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Antibiotics induce sustained dysregulation of intestinal T cell immunity by perturbing macrophage homeostasis

Nicholas A. Scott^{1,2}, Anna Andrusaite³, Peter Andersen⁴, Melissa Lawson⁵, Cristina Alcon-Giner⁵, Charlotte Leclaire⁵, Shabhonam Caim⁵, Gwenaelle Le Gall⁵, Tovah Shaw^{1,2}, James P. R. Connolly³, Andrew J. Roe³, Hannah Wessel³, Alberto Bravo-Blas³, Carolyn A. Thomson³, Verena Kästele³, Ping Wang¹, Daniel A. Peterson^{4,6}, Allison Bancroft^{1,7}, Xuhang Li⁴, Richard Grencis^{1,7}, Allan McI Mowat³, Lindsay J. Hall⁵, Mark A. Travis^{1,2,7}, Simon W. F. Milling^{#3}, and Elizabeth R. Mann^{#1,2,3,†}

¹Lydia Becker Institute of Immunology and Inflammation, University of Manchester, Manchester M13 9PT, UK

²Manchester Collaborative Centre for Inflammation Research, Faculty of Biology, Medicine and Health, Manchester Academic Health Science Centre, University of Manchester, Manchester M13 9PT, UK

³Centre for Immunobiology, Institute of Infection, Immunity and Inflammation, College of Medicine, Veterinary Medicine and Life Sciences, University of Glasgow, Glasgow G12 8TA, UK

⁴Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

⁵Quadram Institute Bioscience, Norwich Research Park, Norwich NR4 7UA, UK

⁶Eli Lilly and Company, Indianapolis, 46285 IN, USA

⁷Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, Manchester M13 9PT, UK

These authors contributed equally to this work.

Abstract

Macrophages in the healthy intestine are highly specialized and usually respond to the gut microbiota without provoking an inflammatory response. A breakdown in this tolerance leads to inflammatory bowel disease (IBD), but the mechanisms by which intestinal macrophages normally

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†Corresponding author. elizabeth.mann@manchester.ac.uk.

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become conditioned to promote microbial tolerance are unclear. Strong epidemiological evidence linking disruption of the gut microbiota by antibiotic use early in life to IBD indicates an important role for the gut microbiota in modulating intestinal immunity. Here, we show that antibiotic use causes intestinal macrophages to become hyperresponsive to bacterial stimulation, producing excess inflammatory cytokines. Re-exposure of antibiotic-treated mice to conventional microbiota induced a long-term, macrophage-dependent increase in inflammatory T helper 1 (T_H1) responses in the colon and sustained dysbiosis. The consequences of this dysregulated macrophage activity for T cell function were demonstrated by increased susceptibility to infections requiring T_H17 and T_H2 responses for clearance (bacterial *Citrobacter rodentium* and helminth *Trichuris muris* infections), corresponding with increased inflammation. Short-chain fatty acids (SCFAs) were depleted during antibiotic administration; supplementation of antibiotics with the SCFA butyrate restored the characteristic hyporesponsiveness of intestinal macrophages and prevented T cell dysfunction. Butyrate altered the metabolic behavior of macrophages to increase oxidative phosphorylation and also promoted alternative macrophage activation. In summary, the gut microbiota is essential to maintain macrophage-dependent intestinal immune homeostasis, mediated by SCFA-dependent pathways. Oral antibiotics disrupt this process to promote sustained T cell-mediated dysfunction and increased susceptibility to infections, highlighting important implications of repeated broad-spectrum antibiotic use.

Introduction

The intestinal immune system has evolved to coexist with the trillions of bacteria comprising the microbiota. Macrophages are critical for maintaining immunological homeostasis in the intestine and are highly adapted to the local environment, recognizing and responding to the gut microbiota without provoking an inflammatory response (1). Under steady-state conditions, intestinal macrophages are hyporesponsive to pattern recognition receptor stimulation (2–4) and shape local CD4⁺ T cell responses by maintaining immunosuppressive regulatory T cells (T_{regs}) (5). These homeostatic processes break down in inflammatory bowel disease (IBD), which is driven by dysregulated CD4⁺ T cell responses against the intestinal microbiota (6, 7) and is characterized by accumulation of inflammatory macrophages in the inflamed intestine (8–11). However, the pathogenesis of IBD remains poorly understood. In particular, the pathways that normally prevent damaging immune responses against the gut microbiota are incompletely defined.

Macrophages are key regulators of immune responses in the intestine and show specialization in the gut to promote tolerance against the microbiota. Thus, intestinal macrophages can promote tolerance via the anti-inflammatory mediators interleukin-10 (IL-10), transforming growth factor β , and retinoic acid (2, 12, 13). The specialization of intestinal macrophages to maintain an “anergic” state is proposed to involve interaction with the gut microbiota and its associated products (14–16). Bacteria residing in the intestine generate short-chain fatty acids (SCFAs) from dietary fiber, with high concentrations of SCFAs found in the colon with proposed anti-inflammatory functions on various immune cells in the intestine [reviewed by Rooks and Garrett (17)]. There is strong epidemiological evidence linking disruption of the gut microbiota by antibiotic use early in life with an increased risk of IBD and other inflammatory diseases (18–21). However, the underlying

mechanisms that promote these proinflammatory effects of antibiotics are poorly understood.

Here, we show that antibiotic-induced bacterial disturbances lead to persistent changes in adaptive immunity in the intestine by interfering with microbiota-dependent regulation of intestinal macrophage function. These immune changes in the intestine after antibiotics promoted susceptibility to intestinal infections and inflammation but could be prevented by supplementation with SCFAs, highlighting important mechanisms by which the microbiota drives intestinal immune tolerance.

Results

Recolonization of antibiotic-treated mice induces infiltration of innate inflammatory cells, followed by sustained T helper 1 responses

To determine the consequences of administering antibiotics and disruption of the microbiota on intestinal immunity, we treated mice with broad-spectrum antibiotics in their drinking water for 1 week. This treatment did not alter the proportions of monocytes, neutrophils, or eosinophils in the colonic lamina propria (LP; Fig. 1A). However, after recolonization with the microbiota from untreated wild-type (WT) mice for 7 days after antibiotic treatment, there were increased infiltrates of monocytes, eosinophils, and neutrophils in the colon compared with nonantibiotic-treated controls (Fig. 1A and fig. S1A). These infiltrates peaked between days 5 and 10 of recolonization but began to normalize around days 10 to 13 of recolonization (Fig. 1B).

