Campylobacter infection promotes IFNγ-dependent intestinal pathology via ILC3 to ILC1 conversion

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Innate lymphoid cells (ILCs) are a heterogeneous family of immune regulators that protect against mucosal pathogens but can also promote intestinal pathology. Although the plasticity between ILCs populations has been described, the role of mucosal pathogens in inducing ILC conversion leading to intestinal pathology remains unclear. Here we demonstrate that IFNγ-producing ILCs are responsible for promoting intestinal pathology in a mouse model of enterocolitis caused by Campylobacter jejuni, a common human enteric pathogen. Phenotypic analysis revealed a distinct population of IFNγ-producing NK1.1+ T-bet+ ILCs that accumulated in the intestine of C. jejuni-infected mice. Adoptive transfer experiments demonstrated their capacity to promote intestinal pathology. Inactivation of T-bet in NKP46− ILCs ameliorated disease. Transcriptome analysis and cell-fate mapping experiments revealed that IFNγ-producing NK1.1+ ILCs correspond to ILC1 profile and develop from RORγT+ progenitors. Collectively, we identified a distinct population of NK1.1+ ex-ILC3s that promotes intestinal pathology through IFNγ production in response to C. jejuni infection.

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INTRODUCTION

Innate lymphoid cells (ILCs) are a recently identified family of immune cells that have emerged as important regulators of immune homeostasis at mucosal surfaces1–5. In the intestine ILCs contribute to immune defense by producing cytokines that protect epithelial barrier integrity and regulate the initiation, maintenance, and resolution of inflammation1–3. Although the protective roles of ILCs during infection or dissemination of commensal bacteria are well documented4–7, mounting evidence from clinical studies, supported by animal studies, suggest that dysregulated ILC responses can promote intestinal pathology8–10. IFNγ-producing ILC1s accumulate in the inflamed tissue of Crohn’s disease patients while ILC3s were diminished, suggesting that ILC1s play a pathogenic role in inflammatory bowel disease (IBD)8,10,11. Experimental evidence in mice lacking adaptive lymphocytes further defined the pathogenic potential of ILC1s8,9. Furthermore, cell fate-mapping experiments in mice provided evidence that IFNγ-producing ILC1s can develop from RORγT-expressing ILC3 progenitors, named as "ex-ILC3s" because of their cellular ontology12,13. However, the conditions that direct the in vivo conversion of ILCs, particularly the role of mucosal pathogens in this process has not been fully analyzed.

Campylobacter is a major human pathogen that infects an estimated 2.5 million people each year resulting in a $1.9 billion economic loss in the U.S.12,13. Among the Campylobacter species, C. jejuni is the primary human pathogen that causes gastroenteritis, which manifests as cramping and diarrhea12,13. In addition to these acute symptoms, mounting epidemiological evidence implicates Campylobacter infection as a cause of long-term intestinal dysfunction such as post-infectious irritable bowel syndrome, which appears to be immune mediated12,13. Experiments in mice support this hypothesis since infection of wild-type mice with C. jejuni causes persistent colonization but does not produce overt symptoms of disease. In contrast, mice lacking IL-10, a key anti-inflammatory cytokine, develop symptoms and pathology that resemble human campylobacteriosis14. Since IL-10 is known to suppress inflammation, and since C. jejuni infection causes disease in IL-10-deficient mice, it is thought that an overly aggressive host response by T cells promotes disease in this model of colitis; however, the role of ILCs in promoting inflammation remains controversial15,16.

In the present study, we investigated the role of ILCs in intestinal inflammation caused by C. jejuni. We found that a population of IFNγ-producing ILCs promoted colitis independently of T cells. These pathogenic ILCs lack NK1.1 and exhibited ILC3 > ILC1 lineage plasticity and, in accordance with the established nomenclature, we designate these cells as NK1.1− ex-ILC3s.
RESULTS

Innate lymphoid cells promote \textit{C. jejuni}-induced colitis

Prior studies established that IL-10\textsuperscript{−/−} mice, but not wild type mice, develop severe colitis when infected by \textit{C. jejuni}\textsuperscript{14,17}. The commensal microbiota strongly influences the development of colitis in IL-10\textsuperscript{−/−} mice\textsuperscript{18}. To exclude the variability associated with the microbiota and to facilitate colonization, IL-10\textsuperscript{−/−} and heterozygous littermates were pre-treated with an antibiotic cocktail in the drinking water prior to oral inoculation with \textit{C. jejuni} and evaluated for weight loss and diarrhea (Fig. 1a). Whereas infected IL-10 heterozygotes were asymptomatic, IL-10\textsuperscript{−/−} mice lost significant weight and began to succumb to \textit{C. jejuni} infection after 10 days (Fig. 1b). Gross examination of the intestine at day 10 revealed marked thickening and inflammation of the cecum and colon (data not shown). Histologically, inflammatory lesions consisted of mixed leukocytic mucosal and submucosal infiltrates with distention of the submucosa. Associated with the infiltrates was mucosal hyperplasia with prominent mitotic figures in the crypts adjacent to regions of inflammation. In the most severely affected sections, many of the crypts contained necrotic cellular debris and mucus (Fig. 1c). Histological scoring of the colon and colonic mass-to-length measurements, an indicator of tissue pathology, confirmed that IL-10\textsuperscript{−/−} mice develop \textit{C. jejuni}-induced colitis whereas IL-10 heterozygous littermates were colonized but did not develop severe pathology (Fig. 1d, e, f). WT mice treated with IL-10Ra blocking mAb developed similar pathology to IL-10\textsuperscript{−/−} mice infected with \textit{C. jejuni} (Fig. 1d–e).

