Early-life antibiotic treatment enhances the pathogenicity of CD4+ T cells during intestinal inflammation

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ABSTRACT
The incidence of inflammatory bowel diseases (IBDs) has steadily increased in recent decades—a phenomenon that cannot be explained by genetic mutations alone. Other factors, including the composition of the intestinal microbiome, are potentially important contributors to the increased occurrence of this group of diseases. Previous reports have shown a correlation between early-life antibiotic (Abx) treatment and an increased incidence of IBD. In this report, we investigated the effects of early-life Abx treatments on the pathogenicity of CD4+ T cells using an experimental T cell transfer model of IBD. Our results show that CD4+ T cells isolated from adult mice that had been treated with Abx during gestation and in early life induced a faster onset of IBD in Rag1-deficient mice compared with CD4+ T cells of untreated mice. Ex vivo functional analyses of IBD-inducing CD4+ T cells did not show significant differences in their immunologic potential ex vivo, despite their in vivo phenotype. However, genome-wide gene-expression analysis revealed that these cells displayed dysregulated expression of genes associated with cell-cycle regulation, metabolism, and cellular stress. Analysis of Abx-treated CD4+ T cell donors showed systemically elevated levels of the stress hormone corticosterone throughout life compared with untreated donors. The cohousing of Abx-treated mice with untreated mice decreased serum corticosterone, and a consequent transfer of the cells from cohoused mice into Rag1-deficient mice restored the onset and severity of disease to that of untreated animals. Thus, our results suggest that early-life Abx treatment results in a stress response with high levels of corticosterone that influences CD4+ T cell function.

Introduction
The incidence of IBDs has significantly increased over the last century and now affects close to 1% of the population in some countries [1]. IBDs are thought to occur as a result of a complex interplay between host genetics and environmental factors, such as the composition of the intestinal microbiota, which in turn, leads to a dysregulated intestinal immune response. Although GWAS have identified >200 genes that affect the onset and severity of IBDs [2], the precise molecular mechanisms leading to IBDs remain unclear.

Of the many genes identified by GWAS, a significant proportion is associated with immune cell signaling, cytokine production, and T cell activation [2]. In animal models of IBD, CD4+ T cells play a major role in the development of disease. For example, in the absence of regulatory mechanisms, such as loss of Tregs or the absence of IL-10, CD4+ T cells are able to react against bacterial antigens and produce high amounts of inflammatory cytokines leading to intestinal inflammation [3]. Changes in the CD4+ T cell compartment by alternative selection, changes in the epigenetic conformation, or changes in the expression levels of critical signaling molecules have been associated with altered behavior of CD4+ T cells during infection, inflammation, or autoimmune diseases [4-6].

Previous work in animal models has shown that the onset of inflammatory diseases later in life correlates to the composition of the microbiota in early life [7], and a study with patients showed a correlation between the development of IBD later in life after exposure to Abx in early life [8]. These studies suggest...
that the interaction between the intestinal microbiome and the developing immune system is critical for shaping the functional capacity of immune cells, such as CD4+ T cells. However, the mechanisms that link changes in the microbiome to changes in immune cell function are not known. In this study, with the use of a mouse model of T cell-dependent intestinal inflammation, we examined the effects of Abx treatment during gestation and in early life on the development of IBD in adult mice. We show that early-life Abx treatment results in increased pathogenicity of CD4+ T cells in a model of intestinal inflammation. This effect is associated with a systemic stress response with high levels of circulating corticosterone. Genome-wide expression analysis of naive CD4+ T cells from Abx-treated mice shows significant changes in genes associated with stress responses, cellular metabolism, cell cycle, and cell death. Furthermore, our results show that this effect is transient, as cohousing of Abx-treated mice with untreated mice after weaning reverses the systemic stress response and reduces the pathogenicity of CD4+ T cells in vivo. Thus, we conclude that early-life Abx treatment results in a stress response, high levels of corticosterone, and dysfunctional CD4+ T cell responses that are reversible later in life. These results provide a link between the regulation of the intestinal microbiome and the developing immune system and identify potential targets for treating IBD.