The normalization of innate cell populations in the colonic LP after 10 to 13 days of recolonization coincided with the beginning of a sustained increase in interferon- γ (IFN γ)-producing CD4⁺ T cells (Fig. 1, B and C, and fig. S1B). The IFN γ -producing CD4⁺ T cells were Ki67⁺ (Fig. 1D), indicating recent local activation of the T helper 1 (T_H1) cells. Furthermore, there were markedly increased proportions of CD4⁺ T cells expressing the T_H1 transcription factor T-bet, together with reduced proportions of FoxP3⁺CD4⁺ T_{regs} (fig. S1C). The enhanced T_H1 responses persisted until at least 60 days after recolonization (Fig. 1E and fig. S1D). No changes were seen in the numbers of IL-17A-producing CD4⁺ T cells (fig. S1B). Thus, microbial recolonization after antibiotic use induces infiltration of innate immune cells, followed by a sustained skewing toward T_H1 CD4⁺ T cell responses.

CCR2⁺ monocyte-macrophages are essential for enhanced T_H1 responses after antibiotic treatment and recolonization

Next, we explored the cellular mechanisms driving the increase in T_H1 cells upon recolonization of antibiotic-treated mice. There were no alterations in the distribution, phenotype, or increased expression of T_H1-associated cytokines among migratory dendritic cell (DC) subsets within the mesenteric lymph nodes of mice recolonized after antibiotic treatment (fig. S2, A to C). Therefore, we focused on monocytes and macrophages as cells that might shape CD4⁺ T cell responses by acting as antigen-presenting cells or by modifying the cytokine environment of T cell polarization. As shown previously (4), the colon of healthy control mice contained Ly6C^{hi}MHC class II⁻ monocytes, Ly6C^{hi}MHC

class II⁺ immature/intermediate macrophages, and mature Ly6C⁺ macrophages expressing major histocompatibility complex (MHC) class II (Fig. 2A). Although there were no differences in the numbers of monocytes and mature macrophages in mice recolonized after antibiotic treatment versus control mice, there was a marked increase in the numbers of Ly6C^{hi}MHC class II⁺ immature/intermediate macrophages in the colon of recolonized mice (Fig. 2A). However, all three populations of colonic monocytes/macrophages from recolonized mice enhanced expression of proinflammatory cytokines, with monocytes showing increased *tnfa* expression, immature/intermediate macrophages showing increased *il6*, *il1β*, and *tnfa* expression, and mature macrophages showing increased *il6* and *il12p35* expression (Fig. 2B). Furthermore, macrophages from recolonized mice expressed higher amounts of *nos2* (associated with classical activation of proinflammatory macrophages) but lower amounts of *cd206* (associated with anti-inflammatory alternative activation; Fig. 2B). Thus, intestinal monocytes/macrophages can not only express MHC class II but also generate a proinflammatory environment that may promote the enhanced T_H1 responses during recolonization after antibiotic treatment.

Although macrophages in the adult intestine are predominantly composed of monocyte-derived cells that enter the intestine as Ly6C⁺ precursors (22), recent evidence demonstrates that some colonic macrophages may be long lived and maintained locally, independent of blood monocytes (23). The markers Tim4 and CD4 allow the fully differentiated intestinal macrophage pool to be divided into three subsets with distinct replenishment rates from blood monocytes. Tim4⁺CD4⁺ gut-resident macrophages are locally maintained independent of monocytes, whereas Tim4⁺CD4⁺ macrophages are replenished slowly by monocytes and Tim4⁺CD4⁺ macrophages are replenished continuously and relatively rapidly by monocytes (23). After confirming this heterogeneity in our control mice, we found that recolonizing after antibiotic treatment led to increased proportions of Tim4⁺CD4⁺ macrophages (Fig. 2C), suggesting that they were derived recently from monocytes and consistent with the expanded populations of Ly6C⁺ cells found in the previous experiments (Fig. 1A). Together, these findings indicate that the proinflammatory changes observed in macrophages after recolonization after antibiotic treatment are associated with an enhanced monocyte-derived macrophage response rather than long-lived, tissue-resident macrophage populations.

To directly examine whether monocyte-derived cells in the intestine influenced T_H1 responses after recolonization of antibiotic-treated mice, we used *Ccr2*^{-/-} mice that are deficient in the Ly6C^{hi} blood monocytes. These cells are the precursors of monocyte-derived intestinal macrophages (21) and contribute to the inflammatory infiltrate during recolonization (Fig. 1A and fig. S1A). As reported previously (21), the colon of steady-state *Ccr2*^{-/-} mice contained a residual population of mature Ly6C⁺MHC class II⁺ macrophages but lacked Ly6C^{hi} monocytes and immature macrophages (fig. S2D). Recent evidence indicates that these residual macrophages in the *Ccr2*^{-/-} intestine are Tim4⁺CD4⁺ embryonically derived cells (23). We found that *Ccr2*^{-/-} mice treated with antibiotics and recolonized with a normal microbiota failed to show the recruitment of Ly6C^{hi} monocytes seen in WT mice (fig. S2D). Their mature macrophages also did not show any alterations in expression of mRNA for *il6*, *il12p35*, *il1β*, *tnfa*, or *cd206* that was seen in recolonized WT mice (Fig. 2D). *Ccr2*^{-/-} mice did, however, display increased infiltrates of eosinophils 7 days after recolonization (Fig. 2E), but importantly, these animals did not display expansion of

IFN γ -producing CD4⁺ T cells 20 days after recolonization (Fig. 2F), the time point where T_H1 cells were expanded consistently in recolonized WT mice. There were no changes in any T cell subsets in *Ccr2*^{-/-} mice after antibiotic treatment and recolonization compared to untreated *Ccr2*^{-/-} mice (Fig. 2F and fig. S2E). Thus, the skewed T_H1 response and dysregulated macrophage function observed in antibiotic-treated and recolonized mice require CCR2-dependent recruitment of monocytes.

Given the differences in function between macrophages located within separate compartments of the intestine [i.e. LP versus muscularis; reviewed by Bogunovic *et al.* (24)], we sought to determine whether the proinflammatory properties of macrophages from recolonized mice may be related to localization of these subsets. The vast majority of macrophages in the colon, however, resided in the LP, with about 10-fold more macrophages within this compartment compared with the muscularis (fig. S2, F and G). Furthermore, there were no differences in numbers of macrophages in either compartment between control and recolonized mice (fig. S2F). Therefore, it is likely that the induced proinflammatory cytokine profile during recolonization is restricted to LP macrophages only.