To delineate the contribution of ILCs and lymphocytes to \textit{C. jejuni}-induced pathology in absence of adaptive immunity, we blocked IL-10Ra in RAG\textsuperscript{−/−} and RAG\textsuperscript{−/−}γc\textsuperscript{−/−} mice prior to infection with \textit{C. jejuni}. Infected RAG\textsuperscript{−/−} mice lost weight, although at a lower rate than IL-10\textsuperscript{−/−} mice and developed similar pathology to IL-10\textsuperscript{−/−} mice (Fig. 1g and data not shown). Surprisingly, RAG\textsuperscript{−/−}γc\textsuperscript{−/−} mice, which lack Thy1.2\textsuperscript{+} ILCs in
Fig. 2  IFNγ contributes to C. jejuni-induced colitis independently of T cells. Antibiotic pre treated TCRβδ−/−IL-10−/− mice were infected with C. jejuni. Ten days after infection colon and MLN were analyzed. a, b Expression of proinflammatory cytokines were measured in colon by real-time PCR. Data combined from two independent experiments with similar results (n = 7–9 mice per group). c Intracellular staining of IFNγ and IL-17A in live, Thy1.2+ gated cells from MLN and cecum after restimulation with PMA and ionomycin. Open black histograms: FMO control; filled histograms: stained panel; red open histograms: stained panel samples without PMA and ionomycin restimulation, blue open histogram: samples after restimulation with PMA and ionomycin. Histograms are a concatenation of four samples. d Intracellular staining of IFNγ in naive (red histogram) and infected (blue histogram) mice after PMA and ionomycin stimulation. e IFNγ-reporter mice were treated with IL-10Rα-blocking and CD4 and CD8 depleting mAbs prior to infection with C. jejuni. IFNγ expression (EYFP) was analyzed in cecal lymphocytes directly ex vivo (without restimulation) 10 days after infection. Flow cytometry plots from four concatenated samples show EYFP+ cells stained for Thy1.2 and CD4 and CD8. f–i TCRβδ−/−IL-10−/− mice were treated with neutralizing mAb or isotype control (Ctrl Ig). Ten days later, disease severity and C. jejuni colonization was evaluated. f Histological examination, g pathology disease scores, h colonic mass-to-length ratio, i enumeration of C. jejuni in colon. Data shown in g–i are pooled from two independent experiments (n = 7–8 mice per group). Bars depict means and SEM. Data represents an individual mouse with the horizontal lines depicting means. Real-time PCR data were normalized to hprt expression. P values were calculated by Mann–Whitney test (a, b) or unpaired Student’s t-test with Welch’s correction when warranted (g–i). *p < 0.05, **p < 0.01, ***p < 0.001.
IFNγ-producing NK1.1+ ILCs accumulate in the inflamed intestine during C. jejuni infection. 

a–f WT mice were treated with IL-10Rα-blocking antibody and infected with C. jejuni (Cj). Ten days after infection, colon LP leukocytes were isolated. Cells were restimulated with PMA and ionomycin and stained for flow cytometry. 

a NK1.1 levels in IFNγ+ ILCs. 
b Frequency (left panel) and absolute number (right panel) of IFNγ+ ILCs. 
c Histograms show expression of indicated markers in IFNγ−CD3−Lin−Thy1+ ILCs (red) relative to FMO control (black). 
d T-bet, RORγt, and Nkp46 expression in IFNγ+ ILCs. Black line: FMO control. 
e Frequency (left panel) and absolute number (right panel) of IFNγ+ NK1.1+ ILCs. 
f Frequency (left panel) and absolute number (right panel) of IFNγ+ NK1.1− ILCs.

g–h IFNγ-reporter mice were depleted of T cells by αCD4 and αCD8 depleting mAbs. Absolute numbers of (g) IFNγ+ NK1.1+ ILCs and (h) IFNγ+ NK1.1− ILCs directly ex vivo (without restimulation). Bars depict means and SEM. Data represent an individual mouse with the horizontal lines depicting means. P values were calculated by unpaired Student’s t-test with Welch’s correction when warranted (b, e–h); ns not significant, **p < 0.01, ***p < 0.001.
addition to T and B cells (Fig. S1a) showed significantly less inflammation and weight loss, and harbored fewer C. jejuni in the colon compared to RAG−/− mice (Fig. 1g, h). These results suggest that Thy1.2+ ILCs promote C. jejuni-induced intestinal pathology.

To further examine the role of Thy1.2+ ILCs in promoting C. jejuni induced colitis independently of T cells, we intercrossed T cell-deficient TCRβδ−/− mice (Fig. S1b, c) and IL-10-deficient mice. We treated these mice with either Thy1.2+ depleting or isotype control mAb prior to inoculation with C. jejuni. Infected TCRβδ−/− IL-10−/− mice developed pathology that was significantly ameliorated by depletion of Thy1.2+ cells (Fig. 1i). Depletion of Thy1.2+ ILCs cells also significantly reduced the numbers of C. jejuni in the colon (Fig. 1j). Collectively, these data suggest that ILCs promote C. jejuni-induced colitis independently of T cells.

IFNγ from ILCs is critical to promote C. jejuni-induced colitis. Several pro- and anti-inflammatory cytokines produced by ILCs and known to regulate intestinal inflammation6,13,19-21. Therefore, we analyzed cytokine expression in colons during C. jejuni infection. IL-17A, IFNγ, TNF, and IL-22 were upregulated in colons of IL-10−/− mice after infection (Fig S2). Interestingly, IFNγ, TNF, and IL-22 but not IL-17A were upregulated in the colon of infected TCRβδ−/− IL-10−/− mice (Fig. 2a) suggesting that mainly T cells were responsible for IL17A production. We also found that C. jejuni infection drove colonic expression of IL-12 and IL-23 (Figs. 2b and S2b), known regulators of IFNγ, TNF, IL-22, and IL-17A production during inflammation22. Together, these results suggest that increased innate production of pro-inflammatory cytokines, such as IFNγ is associated with C. jejuni-induced intestinal pathology.

