Bim by pre-activated CBir1 CD4$^+$ T cells following 24h (D) or 48h (E) co-culture with antigen-pulsed MHCII$^+$ ILC3s in the presence or absence of an anti-MHCII neutralizing antibody. Mean fluorescent intensity (MFI) values are shown in italics. **(F-G)** *Bim*$^{+/+}$ or *Bim*$^{-/-}$ CBir1 T cells were co-cultured with ILC3s in the presence or absence of CBir1 antigen for 48h and **F)** relative cell recovery (%) and **G)** frequencies of Annexin V$^+$ pre-apoptotic cells were quantified. **H)** Heat map of selected candidate genes from mLN-derived CCR6$^+$ ILC3s. **I)** Relative cell recovery (%) of wildtype CBir1 CD4$^+$ T cells co-cultured with antigen-pulsed ILC3s in the presence or absence of exogenous rIL-2 or rIL-7. **(J-K)** Activated CBir1 CD4$^+$ T cells with wildtype or (WT) or constitutively active (CA) STAT-5 signaling were co-cultured with ILC3s in the presence or absence of CBir1 antigen for 48h and **J)** relative cell recovery (%) and **K)** frequencies of

Annexin V^+ pre-apoptotic cells were quantified. **L)** WT CBir1 $CD4^+$ T cells or CBir1 STAT5-CA $CD4^+$ T cells were adoptively transferred into $MHCII^{neg}$ or $MHCII^{ILC3+}$ mice. Mice were administered CBir1 antigen and numbers of $FoxP3^-$ $CD45.1^+$ CBir1 T cells in the cLPL were quantified 9 days post-transfer. *In vitro* assay data are representative of at least 2-3 independent experiments with 2-3 biological replicates per experiment. Array data are representative of a single experiment with 4 biological replicates. All *in vivo* data are representative of at least 2 independent experiments with at least n=3 mice per group. Results are shown as the mean +/- s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (two-tailed students t-test).

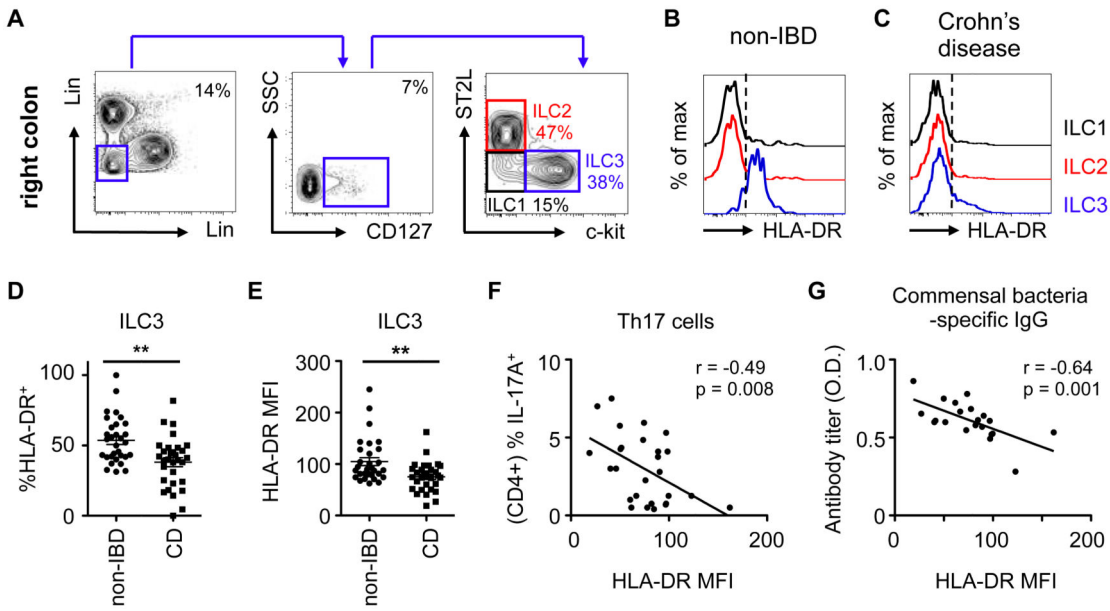

Fig. 4. ILC3-intrinsic MHCII is dysregulated in pediatric Crohn's disease patients and is associated with increased intestinal Th17 cells

A) Lamina propria cells were isolated from colon biopsies from non-IBD control patients and ILCs were identified as $CD45^+$ and lineage (x-axis; CD3, CD5, CD14, FcεRI, y-axis; CD11b CD11c, and CD19) negative, $CD127^+$ and further divided by expression of ST2L (ILC2s; red) and c-kit (ILC3s; blue) or as lacking expression of both markers (ILC1s; black). Expression of MHC class II (HLA-DR) was then determined on ILC subsets in representative biopsies from **B)** non-IBD patients or **C)** pediatric Crohn's disease (CD) patients, and the **D)** frequencies and **E)** mean fluorescent intensity of HLA-DR expression on ILC3s was quantified. ILC3 HLA-DR MFI was correlated with **F)** frequencies of IL-$17A^+$ $CD4^+$ Th17 cells in colon biopsies and **G)** commensal bacterial-specific IgG was quantified in the sera of pediatric Crohn's disease patients. **(B-E)** Representative of n=31 non-IBD and n=31 CD patients or **F)** n=27 and **G)** n=21 pediatric Crohn's disease patients. Results are shown as the mean +/- s.e.m. Statistical analyses between patient groups are performed using a Mann-Whitney test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Correlative analyses were compared by parametric Pearson's rank correlation coefficient (r).