Antibiotic treatment induces a dysregulated cytokine response to lipopolysaccharide in intestinal monocytes and macrophages

Intestinal macrophages are normally refractory to microbial stimulation (2–4). However, monocytes and mature macrophages expressed increased levels of inflammatory cytokines after recolonization of antibiotic-treated mice (Fig. 2B). Thus, antibiotic treatment may have increased intestinal monocyte and macrophage responses to microbial stimulation, disrupting this normal control mechanism. To test this, we stimulated these cells from antibiotic-treated mice *in vitro* with lipopolysaccharide (LPS) and assessed the production of proinflammatory tumor necrosis factor- α (TNF α) and anti-inflammatory IL-10 by intracellular cytokine staining. Immature Ly6C^{hi}MHC class II^{+/-} cells from the steady-state colon, as well as from the colon of antibiotic-treated mice, displayed a robust induction of TNF α in response to LPS (Fig. 3A). Notably, stimulation of colonic Ly6C^{hi} cells from antibiotic-treated mice failed to induce the increased production of IL-10 that was seen after LPS stimulation of steady-state Ly6C^{hi} cells (Fig. 3A).

Treatment with antibiotics led to few differences in gene expression by mature Ly6C⁻MHC class II⁺ macrophages isolated from the colon compared with control macrophages, a finding that was replicated using macrophages from the colon of germ-free (GF) mice (fig. S3). However, mature colonic macrophages from antibiotic-treated mice showed dysregulated cytokine production when challenged with LPS. Unlike Ly6C^{hi} monocytes and immature macrophages, LPS stimulation of mature colonic macrophages from control mice did not increase proportions of TNF α ⁺ or IL-10⁺ cells (Fig. 3B), confirming previous studies (4). In contrast, LPS stimulation of mature macrophages from the colon of antibiotic-treated mice induced an expansion in the proportion of TNF α -producing cells compared with both their nonstimulated counterparts and with LPS-stimulated macrophages from control mice (Fig. 3B). Furthermore, although there were no changes in IL-10 production, higher amounts of TNF α protein were produced from LPS-stimulated macrophages from antibiotic-treated mice compared with control mice (Fig. 3C). Fluorescence-activated cell

sorting (FACS)—sorted intestinal macrophages from antibiotic-treated mice also secreted more proinflammatory IL-6 and MCP-1 (monocyte chemoattractant protein-1) upon LPS stimulation into surrounding supernatants (Fig. 3D). Together, these data demonstrate that antibiotic treatment could cause intestinal monocytes and macrophages to respond inappropriately to bacterial stimulation, generating a proinflammatory cytokine profile.

Sustained immune dysfunction in recolonized mice repolarizes immune responses to infection

To understand the functional consequences of the increased numbers of T_H1 cells observed after antibiotic treatment and recolonization, we examined models of infection that normally require T_H17 (*Citrobacter rodentium*) or T_H2 responses (*Trichuris muris*) for their clearance. *C. rodentium* infection in mice peaked around day 7, as indicated by colony-forming units (CFU) from fecal pellets assessed during the course of infection (Fig. 4A), with robust T_H17 responses being found in the colon on day 10 of *C. rodentium* infection (fig. S4A). Compared with infected control mice, antibiotic-treated mice that had been infected with *C. rodentium* after 20 days of microbiota recolonization had an increased bacterial burden in the cecum 10 days after infection (Fig. 4A). In parallel, recolonized mice infected with *C. rodentium* had lower numbers of T_H17 cells ($ROR\gamma^t^+ CD4^+$ T cells producing IL-17A) than their control *C. rodentium*-infected counterparts (Fig. 4B). Intestinal macrophages from infected recolonized mice also expressed higher amounts of the costimulatory/activation molecule CD80 compared with macrophages from infected control mice (Fig. 4C). Infected recolonized mice also exhibited a reduction in the proportion of $FoxP3^+ CD4^+$ T_{regs} in the colon compared with infected controls (fig. S4B).

We next determined the consequences of antibiotic treatment and recolonization on infection with the intestinal helminth *T. muris*, which is normally expelled via a T_H2 -mediated response and induces inflammation in the colon (25). Compared with control mice, antibiotic-treated mice that were infected with *T. muris* after 20 days of recolonization had higher worm burdens at the peak of infection (Fig. 4D, 21 days after *T. muris* infection). Recolonized mice also had substantial numbers of worms at day 35 after infection, by which time most control mice had cleared the infection (Fig. 4D). Furthermore, recolonized mice demonstrated an impaired ability to generate T_H2 responses in the colon upon *T. muris* infection, with reduced expansion of $CD4^+$ T cells producing IL-13 (Fig. 4E) and expressing the T_H2 transcription factor GATA3 (fig. S4C).

Critically, the dysregulation of protective immunity in recolonized mice was long standing, because these animals were still less able to clear both *C. rodentium* and *T. muris* when infected 60 days after recolonization (Fig. 4F). Thus, these data indicate a long-term disruption of immune function and susceptibility to infections after antibiotic administration.

Recolonization of antibiotic-treated mice causes long-term disruption of the intestinal microbiota and a reduction in SCFAs in the intestine

The intestinal microbiota and intestinal immune system exert profound effects on each other (26, 27). Thus, we sought to determine whether the immune dysfunction that we observed after antibiotic treatment and recolonization was associated with alterations in the

microbiota. To do this, we used 16S ribosomal RNA (rRNA) sequencing to assess the composition of the bacterial communities in the colonic lumen. This analysis revealed sustained disruption of the intestinal microbiota that persisted for at least 60 days after recolonization (Fig. 5, A and B). When sequence data were compared using principal component analysis, PC1 consistently separated samples of recolonized mice from their age-matched control counterparts at all time points (Fig. 5A). Taxonomic profiling revealed an increased ratio of Bacteroidetes/Firmicutes phyla in recolonized mice (Fig. 5, B and C), as observed in human IBD (28). This skewing of bacterial phyla was predominantly accounted for by the notable and consistent reduction in bacteria of the *Allobaculum* genus (Firmicutes phylum) at all time points after recolonization (Fig. 5, B and C). Although little is known about the interactions between *Allobaculum* bacteria and the immune system, these bacteria have regulatory properties that are beneficial during inflammatory disease (29) and are potent generators of SCFAs (30, 31), which have a variety of immunoregulatory effects in the intestine (17).