Both IFNγ and IL-17A have been shown to contribute to C. jejuni infection15. T cells, ILCs and NK cells are the major producers of IFNγ during C. jejuni infection (see also Fig. S3a, b). To identify contribution of ILCs in IFNγ and IL-17A production during C. jejuni-induced colitis, we infected TCRβδ−/− IL-10−/− mice with C. jejuni and isolated cells from the mesenteric lymph nodes (MLN) and cecum. Innate Thy1.2+ cells from both MLN and cecum were robust producers of IFNγ but not IL-17A (Fig. 2c). Furthermore, C. jejuni infection induced high levels of IFNγ production by innate Thy1.2+ cells compared to naive controls (Fig. 2d), indicating that infection is a potent inducer of cytokine production by these cells. To confirm that our findings were not biased by in vitro stimulation and to evaluate the expression of IFNγ directly ex vivo without stimulation, we analyzed cells from IFNγ reporter mice23 after T-cell depletion. Flow cytometry analysis showed that >80% of the cells that express IFNγ during C. jejuni infection were innate Thy1.2+ cells (Fig. 2e) and there were no production of IFNγ in naive mice by these cells (Fig. S3c).

Next, to test whether IFNγ was required to promote pathology during C. jejuni infection, we treated TCRβδ−/− IL-10−/− mice with IFNγ neutralizing mAb prior to C. jejuni infection. Neutralization of IFNγ significantly ameliorated colitis and reduced C. jejuni numbers in the colon (Fig. 2f-i). These results indicate that Thy1.2+ ILCs are a prominent source of IFNγ that contributes to C. jejuni induced intestinal disease.

Distinct population of IFNγ+ NK1.1+ ILCs accumulates in the inflamed intestine. IFNγ-producing ILCs are a heterogeneous population of cells that express the transcription factor T-bet1-3. This group of ILCs includes cytotoxic NK cells and helper-like ILC1s that produce IFNγ and TNF1,3. To characterize the IFNγ-producing ILCs during C. jejuni-induced colitis, we analyzed ILCs (IFNγ+ CD3− Lin Thy1.2+) isolated from MLN and colon lamina propria (cLP) of infected WT mice with disrupted IL-10R signaling. Flow cytometry analysis revealed two distinct populations of IFNγ-producing ILCs: NK1.1+ ILCs and NK1.1+ ILCs (Fig. 3a, b). Murine ILCs are reported to express NKP46 and NK1.1 markers24,25. Interestingly, we found a marked increase of colonic IFNγ-producing NK1.1+ ILCs after infection, whereas the absolute number of IFNγ+ NK1.1+ ILCs was unchanged (Fig. 3b) indicating that C. jejuni infection drives the accumulation of NK1.1+ IFNγ+ ILCs in the colon. Moreover, C. jejuni infection rather than antibiotic treatment or IL-10R blockade induced the accumulation of NK1.1+ IFNγ+ ILCs, since antibiotic-treated or all-10Ra blocked uninfected mice did not display increased number of colonic ILCs (Fig. 3a), in agreement with previous study showing no inflammation in all-10Ra blocked uninfected mice16. Further characterization of IFNγ-producing ILCs revealed that these cells express IL-7Rα (CD127) (Fig. 3c, NKP46 (Fig. 3d), but do not express c-kit, IL-17, IL-22 (Fig 3b) and KLRG1 (Fig. 3h). The absence of KLRG1 expression in IFNγ-producing ILCs indicates that these cells represent ILC1s or ILC3s but not ILC2s. Moreover, IFNγ ILCs also lacked CCR6, the marker associated with LTH-like ILC3s (Fig. 3c).

To define the role of IFNγ+ producing ILCs independently of T cells, we next analyzed NK1.1− and NK1.1+ ILCs in TCRβδ−/− IL-10−/− mice. Consistent with the data obtained in T-cell sufficient mice (Figs. 3b, e and S4d), the frequency of NK1.1+ IFNγ+ ILCs in colon and MLN of TCRβδ−/− IL-10−/− mice was decreased during C. jejuni infection, whereas the frequency of NK1.1− IFNγ+ ILCs was increased (Fig. S5a-d). To confirm our findings without in vitro stimulation, we infected IFNγ reporter mice with C. jejuni and analyzed colonic ILCs by flow cytometry (Fig S5h). C. jejuni infection of IFNγ reporter mice did not impact the number of NK1.1+ ILC1s but enhanced the accumulation of NK1.1+ ILCs to the colon, compared to infected mice (Figs. 3g, h and S4f). Furthermore, depletion of T cells by mAb treatment did not influence the number of NK1.1+ ILCs or NK1.1+ ILCs (Figs. 3g, h and S4), suggesting that the presence of T cells has no impact on accumulation of IFNγ-producing NK1.1+ ILCs to the colon. Accordingly, the number of IFNγ+ NK1.1+ ILCs in the colon was similar between WT and TCRβδ−/− mice (Fig. S5e-g) indicating that the lack of T cells does not influence IFNγ production by NK1.1+ ILCs. Additionally, confocal microscopy detected IFNγ-producing NK1.1+ ILCs clusters in colon of IFNγ-reporter mice treated with CD4, CD8, NK1.1 depleting antibodies (Fig. S5h).