MATERIALS AND METHODS

Mice and Abx treatments
Foxp3 reporter mice on C57BL/6 background (C3H-Foxp3<sup>CreTcR<sup>-</sup>/>) were bred and kept at the animal facility of the Biomedical Research Centre (University of British Columbia, Vancouver, BC, Canada), according to approved protocols. Parent animals were treated with Abx in the drinking water (autoclaved and supplemented with 0.5 g/l of each ampicillin, streptomycin, vancomycin, and metronidazole containing 4 g/l Splenda, 7 d before mating and throughout their lives). Pups of each group (Abx and control) were weaned after 21 d and returned to regular drinking water (without Abx or Splenda) until analysis at wk 8-10. As indicated for some experiments, mice were weaned from Abx-treated parent animals, kept on regular water, and cohoused, 7 d after weaning, with untreated, control mice until analysis. Serum and tissue samples were collected immediately after death, snap frozen in liquid nitrogen, and then stored at −80°C until later analysis of steroids. The term, “early-life Abx treatment” refers to Abx exposure during gestation and until weaning.

T cell transfer colitis
Splenic CD4+ T cells from donor mice (either Abx treated or on regular water) were pre-enriched with the CD4+ T Cell-Negative Isolation Kit (Stemcell Technologies, Vancouver, BC, Canada) and FACS sorted for viable CD4+ CD25<sup>-</sup> Foxp3<sup>-</sup> CD69<sup>+</sup> cells. Cells (4 × 10<sup>7</sup>) were injected intraperitoneally into 8- to 10-wk-old Rag1-deficient mice. Weight loss of >20% was considered the humane endpoint. Proximal colons were processed for histologic H&E staining, loss of epithelial architecture, thickening of colonic wall. Isolated cells were stimulated with 1 μg/ml nCD3/αCD28 overnight; then stimulated with PMA, ionomycin, and Brefeldin A for 4 h; and intracellularly stained for flow cytometry.

Measurement of commensal bacteria-specific T cells
Single-cell suspensions of spleens from donor mice were stained with CBir1-APC tetramer (kind gift from Dr. Timothy Hand, University of Pittsburgh, Pittsburgh, PA, USA) and analyzed as described previously [9, 10].

RNA isolation and real-time qPCR
RNA was isolated from proximal colons by mechanical disruption and the TRIzol method (Thermo Fisher Scientific, Waltham, MA, USA). RNA was purified from sorted CD4+ T cells using RNeasy Mini Kits (Qiagen, Germantown, MD, USA). Reverse transcription was used to generate cDNA, and qPCR was performed using SYBR Green primer sets. Reactions were run on an ABI 7900 Real-Time PCR System (Thermo Fisher Scientific). Samples were normalized relative to expression of β-actin (Actb).

RNA-Seq and bioinformatics
Naive CD4+ T cells, sorted as above, were harvested, and total RNA was extracted using an RNeasy Mini Kit, according to the manufacturer’s instructions (Qiagen). Approximately 500 ng RNA was prepared with an mRNA kit (TruSeq Stranded; Illumina, San Diego, CA, USA) and sequenced on a MiSeq paired-end run (75 × 75, v3; Illumina). Samples were aligned to the mm10 transcript reference using TopHat2, and differential expression was assessed using Cufflinks (Illumina). The RNA-Seq datasets described in this article are available at the National Center for Biotechnology Information (accession number GSE92282).

T cell polarization and flow cytometry
CD4+ T cells were isolated with the CD4+ T Cell-Negative Isolation Kit (Stemcell Technologies) and used in T cell polarization assays, as described previously [6]. For screening of the TCR V<sub>B</sub> use in the CD4+ T cell population, we used the mouse TCR V<sub>B</sub> Screening Panel, according to the manufacturer’s instructions (BD Biosciences, San Jose, CA, USA).

T cell apoptosis assay
Highly enriched CD4+ T cells were cultured under neutral conditions (1 μg/ml each plate-bound αCD3 and αCD28, 10 ng/ml recombinant mouse IL-2) in the presence of indicated concentrations of the corticosteroid DEX. After 16 h, cells were stained and analyzed for early and late apoptotic cells (Annexin V and PI).

Steroid extraction and immunoassay
Steroids were extracted from serum, thymus, proximal colon, and cecum samples using solid-phase extraction with C<sub>18</sub> columns, as described elsewhere [11]. Dried steroid extracts were resuspended in assay diluent with 5% ethanol to aid resuspension, and corticosterone concentrations were measured using a sensitive and specific radioimmunoassay kit (07-120103; MP Biomedicals, Santa Ana, CA, USA), as described previously [11]. Recovery of corticosterone was 78% in serum, 113% in thymus, 109% in proximal colon, and 116% in cecum. Reported tissue corticosterone concentrations are corrected for recovery. In some experiments, only serum corticosterone was measured; therefore, these serum samples were not extracted but diluted in assay diluent and directly quantified by radioimmunoassay.