Other components of the microbiota showed more variability after recolonization, with different genera predominating at different times (Fig. 5B). There was an absence of *Bifidobacteria*, which support the growth of SCFA-generating bacteria (32, 33) in recolonized mice, but there were enhanced levels of IL-12/IFN γ -inducing *Lactobacillus* (34–36). Similar alterations in the bacterial composition in the cecum were observed after recolonization (fig. S5). Thus, recolonization after antibiotic treatment causes differential microbial regrowth, with lower growth of bacteria associated with the generation of SCFAs. These changes persisted for at least 60 days, alongside the observed altered T cell immunity.

To directly determine whether antibiotic treatment (and subsequent recolonization) reduced amounts of SCFAs in the intestine, we used nuclear magnetic resonance (NMR) spectroscopy to determine the amounts of the SCFAs acetate, propionate, and butyrate [usually found at high concentrations in the normal colon (17)]. All three SCFAs were abolished during antibiotic administration, with amounts of butyrate remaining low during the recolonization period whereas acetate and propionate normalized by days 7 and 20, respectively (Fig. 5D). Thus, antibiotic-induced changes in the microbiota caused a reduction of SCFAs in the intestine, with a prolonged reduction in butyrate levels during recolonization.

The SCFA butyrate causes metabolic reprogramming of intestinal macrophages

SCFAs are proposed to be responsible for regulatory effects of the microbiota on intestinal immune cells (17), including macrophages (15, 16). Given the drastic depletion of SCFAs during antibiotic use, and to gain further insight into the mechanisms by which butyrate may act on intestinal macrophages in vivo, we carried out RNA sequencing (RNA-seq) analysis of FACS-sorted macrophages from recolonized mice that were administered antibiotics with or without butyrate before recolonization. Intestinal macrophages from recolonized mice that had received butyrate with antibiotics displayed a distinct gene signature compared with their antibiotic-treated/recolonized and control counterparts (Fig. 6A). Pathway analysis revealed butyrate-induced changes in genes involved in histone and chromatin modification, as expected. However, there was also up-regulation of genes involved in alternative

activation of macrophages (Fig. 6A). To validate these results, we confirmed that butyrate can directly drive alternative activation of macrophages in vitro, as previously reported (37, 38). SCFA-conditioned macrophages were assessed for expression of the alternative activation signature gene *Arginase 1* [*Arg1*; (39)]. Butyrate, but not propionate or acetate, induced a notable up-regulation of *Arg1* expression in macrophages (Fig. 6B), indicating that butyrate can directly drive alternative activation.

We looked for pathways induced by butyrate and associated with alternative activation in macrophages and found that the butyrate-specific gene signature included a notable up-regulation of genes involved in oxidative phosphorylation (OXPHOS) and lipid metabolism (Fig. 6C). Classically activated macrophages have a very different metabolic profile from that of alternatively activated macrophages, using glycolysis for production of proinflammatory cytokines and inducible nitric oxide synthase to enable rapid response to microbial encounter [reviewed by Pearce and Pearce (40) and Galván-Peña and O'Neill (41)]. On the other hand, OXPHOS and lipid metabolism (rather than glycolysis) dominate in alternatively activated macrophages, which exhibit a more anti-inflammatory profile (42, 43). These data therefore suggest that the potential anti-inflammatory effects of butyrate on macrophages may extend to inducing metabolic reprogramming to enable alternative activation of macrophages.

We next sought to determine whether butyrate can act directly on macrophages in vitro to drive metabolic reprogramming. Thus, to determine whether butyrate can directly shape OXPHOS and lipid metabolism in macrophages, cells were conditioned with SCFA and oxidative mitochondrial respiration was measured via oxygen consumption rate (OCR) using Seahorse technology. After addition of carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone [FCCP, which induces maximal respiratory capacity (44)], butyrate-treated macrophages displayed higher levels of OCR than untreated macrophages, indicating enhancement of OXPHOS induction by butyrate (Fig. 6D). Induction of OXPHOS was not observed when macrophages were treated with propionate or acetate, strongly suggesting that the enhancement was specific to butyrate (Fig. 6, D and 6E). Furthermore, in further support of butyrate-mediated metabolic reprogramming, the reduction in OCR after administration of etomoxir, an inhibitor of fatty acid oxidation, was greatest in butyrate-treated macrophages (Fig. 6, D and E), indicating higher levels of lipid metabolism in butyrate-treated macrophages compared with controls or propionate/acetate treatment. These data demonstrate that macrophage metabolism is modulated by butyrate, which can act to promote alternative activation of macrophages (37, 38).

Butyrate prevents antibiotic-associated immune dysfunction

We next explored whether SCFA supplementation during antibiotic treatment could ameliorate the immune dysfunction caused during recolonization. The SCFAs acetate, propionate, and butyrate were administered orally to mice while they were being treated with antibiotics, and the responses of intestinal macrophages to LPS were assessed ex vivo. As before, a higher frequency of mature colonic macrophages from antibiotic-treated mice expressed TNF α after LPS stimulation. Treatment of mice with butyrate, but not propionate or acetate, prevented this increase by intracellular flow cytometry (Fig. 7A) and also at the

level of secreted protein (fig. S6A). There was no effect of SCFA on IL-10 production (Fig. 7A), and TH1 skewing still occurred in recolonized mice deficient in IL-10 (*il10*^{-/-} mice; fig. S7), indicating IL-10 is unlikely to play a role in either the T cell dysfunction during recolonization or the protective effects of SCFA in this system. To determine whether butyrate could act directly on macrophages to reduce TNF α production in response to LPS, we administered butyrate to colonic macrophages in vitro during stimulation with LPS. Butyrate markedly reduced the amounts of TNF α protein secreted by intestinal macrophages after stimulation with LPS as measured by intracellular flow cytometry and also by detection of TNF α in supernatants, secreted by FACS-sorted intestinal macrophages (Fig. 7B). These data demonstrate that butyrate can act directly on intestinal macrophages to reduce inflammatory cytokine production.

Oral administration of butyrate during antibiotic treatment also completely prevented the increase in T_H1 responses in the colon 20 days after recolonization, with no increase in total IFN γ ⁺, IFN γ ⁺ Ki67⁺, and T-bet⁺ CD4⁺ T cell numbers (fig. S6B and Fig. 7C). Therefore, treatment with the SCFA butyrate prevented both the macrophage and T cell dysfunction after antibiotic use, as well as having direct effects on macrophage activation.