T-bet is a key regulator of IFNγ production by ILC11,2,4. In addition to ILC1 subset, T-bet can be co-expressed with RORγt, the signature transcriptional factor for ILC3 subset19. Both NK1.1+ and NK1.1− ILCs expressed T-bet and were the major producers of IFNγ (Figs. 3e, f and S4c). However, in contrast to NK1.1+ ILCs, NK1.1− ILCs did not express Bcl6, indicating their distinct phenotype from NK cells (Figs. 3c and S4c). In agreement with the reduced frequency of NK1.1+ IFNγ+ cells in the colon during C. jejuni infection, we observed the significant reduction of the frequency of T-bet+ NK1.1+ cells, although the absolute number of these cells was not affected (Figs. 3e and S4g). Conversely, T-bet+ NK1.1+ ILCs were significantly increased in cLP of C. jejuni-infected mice compared to naive mice (Fig. 3f), but not in MLN (Fig. S4f). Interestingly, the absolute number of NK1.1+ and NK1.1− IFNγ-producing ILCs that express both T-bet and RORγt was increased in the colon (Fig. 3e, f), indicating their intermediate ILC3-ILC1 phenotype. Together, these results suggest that population of IFNγ-producing Lin+ NK1.1+ ILCs accumulates in the intestine during C. jejuni infection and that these cells are phenotypically distinct from NK1.1+ ILC1s.

NK1.1+ IFNγ-producing ILCs can promote C. jejuni-induced colitis. Our immunophenotyping data identified two populations of IFNγ-producing ILCs that reside in the inflamed intestine during C. jejuni infection, NK1.1+ ILC1s and NK1.1+ ILCs (Fig. 3). Prior studies implicated NK1.1+ ILC1s in promoting colitis in mice8,9. To further define the role of NK1.1+ ILC1s in C. jejuni-induced colitis, we treated TCRβδ−/− IL-10−/− mice with either NK1.1+ depleting or isotype control antibody prior to C. jejuni infection. This strategy
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Fig. 4  Lin^NK1.1^ IFNγ-producing ILCs promote C. jejuni-induced colitis. a-f NK1.1^+^ ILCs are dispensable for C. jejuni-induced colitis. TCRβ/δ^−/−^ IL-10^−/−^ mice were treated with NK depleting (αNK1.1) or isotype control (Ctrl) mAbs. Disease severity, intestinal lymphocytes and IFNγ expression were analyzed 10 days after infection. a The efficacy of in vivo NK1.1 depletion. Histograms depict fully stained panels (filled histograms) relative to FMO controls (outlined) on live, Thy1.2^+^ cells. Each flow cytometry plot is a concatenation of three (Naive) or four (Cj) samples. b Loss of body weight (c) H&E stained colon sections. d Pathology disease scores of colons. e Colonic mass-to-length ratio. f Enumeration of C. jejuni in the colon of infected mice. All data represent an individual mouse from three independent experiments with horizontal lines depicting means. Histopathology score bar graphs depict the mean and SEM of nine (Naive) or 12 (Cj) mice from three independent experiments.

g The capacity for IFNγ secretion by MLN cells after NK1.1 depletion. 1 × 10^6^ mesenteric lymph node cells were restimulated with control (medium alone) or heat-killed C. jejuni for 48 h. After restimulation, IFNγ in culture supernatants was measured by ELISA. Bars represent the mean and SEM of six (naive) or eight (Cj) mice from two independent experiments.

h-k Numbers in the plots show the frequency of cells within the gate. Ten days after infection, disease severity in recipient mice was measured by (j–k) histological examination of H&E stained colon sections (25× magnification) (l) colonic mass-to-length ratio and (m) C. jejuni colonization. Data represent two independent combined experiments (n = 2–3 mice per group). Data represent an individual mouse with bars depicting the mean. P values were calculated by two-way ANOVA (b and g) with Bonferroni’s multiple hypothesis corrections or unpaired Student’s t-test with Welch’s correction when warranted (d-f, k-n). *p < 0.05, **p < 0.01, ***p < 0.001.
has been used successfully to ameliorate ILC1-dependent αCD40-driven colitis. Despite effective depletion of NK1.1+ cells (Figs. 4a and S6), this treatment did not affect colitis or altered C. jejuni numbers in the colon (Fig. 4b). Furthermore, depletion of NK1.1+ ILCs did not impact IFNγ secretion by MLN cells when stimulated in vitro with heat-killed C. jejuni (Fig. 4g). Together these results suggest that NK1.1+ ILC1s are not critical mediators of C. jejuni-induced colitis.

Since depletion of NK1.1+ ILC1s did not impact C. jejuni-induced colitis, we hypothesized that IFNγ-producing NK1.1+ ILCs that accumulate in the inflamed intestine during C. jejuni infection could promote intestinal pathology. To determine the pathogenic potential of these ILCs, we sorted Thy1.2+Lin NK1.1− cells (purity > 95%, as confirmed by flow cytometry, Fig. 4h–i) from the MLN, ceca, and colons of C. jejuni-infected TCRβ/δ−/−IL-10−/− mice. The purified cell population was transferred to RAG−/−γc−/− recipient mice which lack both adaptive and innate lymphocytes and do not show symptoms or pathology after C. jejuni infection (Fig. 1g). One day after receiving either ILCs or vehicle, recipient mice were infected with C. jejuni and disease severity and bacteria burden were evaluated 10 days later. Mice receiving NK1.1− ILCs showed increased histological scores, colonic mass-to-length ratio, and C. jejuni colonization of the colon (Fig. 4j–m). These findings suggest that Lin NK1.1 IFNγ-producing ILCs can promote C. jejuni-induced intestinal pathology.

