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

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**Correspondence/lead contact:** Dr. Elizabeth Mann: Elizabeth.Mann@manchester.ac.uk

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**Deleted: Keywords:** Intestinal macrophages; microbiota; T-cell immunity; antibiotics, intestinal infections; intestinal inflammation; short-chain fatty acids

**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 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 hyper-responsive 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 Th1 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 Th17 and Th2 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 hypo-responsiveness of intestinal macrophages, and prevented T-cell dysfunction. Butyrate altered the metabolic behaviour of macrophages to increase oxidative phosphorylation (OXPHOS) 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.

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## INTRODUCTION

The intestinal immune system has evolved to co-exist 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 hypo-responsive to pattern recognition receptor (PRR) stimulation *(2-4)* and shape local $CD4^+$ T-cell responses by maintaining immuno-suppressive regulatory T-cells (Tregs) *(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 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 (TGFβ), and retinoic acid (RA) *(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 in Rooks et al. *(17)*). There is strong epidemiological evidence linking disruption of the gut microbiota by antibiotic use early in life with increased risk of IBD and other inflammatory diseases *(18-21)*. However, the underlying mechanisms that promote these pro-inflammatory 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 following antibiotics promoted susceptibility to intestinal infections and

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inflammation, but could be prevented by supplementation with SCFAs, highlighting important mechanisms by which the microbiota drives intestinal immune tolerance.

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## RESULTS

### Recolonization of antibiotic-treated mice induces infiltration of innate inflammatory cells followed by sustained Th1 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; Figure 1a). However, following 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 non-antibiotic treated controls (Figure 1a and Supplementary Figure 1a). These infiltrates peaked between days 5-10 of recolonization, but began to normalize around day 10-13 of recolonization (Figure 1b).

The normalization of innate cell populations in the colonic LP after 10-13 days of recolonization coincided with the beginning of a sustained increase in IFNγ-producing $CD4^+$ T-cells (Figure 1b, c and Supplementary Figure 1b). The IFNγ-producing $CD4^+$ T-cells were $Ki67^+$ (Figure 1d), indicating recent local activation of the Th1 cells. Furthermore, there were markedly increased proportions of $CD4^+$ T-cells expressing the Th1 transcription factor T-bet, together with reduced proportions of $FoxP3^+CD4^+$ Tregs (Supplementary Figure 1c). The enhanced Th1 responses persisted until at least 60 days post-recolonization (Figure 1e and Supplementary Figure 1d). No changes were seen in the numbers of IL-17A-producing $CD4^+$ T-cells (Supplementary figure 1b). Thus, microbial recolonization following antibiotic use induces infiltration of innate immune cells followed by a sustained skewing towards Th1 $CD4^+$ T-cell responses.

### $CCR2^+$ monocyte-macrophages are essential for enhanced Th1 responses following antibiotic treatment and recolonization

Next, we explored the cellular mechanisms driving the increase in Th1 cells upon recolonization of antibiotic-treated mice. There were no alterations in the distribution, phenotype, or increased expression of Th1-associated cytokines amongst migratory

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dendritic cell (DC) subsets within the mesenteric lymph nodes (MLN) of mice recolonized after antibiotic treatment (Supplementary figures 2a-2c). Therefore, we focused on monocytes and macrophages as cells that might shape $CD4^+$ T-cell responses by acting as antigen-presenting cells (APCs) or by modifying the cytokine environment of T-cell polarization. As shown previously *(4)*, the colon of healthy control mice contained $Ly6C^{hi}$ $MHCII^-$ monocytes, $Ly6C^{hi}MHC$ Class $II^+$ immature/intermediate macrophages, and mature $Ly6C^-$ macrophages expressing MHC Class II (Figure 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 (Figure 2a). However, all three populations of colonic monocytes/macrophages from recolonized mice enhanced expression of pro-inflammatory cytokines, with monocytes showing increased *tnfα* expression, immature/intermediate macrophages showing increased *il6, il1β* and *tnfα* expression and mature macrophages showing increased *il6* and *il12p35* expression (Figure 2b). Furthermore, macrophages from recolonized mice expressed higher amounts of *nos2* (associated with classical activation of pro-inflammatory macrophages) but lower amounts of *cd206* (associated with anti-inflammatory alternative activation; Figure 2b). Thus intestinal monocytes/macrophages can not only express MHC Class II, but also generate a pro-inflammatory environment that may promote the enhanced Th1 responses during recolonization following antibiotic treatment.