Statistical analysis
Data are presented as means ± sem. Statistical significance was determined by a 2-tailed Student’s <i>t</i> test using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Results were considered statistically significant with <i>P</i> < 0.05.

RESULTS AND DISCUSSION

Early-life treatment with Abx leads to an earlier onset of experimental IBD
To investigate the hypothesis that gestational and early-life treatment with Abx affects the onset and severity of CD4+ T cell-
induced intestinal inflammation later in life, we compared the potency of transferred, naive CD4+ T cells from unmanipulated or Abx-treated mice to induce intestinal inflammation in recipient Rag1-deficient mice. We transferred naïve CD4+ T cells that were isolated either from >8 wk-old wild-type, untreated mice or mice that were treated with Abx in utero and for the first 3 wk of life (Fig. 1A). Strikingly, animals that received CD4+ T cells from Abx-treated mice displayed accelerated weight loss compared with mice that received control CD4+ T cells (Fig. 1B). At d 28 post-transfer, in addition to increased weight loss, we observed significantly increased disease severity (Fig. 1C and D). Analysis of the immune response at d 28 post-transfer revealed a trend toward increased expression of several proinflammatory cytokines in the intestine, which was consistent with the enhanced disease. We found that expression of Tnfa, Ifng, Il1b, and Il17a was increased in the gut of mice that received CD4+ T cells isolated from Abx-treated mice (Fig. 1E). Although the differences in cytokine gene expression did not achieve statistical significance, they were consistently higher over all independent experiments. Thus, early-life Abx treatment resulted in more pathologic CD4+ T cells that caused more severe intestinal inflammation.

Abx treatment does not lead to increased cytokine gene expression in CD4+ T cells

We next sought to understand how early-life Abx treatment led to more pathogenic CD4+ T cells later in life. As we observed a trend toward increased production of proinflammatory cytokines in vivo, we next examined the ability of CD4+ T cells from untreated and Abx-treated mice to produce cytokines under several conditions in vitro by intracellular cytokine staining and flow cytometry. We found equivalent production of IFN-γ, IL-10, IL-13, IL-17A, and TNF-α by CD4+ T cells from both untreated and Abx-treated mice under nonpolarizing or Th1-, Th2-, Th17-, and Treg-differentiating conditions (Fig. 2A). Thus, although early-life Abx treatment leads to increased pathogenicity of CD4+ T cells in vivo, it does not affect the ability of CD4+ T cells to produce cytokines in vitro following polyclonal stimulation.

Abx treatment does not affect TCR use or responsiveness

CD4+ T cells that induce IBD are primarily activated by antigens from intestinal microbes [9, 10, 12], and thus, it has been suggested that one of the benefits of Abx treatment in IBD patients is through the reduction of antigen load [13]. As we could not detect a general dysregulation of cytokine production

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**Figure 1. Early-life Abx treatment enhances the pathogenicity of CD4+ T cells.** (A) Schematic overview of the experiment. (B) Weight change during the course of experimental IBD after injection of naïve T cells from donor mice that were treated with Abx (○) or control (●) mice into recipient Rag1-deficient mice. Results are representative of 16 individual experiments (n = 3 for Abx and n = 6 for control; the last weight of deceased animals was continuously taken into account until the end of the experiment). (C) Histologic analysis of the proximal colons from recipient mice at the time point of analysis of mice shown in B. (D) Characterization of experimental IBD disease markers after analysis of the mice at d 28 (n = 4). (E) Gene-expression analysis of proinflammatory cytokines in the proximal colon of mice analyzed at d 28 post-T cell transfer (n = 4). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Not significant where not indicated. Error bars represent SEM.
in vitro, we hypothesized that the increased disease observed could be a result of a higher frequency of microbiome-specific T cells among the transferred, naive T cell population from Abx-treated mice. However, we found that the expression of TCR Vβ chains on CD4+ T cells from untreated and Abx-treated mice was similar, as assessed by flow cytometric analysis (Fig. 2B), suggesting that there was not a skewed T cell population in general. In addition, we observed no difference in the frequency of commensal bacteria-specific CD4+ T cells in the spleens of untreated or Abx-treated mice, as measured by binding to the CBir1-specific tetramer (Fig. 2C), which is consistent with results seen in germ-free mice [14]. However, we cannot exclude that transferred CD4+ T cells from Abx-treated mice are enriched for a distinct antigen of the microbiome and thus, cause a faster onset of the disease. As CBir1 reactivity is elevated in a subset of Crohn’s disease patients [15], our results suggest that increased frequencies of anti-commensal CD4+ T cells in patients are a consequence rather than the cause of the disease.