IFNγ-producing ILCs predominantly express ILC1 signature genes. Next, to further characterize IFNγ-producing ILCs using an unbiased approach, we sort-purified CD3 Thy1.2+Lin IFNγ+ cells (Fig. 5a) from cLP and MLN of C. jejuni-infected mice treated with anti-IL-10Rα antibodies. Transcriptome profiles were analyzed by
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RNA-seq. Principal component analysis (PCA) was used to simplify the complexity of high-dimensional data. The first Principal component, PC1, which represents 29.9% of variation, distinguished between colon and MLN cells, whereas PC2, which accounted for 21.1% of variation, separated ILCs and T cells (Fig. 5b). Hierarchical clustering of 25 previously reported signature genes of distinct ILC subsets from sILP24 revealed that the major transcripts expressed at higher level in IFNγ-producing ILCs from cLP shown to be characteristic genes for ILC1, such as Ifng, Tbx21, Xcl1, Il22r, Ccl3, Ccl4, and Cds1 (Fig. 5c). Although most upregulated genes were ILC1s signature genes, we also detected upregulation of Il22, Tcf7, Areg, and Ccl1 in cLP ILCs that are characteristic genes for ILC3 and ILC2 subtypes, respectively (Fig. 5c). In contrast, IFNγ-producing ILCs from MLN upregulated predominantly ILC1-characteristic transcripts, but had much lower Il22, Areg, Ccl1 transcript levels compared to cLP ILCs, whereas the expression of Tcf7 gene was the same between MLN and cLP ILCs (Fig. 5c). Thus, the transcriptional identity of IFNγ-producing ILCs that accumulate in the MLN and colon during C. jejuni infection represent predominantly ILC1 subtype.

To define the transcriptional identity of IFNγ-producing ILCs from cLP and MLN, we compared transcriptional profiles of these population with known ILC signature genes described by Gury-BenAri et al.24 using CIBERSORT method28. This statistical deconvolution method provides a useful tool to evaluate the abundances of different cell types in a mixed cell population utilizing gene expression data. To estimate the relative proportion of each ILC subset within IFNγ-producing ILCs in C. jejuni-induced...
colitis, an input matrix of reference gene expression signatures was made based on RNA-seq data generated by Gury-BenAri et al.24. Our analysis revealed that IFN-γ-producing ILCs in colon had mainly ILC1 and ILC3 gene expression profile whereas ILCs from MLN were represented exclusively by ILC1 (Fig. 5c, d). Together, these findings indicate that pathogenic IFN-γ-producing ILCs in colon represent predominantly ILC1 subtype with distinct ILC3 gene signatures.
T-bet+ ILCs are required for C. jejuni-induced colitis

Our data demonstrate that IFNγ-producing ILCs in the inflamed intestine express T-bet (Fig. 3b–d). T-bet is a key regulator of IFNγ production in both ILCs and T cells. To further define the pathogenic role of T-bet+ ILCs in a T-cell sufficient environment, we generated Nkp46-Cre x Tbx21fl/fl (Nkp46-Tbet) mice which are harboring genetic deletion of T-bet in ILC1s, Nkp46+ ILC3s, NK cells and some minor populations of T cells. Following C. jejuni infection, Nkp46-Tbet mice exhibited reduced intestinal pathology compared to WT mice (Fig. 6a–d), although there was no difference in bacterial burden compared to the control group (Fig. 6e). These results indicate that Nkp46 T-bet+ cells had no impact on C. jejuni colonization, yet promoted inflammation.

Given that IFNγ is critical for C. jejuni-induced colitis, we next analyzed IFNγ expression in Nkp46-Tbet and WT mice. As shown in Fig. 6f, IFNγ expression was significantly reduced in colon of Nkp46-Tbet mice on day 10 after C. jejuni infection. Flow cytometry analysis revealed marked reduction of IFNγ by ILCs, whereas IFNγ levels by NK cells were similar to control mice (Fig. 6g, h). Nkp46+ and Nkp46− ILCs isolated from Nkp46-Tbet mice showed significantly reduced IFNγ levels compared to WT mice (Fig. 6i–k), consistent with T-bet expression in these cell populations (Fig. 3d). Together, these data indicate that T-bet+ ILCs contribute to C. jejuni-induced intestinal disease in T-cell sufficient environment.

IFNγ ‘Lin NK1.1’ T-bet-expressing ILCs develop from RORγt progenitors

Our adoptive transfer experiments suggested an important role of Lin NK1.1 IFNγ-producing ILCs in promoting colitis. RNAseq data revealed that ILCs from the colon mostly represent ILC1 subtype, although several ILC3 characteristics transcripts have been revealed. Next, we examined the developmental origin of these cells. In vitro studies with human ILCs and cell fate-mapping studies in mice revealed that RORγt-expressing ILC3s can convert to an ILC1-like phenotype that express T-bet and IFNγ. Therefore, we hypothesized that C. jejuni infection drives the conversion of RORγt+ ILC3s to pathogenic effector IFNγ T-bet+ Lin NK1.1 ILCs.

To test whether RORγt plays a role in the development of IFNγ ‘Lin NK1.1’ ILCs, we infected RORγt−/− and heterozygous littermates on the RAG1−/− background with C. jejuni and evaluated Lin NK1.1 ILCs and intestinal pathology. We found that whereas RORγt heterozygous mice developed severe colitis, RORγt−/− littermates developed only mild inflammation despite being colonized with pathogen at comparable levels (Fig. 7a–e). Flow cytometry analysis revealed that RORγt−/− mice showed reduced frequency and absolute number of IFNγ ‘Lin NK1.1’ ILCs in the cecum compared to heterozygous littermates (Fig. 7f–g). These results indicate that RORγt can participate in development and maintenance of IFNγ ‘Lin NK1.1’ ILCs. However, these results do not exclude a possibility that lack of lymph nodes or gut-associated lymphoid tissues in RORγt−/− mice may affect the course of disease.

To further address the role of RORγt in Nkp46+ ILC3s in C. jejuni-induced colitis, we generated Nkp46-Cre x RORγtfl/fl (Nkp46-RORγt) mice in which RORγt deficiency is selectively restricted to Nkp46+ ILC3 subsets. We did not find significant difference in disease severity (Fig. S7a–c) or IFNγ production by ILCs (Fig. S7d) in Nkp46-RORγt mice compared to controls. The number of colonic Nkp46+ ILC3s in Nkp46-RORγt mice was slightly reduced, whereas the number of Nkp46+ ILC3s was unchanged (Fig. S7e). These data indicate that Nkp46+ ILC3s are not sufficient to drive C. jejuni-induced pathology.