Although macrophages in the adult intestine are predominantly comprised 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

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after antibiotic treatment led to increased proportions of $Tim4^{-}CD4^{-}$ macrophages (Figure 2c), suggesting they were derived recently from monocytes and consistent with the expanded populations of $Ly6C^{+}$ cells found in the previous experiments (Figure 1a). Together, these results indicate that the pro-inflammatory changes observed in macrophages after recolonization following 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 Th1 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 (Figure 1a and Supplementary figure 1a). 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 (Supplementary Figure 2d). Recent evidence indicates 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 (Supplementary Figure 2d). Their mature macrophages also did not show any alterations in expression of mRNA for *il6, il-12p35, il-1β, tnfα* or *cd206* that were seen in recolonized WT mice (Figure 2d). *Ccr2*$^{-/-}$ mice did however display increased infiltrates of eosinophils 7 days after recolonization (Figure 2e) but importantly, these animals did not display expansion of IFNγ-producing $CD4^{+}$ T-cells 20 days after recolonization (Figure 2f), the time point where Th1 cells were expanded consistently in recolonized WT mice. There were no changes in any T-cell subsets in *Ccr2*$^{-/-}$ mice following antibiotic treatment and recolonization, compared to untreated *Ccr2*$^{-/-}$ *mice* (Figure 2f and Supplementary Figure 2e). Thus, the skewed Th1 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. lamina propria versus muscularis; reviewed in

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Bogunovic et al. *(24)*), we sought to determine whether the pro-inflammatory 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 lamina propria, with approximately 10-fold more macrophages within this compartment compared to the muscularis (Supplementary Figures 2f and 2g). Furthermore, there were no differences in numbers of macrophages in either compartment between control and recolonized mice (Supplementary Figure 2f). Therefore, it is likely that the induced pro-inflammatory cytokine profile during recolonization is restricted to lamina propria macrophages only.

**Antibiotic treatment induces a dysregulated cytokine response to LPS 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 following recolonization of antibiotic-treated mice (Figure 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 LPS and assessed the production of pro-inflammatory TNF$\alpha$ and anti-inflammatory IL-10 by intracellular cytokine staining. Immature Ly6C$^{hi}$ MHC Class II$^{+/-}$ cells from the steady-state colon, and from the colon of antibiotic-treated mice, displayed a robust induction of TNF$\alpha$ in response to LPS (Figure 3a). Strikingly, 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 (Figure 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 mice (Supplementary figure 3). 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

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proportions of TNF$\alpha^+$ or IL-10$^+$ cells (Figure 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$\alpha$-producing cells compared with both their non-stimulated counterparts and with LPS-stimulated macrophages from control mice (Figure 3b). Furthermore, although there were no changes in IL-10 production, higher amounts of TNF$\alpha$ protein were produced from LPS-stimulated macrophages from antibiotic treated mice compared with control mice (Figure 3c). FACS-sorted intestinal macrophages from antibiotic-treated mice also secreted more pro-inflammatory IL-6 and MCP-1 upon LPS stimulation into surrounding supernatants (Figure 3d). Together, these data demonstrate that antibiotic treatment could cause intestinal monocytes and macrophages to respond inappropriately to bacterial stimulation, generating a pro-inflammatory cytokine profile.

**Sustained immune dysfunction in recolonized mice repolarizes immune responses to infection**

To understand the functional consequences of the increased numbers of Th1 cells observed following antibiotic treatment and recolonization, we examined models of infection that normally require Th17 (*Citrobacter rodentium)* or Th2 responses (*Trichuris muris*) for their clearance. *C. rodentium* infection in mice peaked around day 7 as indicated by colony forming units (CFU) from faecal pellets assessed during the course of infection (Figure 4a), with robust Th17 responses being found in the colon on day 10 of *C. rod* infection (Supplementary Figure 4a). 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 caecum 10 days after infection (Figure 4a). In parallel, recolonized mice infected with *C. rodentium* had lower numbers of Th17 cells (ROR$\gamma t^+$ CD4$^+$ T-cells producing IL-17A) than their control *C. rodentium*-infected counterparts (Figure 4b). Intestinal macrophages from infected recolonized mice also expressed higher amounts of the co-stimulatory/activation molecule CD80 compared to macrophages from infected control mice (Figure 4c). Infected recolonized mice also exhibited a reduction in the proportion of FoxP3$^+$CD4$^+$ Tregs in the colon compared with infected controls (Supplementary Figure 4b).