Thus, together, our results not only highlight crucial mechanisms regulating intestinal immune responses that are disrupted by antibiotic treatment but also provide strategies in which SCFAs maintain intestinal immune homeostasis, underpinning the anti-inflammatory properties of butyrate in the intestine.

Discussion

Here, we demonstrate that the gut microbiota is essential for the regulatory functions of intestinal macrophages, which is at least in part due to the immunoregulatory actions of the SCFA butyrate. These data provide a mechanistic link between antibiotic use and predisposition to intestinal inflammation, via sustained dysregulation of intestinal immunity. Oral antibiotics cause a hyperactive state in intestinal macrophages to promote sustained T cell-mediated dysfunction and increased susceptibility to infections, highlighting important implications of repeated broad-spectrum antibiotic use.

Intestinal macrophages normally prevent active immune responses against the commensal microbiota by acquiring a state of hyporesponsiveness to bacterial stimulation (22). We show here that this regulatory process is disrupted by antibiotic treatment, confirming that the intestinal microbiota plays a crucial role in shaping intestinal macrophage homeostasis to prevent inflammation. The dysregulated gene expression profile in macrophages from recolonized mice was restricted to monocyte-derived macrophages, with the tissue-resident embryonic-derived Tim4⁺CD4⁺ macrophages that dominate the intestinal mucosa in *Ccr2*^{-/-} mice (23) being unaffected by recolonization. These data suggest a marked plasticity in macrophage function during differentiation from monocytes that is susceptible to microbial disruption and indicate that differentially targeting monocyte-derived rather than tissue-resident macrophages during intestinal inflammation may be beneficial therapeutically.

It is not clear how intestinal macrophages normally become conditioned to promote tolerance against the microbiota, although evidence indicates an important role for IL-10 in shaping the regulatory properties of intestinal macrophages (2, 12, 13). However, in the absence of IL-10 (*il-10^{-/-}* mice), the macrophage-dependent T_H1 cell skewing still occurs in recolonized mice after antibiotic administration, indicating that antibiotics disrupt T cell polarization independent of IL-10.

SCFAs also have potent immunoregulatory effects on intestinal macrophages (15, 16) and are usually found at high concentrations in the healthy colon (17). SCFAs were diminished by antibiotic administration, with butyrate levels remaining low during recolonization, and the importance of SCFAs in our model was shown by the ability of butyrate to prevent the hyperresponsiveness of intestinal macrophages to microbial stimulation and to prevent the aberrant T_H1 response during recolonization. This may at least in part be due to inhibition of histone deacetylase activity by butyrate, which suppresses inflammatory cytokine production by intestinal macrophages (15), although butyrate can also act directly on these cells via its receptor, Gpr109a (16). SCFAs also shape the immunomodulatory functions of various other immune cells including epithelial cells, DCs, neutrophils, and T cells [reviewed by Rooks and Garrett (17)]. Thus, although we cannot rule out the possibility that the beneficial effects of butyrate in vivo in preventing antibiotic-associated immune dysfunction may be partially due to indirect effects on other cells, we have demonstrated that butyrate can directly reduce inflammatory cytokine production in intestinal macrophages. Our data also highlight an important new mechanism by which butyrate can regulate macrophage function via increasing levels of OXPHOS and lipid metabolism. These metabolic events play an important role in alternative activation of macrophages (37, 38), and butyrate may shape the metabolic profile of intestinal macrophages by acting directly as a metabolic substrate itself (45), via G protein (heterotrimeric guanine nucleotide-binding protein)-coupled receptor signaling or via inhibition of histone deacetylases.

These data have direct relevance to IBD, linking the aberrant T_H1 responses that dominate the inflamed mucosa in human Crohn's disease (7) with the immature inflammatory macrophages that accumulate under these conditions, where they produce inflammatory cytokines (8, 9). Furthermore, *Escherichia coli* infections (which *C. rodentium* models in mice) and other intestinal pathogenic infections requiring T_H17 responses for their clearance (*Salmonella* and *Campylobacter jejuni*) are high-risk factors associated with triggering the onset of IBD in genetically susceptible individuals (46). Thus, these data also provide a mechanistic link between antibiotic use and predisposition to intestinal inflammation, not only via dysregulated T cell immunity but also by enhancing susceptibility to infections that may act as triggering factors for the onset of IBD.

This work provides proof of principle that antibiotic-induced microbial disruption can have sustained effects on intestinal immune homeostasis, although limitations include the fact that the effects of individual antibiotics used in routine clinical practice have not been investigated. Furthermore, it is not known exactly how long macrophage function is disrupted during recolonization after antibiotic use or the precise mechanisms by which macrophages skew adaptive immunity toward T_H1 activation. Although it seems likely that this involves production of polarizing cytokines such as IL-12 in mice (47), which would

implicate macrophages rather than monocytes in driving the T_H1 response (only macrophages expressed increased amounts of IL-12 during recolonization), this remains to be proven directly. Furthermore, the possibility that other functions such as direct presentation of antigen by MHC class II-expressing macrophages skew immunity toward T_H1 activation cannot be excluded. It is also difficult to be precise about the exact cellular targets of butyrate in vivo and, particularly, whether butyrate may induce metabolic changes or alternative activation in monocytes and macrophages. The small numbers of monocytes present in the murine intestine make it difficult to perform in vitro assays or metabolic assays with these cells. Last, it is possible that the T cell alterations may not have solely been caused by a lack of butyrate only. For example, the altered (and more dynamic) microbial community during recolonization may contribute to influencing T cell responses independently of butyrate. Nonetheless, both the antibiotic-associated macrophage and T cell dysfunction were totally prevented by butyrate supplementation, underlying the potent immunoregulatory effects of this mediator.

In summary, the gut microbiota is essential for maintaining macrophage-mediated intestinal immune homeostasis through SCFA-dependent pathways. Oral broad-spectrum antibiotics can disrupt these pathways and promote sustained immune dysfunction. This not only leads to a local inflammatory state but also can polarize local T cell responses and influence responses to infection. Thus, we have highlighted the potential impact of interfering with microbiota-dependent regulation of intestinal macrophage function, with long-term detrimental implications for adaptive immunity and susceptibility to infections and inflammation. Targeting restoration of macrophage homeostasis after antibiotic treatment may be a novel and productive strategy for preventing sustained immune dysfunction.