Our data suggest that NK1.1 ILCs are the major producers of IFNγ during C. jejuni infection. Next, we determined the developmental origin of these IFNγ ‘Lin NK1.1’ ILCs using RORγt cell fate map mice. Mice expressing EYFP that is preceded by a loxP-flanked STOP sequence in the Rosa26 locus were crossed to mice expressing Cre recombinase under the RORγt promoter to generate RORγtEYFP mice. In these mice, cells that express RORγt are permanently marked by EYFP expression, allowing the identification of the cells that have unstable or transient expression of RORγt. We found that the majority of NK1.1 ILCs (Thy1.2+ Lin IFNγ+) from the intestine and MLN were EYFP+ compared to uninfected controls (Fig. 7h, i), indicating that C. jejuni infection promotes ILC3 plasticity. Moreover, T cells, which express RORγt in the thymus, displayed efficient recombainse activity in RORγtEYFP mice since >94% of T cells in the cecum and MLN were EYFP+ as well as 89% of cecal IFNγ ‘Lin NK1.1’ ILCs. Whereas EYFP+ T cells (Thy1.2+ Lin CD4+ or CD8+) predominantly expressed T-bet, EYFP− ILCs (Thy1.2+ Lin CD4− CD8−) were heterogeneous in their RORγt and T-bet expression (Fig. 7j), suggesting their plasticity. Together, these findings indicate that IFNγ ‘Lin NK1.1’ ILCs develop from RORγt-expressing progenitors during C. jejuni infection and represent NK1.1+ ex-ILCs.

DISCUSSION

The discovery of ILCs has greatly expanded our understanding of effector immune cells that contribute to host defense against pathogens and promote tissue repair after injury. Nevertheless, mounting clinical evidence suggests that dysregulated ILCs responses can promote chronic inflammatory pathologies such as IBD. How ILCs become dysregulated and contribute to disease remains unclear. Using a mouse model of campylobacteriosis, we demonstrate that C. jejuni infection induces a population of IFNγ ‘Lin NK1.1’ ILCs that exhibits ILC3 > ILC1 phenotypic plasticity and promotes IFNγ-dependent intestinal inflammation.

C. jejuni is a major foodborne pathogen and a significant cause of immune-mediated post-infectious sequelae. IL-10-deficient mice develop severe colitis with an accumulation of lymphocytes in the intestine when associated with members of the bacterial order Campylobacterales, particularly Helicobacter hepaticus and C. jejuni. Depletion of neutrophils or Thy1+ lymphocytes significantly ameliorates colonic inflammation in IL-10−/− mice.
infected with *C. jejuni*, revealing the pathogenic role of dysregulated host responses during infection by this pathogen. In our study, depletion of Thy1+ cells in T-cell deficient TCRβ/δ−/− IL-10−/− mice markedly reduced pathology after *C. jejuni* infection, suggesting a role for dysregulated ILCs in promoting colitis. Consistent with this, RAG−/−/− mice that are deficient for both adaptive immune cells and all ILCs were better protected from *C. jejuni*-induced colitis compared to RAG−/−/− mice. Therefore, we conclude that ILCs can promote *C. jejuni*-induced intestinal disease.

IFNγ is a pro-inflammatory cytokine that has important functions in immune defense against intracellular pathogens including the upregulation of antigen presenting MHC molecules, the induction of anti-microbial immunity and the production of chemokines that facilitate leukocyte trafficking.[18] In clinical trials, IFNγ levels correlated with disease resistance when people were rechallenged with *C. jejuni*, suggesting that IFNγ plays a protective role during repeated exposure to this pathogen. Despite IFNγ role in combating infection, its excessive production is implicated in the etiology of IBD and the number of IFNγ-producing ILCs are increased in the colon of Crohn’s disease patients compared to healthy cohorts.[9,10] IFNγ-producing lymphocytes also accumulate in intestine during experimental infection with *H. hepaticus* or *C. jejuni* and neutralization of IFNγ ameliorated disease in these models of colitis.[11] In our experiments, neutralization of IFNγ in TCRβ/δ−/− IL-10−/− mice significantly ameliorated *C. jejuni*-induced colitis and reduced bacterial counts in the colon, suggesting that IFNγ-producing ILCs contribute to intestinal pathology.

IFNγ-producing ILCs include cytotoxic NK cells and helper-like ILC1s that produce cytokines.[1] To date, murine ILC1s are defined by their expression of the transcription factor T-bet and surface markers NK1.1 and NKp46.[12,13] Although IFNγ production by NK1.1+ ILC1s has been implicated in protective responses to intracellular pathogens, dysregulated production of IFNγ by ILCs could also promote immune-mediated pathology.[14,15] In addition to ILC1, NKp46− and NKp46−CCR6− ILC3 can also produce IFNγ in response to environmental cues or bacterial infection.[6,19] Anti-NK1.1 antibody cell depletion studies demonstrated the pathogenic potential of murine ILC1s in oCDA40 experimental innate colitis model.[9,9] In our study we revealed two populations of IFNγ-producing ILCs in the colons of *C. jejuni* infected mice, one that expresses markers consistent with previously described murine NK1.1+ ILC1s and another one that does not express NK1.1 (NK1.1− ILC1s). Both of these ILCs populations expressed T-bet and RORγt. Surprisingly, depletion of NK1.1+ ILCs did not impact *C. jejuni*-induced intestinal pathology, suggesting that NK1.1+ ILC1s are dispensable for *C. jejuni*-induced intestinal disease. We next therefore focused on the role of IFNγ-producing NK1.1+ ILCs which are greatly increased in the inflamed mouse intestine during *C. jejuni* infection. Using an adoptive transfer strategy, we confirmed that Lin−NK1.1+ ILCs can facilitate *C. jejuni*-induced pathology. Our data demonstrate the importance of Lin− NK1.1+ ILCs for the development of *C. jejuni*-induced colitis. In line with our study, recent report demonstrated that Lin− NK1.1+ ILCs are the major producer of IFNγ during *Yersinia enterocolitica* infection and can contribute to protection.[16] Infection or inflammation can affect local ILCs populations. In fact, the number of ILC1s is increased in the intestinal inflamed mucosa of CD patients.[5,30] Previous studies showed that IFNγ-producing ILC1s can arise from CCR6+ RORγt+ ILC3 population that upregulates T-bet expression in response to signals from the environmental cues.[9,19] Our data suggest that colonic IFNγ-producing ILCs mostly have transcriptional signatures of ILC1s with distinct signature genes of ILC3s during *C. jejuni* infection, whereas in MLN IFNγ-producing ILCs exclusively display transcriptional signatures of ILC1s. This difference in transcriptional profiles can depend on the local cytokine microenvironment and the ability of ILCs to migrate within the organs. A recent report indicates that ILCs can migrate between the MLN and intestine.[41]