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We next determined the consequences of antibiotic treatment and recolonization on infection with the intestinal helminth *Trichuris muris*, which is normally expelled via a Th2-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 (Figure 4d). Furthermore, recolonized mice demonstrated an impaired ability to generate Th2 responses in the colon upon *T. muris* infection, with reduced expansion of $CD4^+$ T-cells producing IL-13 (Figure 4e) and expressing the Th2 transcription factor GATA3 (Supplementary Figure 4c).

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

**Recolonization of antibiotic-treated mice causes long-term disruption of the intestinal microbiota and a reduction in short-chain fatty acids 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 we observed after antibiotic treatment and recolonization was associated with alterations in the microbiota. To do this, we used 16S 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 (Figure 5a and 5b). 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 (Figure 5a). Taxonomic profiling revealed an increased ratio of Bacteroidetes: Firmicutes phyla in recolonized mice (Figures 5b and 5c), as observed in human IBD *(28)*. This skewing of bacterial phyla was predominantly accounted for by the striking and consistent reduction in bacteria of the *Allobaculum* genus (Firmicutes phylum) at

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all time points after recolonization (Figures 5b and 5c). 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 immuno-regulatory effects in the intestine *(17)*.

Other components of the microbiota showed more variability after recolonization, with different genera predominating at different times (Figure 5b). Interestingly, there was an absence of *Bifidobacteria* which support the growth of SCFA-generating bacteria *(32,33)* in recolonized mice, but enhanced levels of IL-12/IFNγ-inducing *Lactobacillus* *(34-36)*. Similar alterations in the bacterial composition in the caecum were observed after recolonization (Supplementary Figure 5). Thus, recolonization following antibiotic treatment causes differential microbial re-growth, 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 decreased during the recolonization period whilst acetate and propionate normalized by days 7 and 20, respectively (Figure 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 short-chain fatty acid 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 RNAseq analysis of FACS-sorted macrophages from recolonized mice

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that were administered antibiotics with or without butyrate, prior to recolonization. Intestinal macrophages from recolonized mice that had received butyrate with antibiotics displayed a distinct gene signature compared to their antibiotic-treated/recolonized and control counterparts (Figure 6a). Pathway analysis revealed butyrate-induced changes in genes involved in histone and chromatin modification as expected. However, there was also upregulation of genes involved in alternative activation of macrophages (Figure 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).* Butyrate, but not propionate or acetate, induced a striking upregulation of *Arg1* expression in macrophages (Figure 6b), indicating 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 striking upregulation of genes involved in oxidative phosphorylation (OXPHOS) and lipid metabolism (Figure 6c). Classically activated macrophages have a very different metabolic profile from that of alternatively activated macrophages, utilizing glycolysis for production of pro-inflammatory cytokines and iNOS to enable rapid response to microbial encounter (reviewed by Pearce et al. *(39)* and Galván-Peña et al. *(40)*). On the other hand, OXPHOS and lipid metabolism (rather than glycolysis) dominate in alternatively activated macrophages, which exhibit a more anti-inflammatory profile *(41, 42)*. 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. Following addition of carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP, which induces maximal respiratory capacity *(43)*) butyrate-treated macrophages displayed higher levels of OCR than untreated macrophages indicating enhancement of OXPHOS