Another explanation for the heightened disease induced by CD4+ T cells from Abx-treated mice is increased TCR responsiveness, which could possibly lead to a faster or stronger T cell response and enhanced pathogenicity. To address this, we examined calcium flux following TCR stimulation using a fluorescence-based assay (Fig. 2D). Following stimulation of CD4+ T cells with antibodies against CD3, we observed that the calcium-sensitive Fluo-4/Fura Red signal was indistinguishable between T cells that were isolated from Abx-treated and those of control mice. Thus, although Abx treatment leads to T cells with increased pathogenic potential, Abx treatment does not affect TCR diversity or responsiveness.

**Unbiased analysis of the donor T cells reveals a differential gene-expression profile**

We next performed an unbiased gene-expression analysis (RNA-Seq) of naïve CD4+ T cells isolated from untreated and Abx-treated mice. RNA-Seq analysis of the naïve CD4+ T cells revealed an aberrant RNA expression pattern in T cells from Abx-treated mice (Fig. 3A). Strikingly, PCA revealed that although the naïve CD4+ T cells from untreated mice clustered together, CD4+ T cells from Abx-treated mice did not cluster (Fig. 3B). This could suggest that there is not a stereotypical response to Abx treatment and that CD4+ T cells from individual animals might respond distinctly to a loss of commensal microbes. Nevertheless, we did observe some consistent changes in gene expression between the samples (Fig. 3C). Gene-set enrichment analysis revealed that changes in gene expression were most significantly
associated with cellular metabolism, stress response, cell-cycle regulation, and cell death (Table 1). Thus, Abx treatment in early life led to changes in gene expression in naive CD4+ T cells from adult mice.

**Cohousing of untreated and Abx-treated mice abrogates pathology**

We next examined if the changes in the pathogenicity of CD4+ T cells associated with Abx treatment in early life were stable or plastic. We directly assessed this by cohousing Abx-treated mice with conventionally treated mice, 1 wk after weaning, and analyzed the cohoused mice at wk 8 postweaning. We found that cohousing the Abx-treated mice with conventionally treated mice reverted the faster onset of experimental IBD caused by CD4+ T cells from Abx-treated mice (Fig. 4A and B), suggesting that the Abx-associated changes in CD4+ T cell function were not fixed. We also observed trends toward reductions in expression of Cd4, Tnfα, and Ifng in the proximal colons of cohoused mice compared with Abx-treated mice with similar expression levels compared with control mice (Fig. 4C). Thus, these results suggest that the increased pathogenicity of CD4+ T cells mediated by early-life Abx treatment was
Our gene-expression analysis identified several gene sets that were dysregulated following Abx treatment, including stress, metabolism, proliferation, and death. As stress hormones, such as the glucocorticoid corticosterone, have been shown to affect all of these aspects of cell function [16], we conclude that an early-life Abx treatment has the potential to change the behavior of naive CD4+ T cells to induce a faster stress response associated with high levels of corticosterone, which impacts upon CD4+ T cell function and not resistance to corticosterone-induced apoptosis.

We show that CD4+ T cells from Abx-treated mice have an altered gene-expression profile that suggests a stressed phenotype. However, this phenotype could be reverted by cohousing Abx-treated mice with untreated mice. From these results, we conclude that an early-life Abx treatment has the potential to change the behavior of naive CD4+ T cells to induce a faster onset of experimental IBD. This finding is in line with previous reports of changes in the phenotypical behavior of CD4+ T cells after early-life Abx treatment in a direct [17] or indirect way [18] or of gnotobiotic mice [19]. However, results from these studies demonstrate that Abx-induced changes are not permanent and can be modified, suggesting that intervention to manipulate the microbiome may provide a mechanism to affect the development of IBD later in life.