The contribution of ILCs to pathology during bacterial infection in immune sufficient host can be easily overlooked due to contribution of T cells. Importantly, our data demonstrate that T-bet deficiency in NKp46+ cells (mainly ILC1, NKp46+ ILC3, and NK cells) reduced pathology and IFNγ production in the colon highlighting the role of IFNγ production by ILCs in the early stages of infection in a T-cell sufficient environment and intact development of gut-associated lymphoid tissues. Although impaired IFNγ production was observed in both NK1.1+ ILCs and NK1.1− ILCs, our NK1.1 depletion experiments indicate that NK1.1+ ILCs rather then NK1.1− cells have a major impact in pathogenesis of *C. jejuni*-induced colitis. Dissecting the relative contribution of ILCs and NK cells in mice with selective NK cell deficiency will be important for further studies of campylobacteriosis.

Transcriptional analyses revealed extensive heterogeneity and plasticity among ILC subsets.[24,26,31] Although the in vivo conditions that promote ILC conversion are still poorly understood, in vitro experiments showed that ILC3 > ILC1 transition can be stimulated by IL-15, IL-18, and IL-12.[19,30] Although our results with NKp46− RORγt mice suggest that NKp46− ILCs are not sufficient to drive *C. jejuni*-induced pathology, cell fate mapping experiments indicate that majority of IFNγ-producing Lin− NK1.1+ ILCs have a history of RORγt expression, and therefore represent “ex-ILC3s.” Surprisingly, these cells lack NK1.1 and only partially express NKp46 despite increased T-bet and reduced RORγt expression. In addition to ex-ILC3s, we also do not exclude the role of IFNγ-producing ILC1s in *C. jejuni*-induced intestinal pathology. In line with our results, recent studies demonstrate that NKp46 expression in RORγt+ ILC3s is unstable and can be lost in adult mice during antibiotic treatment suggesting that signals from commensal microbiota may regulate this cell population.[12,42,43] Additionally, previous study described that both NKp46− RORγt+ and NKp46− RORγt+ ILCs can downregulate RORγt to induce T-bet-dependent IFNγ production in response to *Salmonella enterica* infection.[44]

In conclusion, we have described the plasticity between RORγt+ ILC3 and NK1.1+ ILC1 populations induced by *C. jejuni*. We propose that induction of IL-12, IL-18, and IL-15 cytokines by *C. jejuni* infection can drive the conversion of RORγt+ ILC3s towards IFNγ-producing NK1.1+ T-bet+ ILC3s (“ex-ILC3s”) by upregulating T-bet expression and downregulating RORγt expression. Thus, ex-ILC3s produce IFNγ thereby promoting intestinal inflammation. It is tempting to speculate that *C. jejuni*-induced dysregulation of ILCs is beneficial for the pathogen to induce inflammation, tissue damage, and diarrhea, thereby facilitating dissemination and spread to new hosts.

Stimulation of RORγt+ ILC3-facilitated protective responses mediated by IL-22 and IL-2 may provide a therapeutic potential to ameliorate the intestinal inflammation and promote mucosal healing in IBD.[14,44] On the other side, ILC3 plasticity induced in response to mucosal bacterial infection can promote ILC3 to ILC1 conversion leading to IFNγ-dependent intestinal pathology.[12,19] Therefore, context-dependent strategies are required for optimal targeting of ILCs responses in intestinal disease. Thus, our findings demonstrate that Campylobacter infection induces a population of IFNγ NK1.1+ ILCs that exhibits ILC3 to ILC1 phenotypic plasticity and promotes IFNγ-dependent intestinal pathology.

**Materials and Methods**

Mice

All animal studies were conducted in accordance with the Trudeau Institute and the University of Texas Health Science Center at San...
Antonio Animal Care and Use guidelines. Six to twelve-week-old male and female mice were used for experiments. The following mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions: C57Black/6 wild-type (WT), B6.1295-Rag1<sup>tm1Min</sup>/J<sup>23,45</sup> B6.129SA-Ifnγ<sup>tm3.Jkr</sup>/J<sup>23,45</sup> B6.129P2-Tcrγ<sup>tm1Min</sup>/J<sup>23,45</sup> B6.129P2-Tcrδ<sup>tm1Min</sup>/J<sup>23,45</sup> B6.129P2-Lt<sup>tm1Cgn</sup>/J<sup>23,45</sup> RORγt<sup>Cre</sup>-<sup>tm1Mom</sup>/J<sup>23,45</sup> ROSA26-YFP<sup>tm1</sup>/J<sup>23,45</sup> Tbx21<sup>tm2.Znr7</sup> Rorc<sup>tm3.1Lky</sup> RAG<sup>γδ</sup>-<sup>-</sup>/<sup>-</sup> mice (B10; B6-<sup>-</sup>/J). NKp46-Cre mice were previously described<sup>49</sup>. Isolation of lamina propria leukocytes

The cecum and colon were cut open and rinsed twice in PBS to remove feces. The tissue was finely chopped and incubated in complete medium (DMEM supplemented with 10% FCS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1 mM penicillin-streptomycin, 55 μM 2-mercaptoethanol) containing 2 mM EDTA for 30 min at 37 °C. After vortexing, the remaining tissue was collected and digested in serum-free medium containing 375U Type XI collagenase for 40 min at 37 °C. The digested tissue was passed through a mesh screen, washed with PBS containing 2% FCS and separated by a 60/40 Percoll gradient. Cells were collected at the interface, washed, and viable cells counted by Trypan blue exclusion.