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induction by butyrate (Figure 6d). Induction of OXPHOS was not observed when macrophages were treated with propionate or acetate, strongly suggesting the enhancement was specific to butyrate (Figure 6d and 6e). Furthermore, in further support of butyrate-mediated metabolic reprogramming, the reduction in OCR following administration of etomoxir, an inhibitor of fatty acid oxidation, was greatest in butyrate-treated macrophages (Figure 6d and 6e) 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 or 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 TNFα after LPS stimulation. Treatment of mice with butyrate, but not propionate or acetate, prevented this increase by intracellular flow cytometry (Figure 7a) and also at the level of secreted protein (Supplementary figure 6a). There was no effect of SCFA on IL-10 production (Figure 7a). To determine whether butyrate could act directly on macrophages to reduce TNFα production in response to LPS, we administered butyrate to colonic macrophages *in vitro* during stimulation with LPS. Butyrate markedly reduced the amounts of TNFα protein secreted by intestinal macrophages after stimulation with LPS as measured by intracellular flow cytometry and also by detection of TNFα in supernatants, secreted by FACS-sorted intestinal macrophages (Figure 7b). These data demonstrate that butyrate can act directly on intestinal macrophages to reduce inflammatory cytokine production.

Importantly, oral administration of butyrate during antibiotic treatment also completely prevented the increase in Th1 responses in the colon 20 days after recolonization, with no increase in total IFN$\gamma^+$, IFN$\gamma^+$ Ki67$^+$ and T-bet$^+$ CD4$^+$ T-cell

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numbers (Supplementary figure 6b and Figure 7c). Therefore, treatment with the SCFA butyrate prevented both the macrophage and T-cell dysfunction following 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.

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## 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 immuno-regulatory actions of the short-chain fatty acid 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 hypo-responsiveness 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. Interestingly, 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 Th1 cell skewing still occurs in recolonized mice following antibiotic administration indicating antibiotics disrupt T-cell polarisation independent of IL-10.

SCFAs also have potent immuno-regulatory effects on intestinal macrophages *(15,16)*, and are usually found at high concentrations in the healthy colon *(17)*.

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Discussion is a bit on the long side; please try to condense/remove sentences that repeat results.

Instead of focusing on monocyte subsets, could this be tied back to the impact of antibiotics on human gut immunity?

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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 hyper-responsiveness of intestinal macrophages to microbial stimulation as well as preventing the aberrant Th1 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, dendritic cells, neutrophils and T-cells (reviewed in Rooks et al. *(17)*). Thus, although we cannot rule out 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 coupled receptor signalling or via inhibition of histone deacetylases.

These data have direct relevance to IBD, linking the aberrant Th1 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 Th17 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

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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 towards Th1 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 Th1 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 cannot be excluded. It is also difficult to be precise about the exact cellular targets of butyrate *in vivo* and in particular whether butyrate may induce metabolic changes or alternative activation in monocytes as well as macrophages. The small numbers of monocytes present in the murine intestine makes it difficult to perform *in vitro* assays or metabolic assays with these cells. Finally, it is possible that the T-cell alterations may not have solely been caused by 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 immuno-regulatory 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 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.

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## 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 impact intestinal immune responses. Mice were administered antibiotics to disrupt the microbiota, and in some cases subsequently recolonized with faeces from untreated control mice to assess how the intestinal immune system responds when re-introduced to the microbiota following 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 following 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 4-6 mice per group will be needed to detect a 20% difference between groups with approximately 10% standard deviation. No blinding was carried out. Primary data are located in table S4.

### Mice

Wild-type 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. Germ free (GF) mice were maintained in the University of Manchester Gnotobiotic Mouse Facility (Manchester, UK), and in the Johns Hopkins Medicine Gnotobiotic Mouse Facility (Baltimore, US). GF mice were confirmed to be free of all culturable bacteria prior to 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 (8-10 week 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 UK Home Office. All experiments were

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carried out according to UK 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/L), metronidazole (1g/L), neomycin (1g/L), gentamicin (1g/L) and vancomycin (0.5g/L) in drinking water for 7 days, which was replaced once, after 3-4 days.

**SCFA treatment**

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

**Re-colonization of mice**

Faeces 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 faeces from several cages of untreated (non-experimental) control mice were mixed together and distributed evenly between experimental control groups and experimental antibiotic-treated groups. Thus, mice were recolonized for variable time periods following 7-day antibiotic treatment. Cages and faeces were replaced every 7 days.