Materials and Methods

Study design

The overall objective of this study was to determine how disruption of the intestinal microbiota with broad-spectrum antibiotic use may affect intestinal immune responses. Mice were administered antibiotics to disrupt the microbiota and, in some cases, subsequently recolonized with feces from untreated control mice to assess how the intestinal immune system responds when reintroduced to the microbiota after antibiotic administration. Some mice were infected with intestinal pathogens during the recolonization period to assess ability to clear infections. Immune characterization, bacterial sequencing, and metabolite analysis were carried out to assess alterations in the intestinal environment and immune responses during the recolonization period. End points were determined by defined periods of recolonization or after an appropriate number of days after pathogen infection. Animals were randomly assigned to treatment groups. Sampling and replicates differed between experiments and are stated in the figure legends. For all experiments, power calculations were used to determine that at least four to six mice per group would be needed to detect a 20% difference between groups with about 10% SD. No blinding was carried out. Primary data are located in table S4.

Mice

WT C57BL/6 mice (Envigo) were maintained under specific pathogen-free conditions at the University of Glasgow, UK, and the University of Manchester, UK. *Cx3cr1^{+/gfp}* and *Ccr2^{-/-}* mice were maintained under specific pathogen-free conditions at the University of Glasgow, UK. GF mice were maintained in the University of Manchester Gnotobiotic Mouse Facility (Manchester, UK) and in the Johns Hopkins Medicine Gnotobiotic Mouse Facility (Baltimore, USA). GF mice were confirmed to be free of all culturable bacteria before and at the culmination of all experiments. Conventionally housed counterparts were bred and maintained at Johns Hopkins and the University of Manchester. Age- and gender-matched adult (8 to 10 weeks old, male) animals were used in all experiments, approved by the University of Glasgow and University of Manchester Animal Welfare Ethical Review Boards and performed under licenses issued by the U.K. Home Office. All experiments were carried out according to U.K. Home Office regulations or in agreement with Johns Hopkins Medicine Institutional Animal Care and Use Committee-approved protocols. For each experiment, littermate mice were split into control and antibiotic-treated groups.

Antibiotic treatment

Mice received an antibiotic cocktail containing ampicillin (1g/liter), metronidazole (1g/liter), neomycin (1g/liter), gentamicin (1g/liter), and vancomycin (0.5g/liter) in drinking water for 7 days, which was replaced once, after 3 to 4 days.

SCFA treatment

Mice were administered acetate, propionate, or butyrate in their drinking water for 7 days at a concentration of 200 mM. SCFAs were added to LP cells in vitro at a concentration of 1 mM for 4 hours. These concentrations of SCFAs were based on published work (15, 17) and pilot experiments (no in vivo effects on macrophages <100 mM and decreased oral intake >300 mM; in vitro concentrations assessed for efficacy versus toxicity). The administered in vivo amount of 200 mM is higher than the physiological concentrations in the colon (20 to 50 mM) to take into account the absorption in the small intestine that will reduce the amount of SCFA reaching the colon. For in vitro experiments, 1 mM butyrate was used to take into account amounts of butyrate that might reach macrophages in the LP from the colonic lumen, where it is known that a substantial amount would be taken up by epithelial cells.

Recolonization of mice

Feces from control mice (housed separately from experimental mice) were administered to cages of both the experimental control group and the experimental antibiotic-treated group. Specifically, sawdust and feces from several cages of untreated (non-experimental) control mice were mixed together and distributed evenly between the experimental control group and the experimental antibiotic-treated group. Thus, mice were recolonized for variable time periods after 7-day antibiotic treatment. Cages and feces were replaced every 7 days.

Statistical analysis

On the basis of analyses of preliminary experiments, group sizes were chosen to ensure a 20% difference with about a 10% error rate, with variance being similar between groups to

be compared. Results are presented as individual data points with means, and groups were compared using a Mann-Whitney test (paired or unpaired) or Kruskal-Wallis test (for multiple groups) using Prism 7 software (GraphPad). $P < 0.05$ was considered significant. For microbiota analysis, analysis software R was used. Average community profile comparison of two groups was analyzed and compared using MEGAN. Abundance matrices are depicted using boxplots in R for each taxon, showing comparison of two groups at each time point (control versus antibiotics or control versus recolonized).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Recolonization of antibiotic-treated mice induces infiltration of innate inflammatory cells, followed by sustained TH1 responses.

(A) Colonic monocytes (CD11b⁺Ly6C⁺SiglecF⁺Ly6G⁻), eosinophils (SiglecF⁺MHC class II⁻Ly6C⁻), and neutrophils (SiglecF⁻MHC class II⁻Ly6G⁺Ly6C⁻) were identified from live CD45⁺ cells by flow cytometry in mice that had been recolonized with microbiota from WT mice for 7 days (7d) after 7 days of antibiotic treatment. Pooled data are shown [$*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Kruskal-Wallis test; $n = 15$ to 17 (control; Ctrl), $n = 9$ (antibiotics; Abx), $n = 6$ to 8 (recolonized)]. (B) Proportions of monocytes, neutrophils, and

eosinophils after antibiotic treatment and subsequent recolonization at time points up to 23 days of recolonization after antibiotic treatment (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Kruskal-Wallis test; $n = 3$ for all time points). (C) Proportions and numbers of CD3⁺CD4⁺ T cells and IFN γ production by CD3⁺CD4⁺ T cells in control versus recolonized mice were determined by flow cytometry, with representative flow cytometry plots [from day 13 (d13) of recolonization] and pooled data at time points shown (* $P < 0.05$, ** $P < 0.01$, Kruskal-Wallis test; $n = 3$ for all time points). (D) Ki67 expression by IFN γ -producing CD3⁺CD4⁺ T cells and T-bet expression by CD3⁺CD4⁺ T cells at day 20 of recolonization (** $P < 0.01$, *** $P < 0.001$, unpaired Mann-Whitney test; $n = 9$ to 12). (E) Analysis of CD3⁺CD4⁺ T cells producing IFN γ or expressing T-bet in mice that had been recolonized for 20 or 60 days after the end of antibiotic treatment. Data at each time point were compared to age-matched controls set up in parallel (* $P < 0.05$, *** $P < 0.001$, unpaired Mann-Whitney test; $n = 7$ to 20 per time point). All data represent at least two independent experiments.

Fig. 2. CCR2⁺ monocyte-macrophages are essential for enhanced TH1 responses after antibiotic treatment and recolonization.