Flow cytometry

Antibodies used for flow cytometry are listed in Supplementary Table 1. A lineage cocktail included antibodies against CD5, CD3, CD11b, CD11c, B220, GR1, Ter119, unless specified differently. Single-cell suspensions (1 × 10<sup>6</sup> cells) were stimulated with 100 ng/ml PMA and 1.5 μg/ml ionomycin with 5 μg/ml brefeldin A (all from Sigma-Aldrich) in complete medium for 4 h. After stimulation, cells were stained for viability with LIVE/DEAD<sup>®</sup> (Invitrogen, Carlsbad, CA) for 20 min on ice. After washing, the FcR was blocked with 1 μg of mAb clone 2.4G2 (BioXCell) and stained for surface antigens for 30 min on ice. For intracellular cytokine staining, cells were fixed in 4% paraformaldehyde for 20 min on ice, washed, then stained with cytokine antibodies in permeabilization buffer (eBioscience) for 1 h at 4 °C. Data was acquired on either a FACS Canto II or LSRII (BD Biosciences) and analyzed with FlowJo (FlowJo LLC).

Adaptive transfer of ILCs

Isolated ILCs from cecum and MLN from <i>C. jejuni</i>-infected TCRB<sup>-/-</sup>/IL10<sup>-/-</sup> mice were sorted and 1 × 10<sup>5</sup> purified ILCs were adaptively transferred by i.v. injection into RAG<sup>γδ</sup>-<sup>-</sup>/<sup>-</sup> recipients treated with IL-10Ra-blocking mAb one day prior to <i>C. jejuni</i> infection.

RNA-seq analysis

cLP and MLN cells were isolated from IFNγ-reporter mice as described above. ILCs, defined as CD4<sup>+</sup> Lin<sup>-</sup> CD3<sup>-</sup> CD90<sup>+</sup> and IFNγ<sup>+</sup> (Lineage: CD11c, B220, GR1; Ter119) were sort purified using FACS Aria (BD Biosciences) and RNAs from the sorted cells were isolated using RNeasy Micro kit (Qiagen), following the manufacturer’s instructions. RNA integrity was determined using Fragment Analyzer (Agilent, Santa Clara, CA) prior to library preparation. RNA-seq libraries were prepared according to SMART-seq2 protocol<sup>32</sup> with the following modifications: PCR preamplification to 15 cycles, two rounds beads cleanup with 1:1 ratio after cDNA synthesis, and 0.6-0.8 dual beads cleanup for Nextera XT DNA-seq library purification. RNA-seq libraries were sequenced using Illumina HiSeq 3000 system (Illumina, San Diego, CA) with 54 bp single-read sequencing module. Upon sequencing completion, short-read sequences from RNAseq were first aligned to UCSC mm9 genome build using TopHat2 aligner<sup>23</sup> and then quantified for gene expression by HTSeq<sup>24</sup> to obtain raw read counts per gene, and then converted to RPKM (Read Per Kilobase of gene length per Million reads of the library). The transcriptional identity of IFNγ<sup>+</sup> ILCs was analyzed in comparison with available RNAseq data (GSE85154)<sup>24</sup>. Signature genes of different ILC subsets from intestinem<sup>24</sup> were analyzed using CIBERSORT method<sup>28</sup>.
Real-time reverse transcriptase PCR analysis RNA from colon was isolated using E.Z.N.A. Total RNA kit I (Omega Bio-tek). cDNA synthesis and real-time RT-PCR were performed as described previously. PCR primers are listed in Supplementary Table 2.

Quantification of secreted cytokines MLNs were collected and processed into single-cell suspensions by passing through a fine gauge screen. Antigens for restimulation were prepared by harvesting C. jejuni bacterial lawns in PBS and heat inactivated by incubation at 65 °C for 1.5 h. Protein concentration was determined by bicinchoninic acid assay (Thermo Scientific, Rockford IL). Bacteria were confirmed to be non-culturable by plating on agar. 1 × 10⁶ test cells were cultured with an equal number of heat-killed bacteria (10 μg/ml of protein). Cytokines secreted into the supernatant were assayed by capture ELISA after 48 h (IFNy) using commercially available antibodies (IFNy: BD Biosciences, San Jose CA).

Statistical analysis Results are expressed as mean±S.E.M. Differences between groups were evaluated by two-tailed t test after assessing assumptions of normality and variance by D’Agostino Omnibus and F tests, and visualization of residual and Q-Q plots. Student’s t-test or two-way analysis of variance (ANOVA) were performed when the dataset met statistical assumptions. Nonparametric analyses (Mann–Whitney and Welch’s correction) were performed when statistical assumptions were not valid. All statistical computations were performed using GraphPad Prism 8 program. ns – not significant, *p < 0.05, **p < 0.01, ***p < 0.001.

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AUTHOR CONTRIBUTIONS Study concept and design: WTM and AVT. Designed and performed experiments, analyzed data, wrote manuscript: WTM, AAK, EPK, and AVT. Performed experiments: QX, SAS, XJ, and LWK. Provided important experimental materials: MM and EV. Performed RNA seq and bioinformatics analysis: ZL, KW, L-JW, and YC.

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