**Statistical analysis**

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Based on analyses of preliminary experiments, group sizes were chosen to ensure a 20% difference with approximately 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 (paired or unpaired) or for multiple groups, Kruskal-Wallis tests 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 were analysed and compared using MEGAN. Abundance matrices are depicted using boxplots in R for each taxa showing comparison of two groups at each time point (control versus antibiotics or control versus recolonized).

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**SUPPLEMENTARY MATERIALS**

Fig S1: Monocytes, neutrophils, eosinophils and T cells in recolonized mice.

Fig S2. Characterization of dendritic cells, and distribution of monocytes and macrophages in *Ccr2*$^{-/-}$ mice.

Fig S3. Gene expression profiles of mature colonic macrophages from antibiotic-treated and germ free mice.

Fig S4. Sustained immune dysfunction in recolonized mice repolarizes immune responses to infection.

Fig S5. Recolonization of antibiotic-treated mice causes long-term disruption of the intestinal microbiota.

Fig S6. The short-chain fatty acid butyrate prevents antibiotic-associated immune dysfunction.

Fig S7. Th1 polarization in *il-10*$^{-/-}$ recolonized mice.

Supplementary materials and methods.

Table S1. Antibodies.

Table S2. qPCR primer sequences for macrophage gene expression.

Table S3. Primer sequences for 16S bacterial sequencing.

Table S4. Primary data

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## AUTHOR CONTRIBUTIONS

N.S., A.A., M.L., C.A.G., C.L., S.C., G.L.G., T.S., H.W., A.B., V.K., and A.B. carried out experiments, data acquisition and analysis. P.A. taught critical experimental techniques and edited manuscript. J.C. and A.R. provided critical material, edited manuscript. C.A.T. taught critical experimental techniques. P.W. assisted with data interpretation and analysis. D.A.P. provided critical material, edited manuscript. X.L. provided critical expertise and reagents. R.G. and M.A.T. provided critical expertise and reagents, edited manuscript. A.M.M. and L.J.H. provided critical expertise, edited manuscript. S.W.F.M. performed experimental and conceptual design, provided critical expertise, edited manuscript. E.R.M. performed experimental and

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conceptual design, carried out experiments, data acquisition and analysis, wrote manuscript.

**Data and Materials availability: All data associated with this study are present in the paper or Supplementary Materials.** RNA sequencing data were deposited in the ArrayExpress public database under accession number E-MTAB-7132.

## FIGURE LEGENDS

**Figure 1. Recolonization of antibiotic-treated mice induces infiltration of innate inflammatory cells followed by sustained Th1 responses.**

**a)** Colonic monocytes ($CD11b^+Ly6C^+$Siglec $F^-Ly6G^-$), eosinophils (Siglec $F^+$ MHC Class $II^-$ $Ly6C^-$) and neutrophils (Siglec $F^-$ MHC Class $II^-$ $Ly6G^+$ $Ly6C^-$) were identified from live $CD45^+$ cells by flow cytometry, in mice that had been recolonized with microbiota from wild-type mice for 7 days following 7 days of antibiotic treatment. Pooled data are shown, (*P<0.05, **P<0.01, ***P<0.001, Kruskal-Wallis test, n=15-17 (control), n=9 (antibiotics), n=6-8 (recolonized). **b)** Proportions of monocytes, neutrophils and eosinophils following antibiotic treatment and subsequent recolonization at time points up to 23 days of recolonization following 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 IFNγ-production by $CD3^+CD4^+$ T-cells in control versus recolonized mice was determined by flow cytometry, with representative flow cytometry plots (from day 13 of recolonization) and pooled data at time points shown. (*P<0.05, **P<0.01, ***P<0.001, Kruskal-Wallis test, n≥3 for all time points). **d)** Ki67 expression by IFNγ-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=9-12). **e)** Analysis of $CD3^+CD4^+$ T-cells producing IFNγ 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=7-20 per time point). All data represent at least two independent experiments.