(A) Live CD45⁺SiglecF⁺Ly6G⁺CD11b⁺CD64⁺ colonic LP cells were analyzed by flow cytometry, and monocytes (Ly6C⁺MHC class II⁻), intermediates (Ly6C⁺MHC class II⁺), and macrophages (Ly6C⁻MHC class II⁺) were identified in mice that had been recolonized for 7 days after antibiotic treatment. Representative flow cytometry plots and pooled data (numbers) are depicted (**P < 0.01, unpaired Mann-Whitney test; n = 8 to 10). (B) Ly6C⁺MHC class II⁻ monocytes, Ly6C⁺MHC class II⁺ intermediates, and Ly6C⁻MHC class II⁺

macrophages were sorted by flow cytometry, and mRNA expression for *il6*, *il12p35*, *TNF α* , *il1 β* , *nos2*, and *cd206* was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Results are shown as mean expression normalized to TATA binding protein and are shown relative to mean of control mice in all cases (* $P < 0.05$, ** $P < 0.01$, unpaired Mann-Whitney tests; $n = 4$ to 9). NOS2, nitric oxide synthase 2. (C) Expression of Tim4 and CD4 by colonic Ly6C⁺MHC class II⁺-differentiated macrophages of control or recolonized (day 7) mice was analyzed by flow cytometry, with representative flow cytometry plots and pooled data depicted (* $P < 0.05$, *** $P < 0.001$, unpaired Mann-Whitney tests; $n = 9$). (D) Ly6C⁺MHC class II⁺ macrophages from *Ccr2*^{-/-} mice were sorted by flow cytometry, and mRNA expression for *il6*, *il12p35*, *TNF α* , *il1 β* , and *cd206* was analyzed by qRT-PCR. Results are shown as mean expression normalized to TATA binding protein and shown relative to mean of *Ccr2*^{-/-} control mice in all cases (unpaired Mann-Whitney tests; $n = 4$). (E) Eosinophils (SiglecF⁺ MHC class II⁺Ly6C⁺) and neutrophils (SiglecF⁺ MHC class II⁺Ly6G⁺Ly6C⁺) were identified from live CD45⁺ cells by flow cytometry in *Ccr2*^{-/-} mice that had been recolonized for 7 days after antibiotic treatment. Pooled data are depicted (* $P < 0.05$, unpaired Mann-Whitney test; $n = 4$). (F) Production of IFN γ and IL-17A and expression of Ki67, T-bet, and FoxP3 by CD3⁺CD4⁺ cells from the colonic LP of control or recolonized (day 20) *Ccr2*^{-/-} mice were analyzed by flow cytometry, with pooled data depicted (** $P < 0.01$, *** $P < 0.001$, unpaired Mann-Whitney tests; $n = 5$ to 18). WT mice (control and recolonized) are also shown as a reference. All data shown are representative of at least two independent experiments.

Fig. 3. Antibiotic treatment induces a dysregulated cytokine response to LPS in intestinal monocytes and macrophages. Live CD45⁺SiglecF⁻Ly6G⁻CD11b⁺CD64⁺ colonic LP cells from control and antibiotic-treated mice (7 days) were further subdivided into (A) Ly6C^{hi} (monocytes and intermediates) and (B) Ly6C⁺MHC class II⁺ cells (mature macrophages). Response to in vitro LPS (1 μ g/ml) stimulation was assessed by measuring intracellular IL-10 and TNF α production by flow cytometry. Representative flow cytometry plots and pooled data (proportions of cytokine-producing cells) are depicted (* P < 0.05, ** P < 0.01, *** P < 0.001,

paired and unpaired Mann-Whitney tests; $n = 8$ to 10). (C) Pooled data depicting TNF α protein production by mature macrophages from control and antibiotic-treated mice, as assessed by mean fluorescence intensity (MFI; $*P < 0.05$, unpaired Mann-Whitney test; $n = 4$). (D) FACS-sorted colonic macrophages (live CD45 $^{+}$ SiglecF $^{-}$ Ly6G $^{-}$ CD11b $^{+}$ CD64 $^{+}$ Ly6C $^{-}$ MHC class II $^{+}$ cells) were cultured for 18 hours with LPS (100 ng/ml), and secreted protein was measured in supernatants by cytometric bead array ($*P < 0.05$, unpaired Mann-Whitney test; $n = 5$). Data in (C) are representative of one of three individual experiments with similar results. All other data are representative of at least two independent experiments.

Fig. 4. Sustained immune dysfunction in recolonized mice repolarizes immune responses to infection.

(A) Mice were treated with antibiotics and subsequently recolonized for 20 days before infection with *C. rodentium*, alongside nonantibiotic-treated mice (controls). CFU were grown overnight from fecal pellets harvested during infection for assessment of kinetics and from cecal homogenates 10 days after infection (* $P < 0.05$, nonpaired Mann-Whitney test; data shown as $n = 5$ but representative of two individual experiments with similar results).

(B) Live CD45⁺CD3⁺CD4⁺ colonic T cells were identified by flow cytometry from control

and recolonized mice infected with *C. rodentium* and characterized for expression of ROR γ t and for production of IL-17A. Pooled data are depicted (* $P < 0.05$, nonpaired Mann-Whitney test; $n = 8$). (C) Mature macrophages were identified from colonic LP cells as live CD45⁺SiglecF⁻Ly6G⁻CD11b⁺ CD64⁺Ly6C⁻MHC class II⁺ cells, and surface expression of CD80 was characterized by flow cytometry. Representative flow cytometry plots and pooled data are depicted (* $P < 0.05$, unpaired Mann-Whitney test; $n = 5$). (D) Mice were treated with antibiotics and subsequently recolonized for 20 days before infection with the intestinal helminth *T. muris*, alongside nonantibiotic-treated mice. Ceca and proximal colons were harvested at day 21 or day 35 after *T. muris* infection. Pooled data depict worm counts from the cecum of *T. muris*-infected control (nonantibiotic-treated) mice and from infected recolonized mice (* $P < 0.05$, nonpaired Mann-Whitney test; $n = 5$ at both time points). (E) Live CD45⁺CD3⁺CD4⁺ T cells from the proximal colon were identified by flow cytometry and characterized for production of IL-13. Representative flow cytometry plots and pooled data are depicted (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Kruskal-Wallis test; $n = 5$ at both time points). (F) Mice were treated with antibiotics and subsequently recolonized for 60 days before infection with either *C. rodentium* or *T. muris*, alongside nonantibiotic-treated control mice. For *C. rodentium* infections, 10 days after infection, tissue homogenates from the ceca were used to grow CFU overnight (* $P < 0.05$, unpaired Mann-Whitney test; $n = 5$). For *T. muris* infections, ceca were harvested at day 21 or day 35 after *T. muris* infection, and worms were counted (* $P < 0.05$, unpaired Mann-Whitney test; $n = 5$). Data shown in all experiments are representative of at least two individual experiments.