### TABLE 1. Gene set enrichment analysis of differentially expressed genes between naive CD4+ T cells isolated from untreated and Abx-treated mice

<table>
<thead>
<tr>
<th>Gene set</th>
<th># of Genes</th>
<th>P</th>
<th>FDR</th>
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<tr>
<td>Stress</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Response to stress</td>
<td>16</td>
<td>1.19 x 10^-8</td>
<td>9.80 x 10^-7</td>
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<tr>
<td>Metabolic process</td>
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<td></td>
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<td>Nucleic acids</td>
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<td>8.52 x 10^-14</td>
<td>7.03 x 10^-11</td>
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<tr>
<td>Biopolymer</td>
<td>35</td>
<td>2.39 x 10^-12</td>
<td>9.84 x 10^-10</td>
</tr>
<tr>
<td>Cellular protein</td>
<td>25</td>
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<tr>
<td>Macromolecules</td>
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<td>2.35 x 10^-7</td>
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<td>4.76 x 10^-6</td>
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<td>1.12 x 10^-7</td>
<td>5.42 x 10^-6</td>
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<tr>
<td>Regulation</td>
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<td>1.70 x 10^-7</td>
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<tr>
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<td></td>
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<tr>
<td>Mitotic cell cycle</td>
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<td>7.96 x 10^-9</td>
<td>9.24 x 10^-7</td>
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<td>M Phase</td>
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<td>Mitosis</td>
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<td>Regulation</td>
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Gene set enrichment analysis on the top 100 up-regulated and 100 down-regulated genes was carried out using Molecular Signatures Database (MSigDB; software.broadinstitute.org/gsea/msigdb). FDR, False-discovery rate.

not stable and could be modified by re-exposure to a wild-type microbiome.

**Abx-treated mice show higher levels of serum corticosterone**

Our gene-expression analysis identified several gene sets that were dysregulated following Abx treatment, including stress, metabolism, proliferation, and death. As stress hormones, such as the glucocorticoid corticosterone, have been shown to affect all of these aspects of cell function [16], we first addressed whether the Abx-treated mice showed higher glucocorticoid levels compared with untreated mice. Indeed, corticosterone levels were higher in the serum of 3- and 6-wk-old Abx-treated mice and were still increased at the time point of analysis and transfer at 8 wk (Fig. 4D). In addition, we found heightened levels of corticosterone in the thymus, proximal colon, and cecum (Fig. 4E). The increased concentrations of corticosterone in the periphery of the Abx-treated mice did not have results in any increased sensitivity or resistance against corticosterone-induced apoptosis ex vivo (Fig. 4F). Thus, in addition to our results showing equivalent production of cytokines (Fig. 2A), these results suggest that the heightened levels of corticosterone in Abx-treated mice have minimal bystander-immunosuppressive effects on CD4+ T cells. Taken together, Abx treatment in early life leads to increased levels of circulating corticosterone that is maintained during aging. Importantly, the cohousing of untreated and Abx-treated mice resulted in a normalization of corticosterone levels (Fig. 4D). As we observed a reduction in disease in cohoused mice (Fig. 4A), these results suggest that early-life Abx treatment leads to a stress response associated with high levels of corticosterone, which impacts upon CD4+ T cell function and not resistance to corticosterone-induced apoptosis.
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Figure 4. The cohousing of Abx-treated mice with untreated mice leads to reduced pathogenicity of CD4+ T cells. (A) Weight changes of mice with experimental IBD after transfer of splenic, naive CD4+ T cells from Abx-treated, control, and cohoused mice \( (n = 6/\text{group}) \). (B) Disease score of mice in A. (C) Expression analysis of the indicated genes in the proximal colon of mice in A. (D) Serum corticosterone levels of mice that were treated with Abx in utero and for the first 3 wk of life (Abx), control mice on regular water, and Abx-treated mice that were cohoused after 28 d with control mice (cohoused mice; \( n = 3-12/\text{time point} \)). (E) Corticosterone levels in the thymus, proximal colon, and cecum (same mice as in D). (F) Analysis of corticosterone-induced apoptosis of CD4+ T cells. CD4+ T cells from Abx and control mice were incubated with indicated concentrations of the corticosteroid DEX and analyzed after 16 h for early (Annexin V+, P I+ ) and late (Annexin V+, P I+ ) apoptotic cells \( (n = 3) \). Data shown are representative of 2 individual experiments. *\( P \leq 0.05 \), **\( P \leq 0.01 \), ***\( P \leq 0.001 \). Error bars represent SEM.

AUTHORSHIP


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**DISCLOSURES**

The authors declare no conflicts of interest.

**REFERENCES**


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