Fig. 5. Recolonization of antibiotic-treated mice causes long-term disruption of the intestinal microbiota and a reduction in SCFAs in the intestine.

(A) Principal component analysis using Bray-Curtis metrics performed on the basis of the taxonomic assignments obtained from the 16S rRNA gene sequencing libraries analyzed from colonic stool samples of recolonized mice (red, A samples) at days 13, 20, and 30 and 60 days after antibiotic treatment and age-matched controls (dark blue, C samples). Results are shown as three mice per group. (B) Bar charts represent the relative abundance of the bacterial taxa. Bar colors represent different genus taxa, and bar heights signify the relative abundance of each taxon in colonic stool samples from control and recolonized mice (days

13, 20, 30, and 60). **(C)** Pooled data depicting ratios of phyla Bacteroidetes/Firmicutes in colonic stool samples from control and recolonized mice and number of reads from *Allobaculum* genus as a proportion of total number of reads per sample, in control and recolonized mice ($n = 3$). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. **(D)** Metabolite analysis was carried out via NMR on colonic fecal samples from control and recolonized mice and was normalized to fecal weight to determine absolute levels of SCFAs in the intestine. Pooled data depicting heatmaps made from Z -scores (relative) and summary graphs representing normalized amounts of SCFAs are shown [$*P < 0.05$, $***P < 0.001$, Kruskal-Wallis test; $n = 16$ (controls) and $n = 4$ (recolonized)].

Fig. 6. Butyrate causes metabolic reprogramming of intestinal macrophages.

(A) RNA-seq results were generated from FACS-sorted mature colonic macrophages (live CD45⁺SiglecF⁻Ly6G⁻CD11b⁺ CD64⁺Ly6C⁻MHC class II⁺ cells) from control mice or mice that had been treated with antibiotics ($\pm$ 200 mM butyrate) before recolonization (7 days). Main heatmap is shown depicting relative gene expression (Z-scores) from individual samples, showing all differentially expressed genes. Individual heatmaps show the gene expression profiles of genes involved in histone/chromatin modification and alternative activation as identified by pathway [Kyoto Encyclopedia of Genes and Genomes (KEGG)]

analysis. **(B)** Bone marrow–derived macrophages were conditioned with SCFAs during development and mRNA for *arg1* was analyzed by qRT-PCR. Results are shown as mean expression normalized to TATA binding protein and shown relative to mean of the control group in all cases (* $P < 0.05$, Kruskal-Wallis test; $n = 4$). **(C)** Heatmaps showing gene expression profiles of genes involved in OXPHOS and lipid metabolism as identified by pathway (KEGG) analysis, after RNA-seq of colonic macrophages from control mice or mice recolonized after antibiotic treatment $\pm$ 200 mM butyrate. **(D)** Oxygen consumption rate (OCR) of bone marrow–derived macrophages conditioned with SCFAs (1 mM) during development, shown at baseline and after sequential treatment with oligomycin (Oligo), FCCP, etomoxir (Eto), and rotenone plus antimycin (R + A). Results were normalized to cellular protein levels. **(E)** OCR of macrophages after FCCP administration (mitochondrial stress). Results were normalized to protein levels (* $P < 0.05$, ** $P < 0.01$, Kruskal-Wallis test; $n = 4$). Lipid metabolism is shown as the fold change (reduction) in OCR in macrophages after etomoxir administration, indicating measures of reliance on fatty acid oxidation (** $P < 0.01$, Kruskal-Wallis test; $n = 4$). All data shown are representative of at least two independent experiments. All metabolic assays were performed in quadruplet per data point.

Fig. 7. The SCFA butyrate prevents antibiotic-associated immune dysfunction.

(A) Live CD45⁺ SiglecF⁻ Ly6G⁻CD11b⁺ CD64⁺Ly6C⁻MHC class II⁺ mature colonic macrophages were characterized from control mice, antibiotic-treated mice, and mice treated with antibiotics that had been supplemented with butyrate (But), propionate (Prop), or acetate (Ace) for 7 days (200 mM each). After in vitro stimulation with LPS (1 μ g/ml), intracellular IL-10 and TNF α production were assessed by flow cytometry. Representative flow cytometry plots and pooled data (proportions of cytokine-producing cells) are depicted [$*P < 0.05$, $**P < 0.01$, paired Mann-Whitney test and Kruskal-Wallis test; $n = 10$ (control

and antibiotics), $n = 7$ (antibiotics + butyrate, antibiotics + propionate), and $n = 4$ (antibiotics + acetate)]. **(B)** Total colonic LP cells were stimulated in vitro with LPS (1 $\mu\text{g/ml}$) in the presence or absence of butyrate (1 mM), and mature colonic macrophages were identified by flow cytometry and assessed for intracellular TNF α production. Representative flow cytometry plots demonstrating intracellular TNF α protein (as assessed by MFI) and pooled data are depicted ($*P < 0.05$, paired Mann-Whitney test; $n = 6$). In other experiments, colonic mature macrophages were FACS-sorted and cultured in vitro with LPS (100 ng/ml) for 18 hours, and TNF α secretion into surrounding supernatant was assessed by cytometric bead array ($*P < 0.05$, unpaired Mann-Whitney test; $n = 4$). **(C)** Live CD45 $^{+}$ CD3 $^{+}$ CD4 $^{+}$ colonic T cells were characterized from control mice and mice that had been recolonized for 20 days after antibiotic treatment. In one group, antibiotics were supplemented with butyrate (200 mM). CD3 $^{+}$ CD4 $^{+}$ T cell production of IFN γ and expression of Ki67 and T-bet were determined by flow cytometry. Representative flow cytometry plots and pooled data are depicted [$*P < 0.05$, $***P < 0.001$, Kruskal-Wallis test; $n = 12$ (control and recolonized) and $n = 6$ (butyrate)]. All data shown are representative of at least two independent experiments.