**Figure 2. $CCR2^+$ monocyte-macrophages are essential for enhanced Th1 responses following antibiotic treatment and recolonization.**

**a)** Live $CD45^+$Siglec$F^-Ly6G^-CD11b^+CD64^+$ colonic lamina propria cells were analysed by flow cytometry and monocytes ($Ly6C^+$MHC Class $II^-$), intermediates ($Ly6C^+$MHC Class $II^+$) and macrophages ($Ly6C^-$MHC Class $II^+$) identified in mice that had been recolonized for 7 days following antibiotic treatment. Representative flow cytometry plots and pooled data (numbers) are depicted (**P<0.01, unpaired Mann-Whitney test, n=8-10). **b)** $Ly6C^+$MHC class $II^-$ monocytes, $Ly6C^+$MHC class $II^+$

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intermediates and Ly6C$^-$MHC class II$^+$ macrophages were sorted by flow cytometry and mRNA for *il6, il12p35, TNFα, il1β, nos2* and *cd206* analysed by quantitative real-time PCR (qRT-PCR). Results are shown as mean expression normalized to TATA binding protein and shown relative to mean of control mice in all cases (unpaired Mann-Whitney tests, n=4-9). **c)** Expression of Tim4 and CD4 by colonic Ly6C$^-$MHC Class II$^+$ differentiated macrophages of control or recolonized (day 7) mice was analysed 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 for *il6, il12p35, TNFα, il1β* and *cd206* analysed by quantitative real-time PCR (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 (Siglec F$^+$ MHC Class II$^-$ Ly6C$^-$) and neutrophils (Siglec F$^-$ MHC Class II$^-$ Ly6G$^+$ Ly6C$^-$) were identified from live CD45$^+$ cells by flow cytometry in *Ccr2*$^{-/-}$ mice that had been recolonized for 7 days following antibiotic treatment. Pooled data are depicted (*P<0.05, unpaired Mann-Whitney test, n=4). **f)** Production of IFNγ 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 was analysed by flow cytometry, with pooled data depicted (unpaired Mann-Whitney tests, n=5-18). WT mice (control and recolonized) are also shown as a reference. All data shown are representative of at least two independent experiments.

**Figure 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 (1μg/ml) stimulation was assessed by measuring intracellular IL-10 and TNFα 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=8-10. **c)** Pooled data depicting TNFα protein production by mature macrophages from control and

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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 $CD45^+$ Siglec $F^-$ $Ly6G^-$ $CD11b^+$ $CD64^+$ $Ly6C^-$ MHC Class $II^+$ cells) were cultured for 18 hours with 100 ng/ml LPS 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.

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

**a)** Mice were treated with antibiotics and subsequently recolonized for 20 days prior to infection with *Citrobacter rodentium*, alongside non antibiotic-treated mice (controls). Colony forming units (CFU) were grown overnight from faecal pellets harvested during infection for assessment of kinetics, and from caecal homogenates ten days after infection (*P<0.05, non-paired Mann-Whitney test, data shown 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 RORγt and for production of IL-17A. Pooled data are depicted (*P<0.05, non-paired Mann-Whitney test, n=8). **c)** Mature macrophages were identified from colonic LP cells as live $CD45^+$ Siglec $F^-$ $Ly6G^-$ $CD11b^+$ $CD64^+$ $Ly6C^-$ 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 prior to infection with the intestinal helminth *Trichuris muris,* alongside non-antibiotic treated mice. Caeca and proximal colons were harvested at days 21 or days 35 after *T. muris* infection. Pooled data depicting worm counts from the caecum of *T. muris*-infected control (non-antibiotic treated) mice, and from infected recolonized mice (*P<0.05, non-paired 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

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recolonized for 60 days prior to infection with either *C. rodentium* or *T. muris*, alongside non-antibiotic treated control mice. For *C. rodentium* infections, ten day after infection, tissue homogenates from the caeca were used to grow CFU overnight (*P<0.05, unpaired Mann-Whitney test, n=5). For *T. muris* infections, caeca were harvested at days 21 or days 35 after *T. muris* infection and worms counted (*P<0.05, unpaired Mann-Whitney test, n=5). Data shown in all experiments are representative of at least 2 individual experiments.

**Figure 5. Recolonization of antibiotic-treated mice causes long-term disruption of the intestinal microbiota and a reduction in short-chain fatty acids in the intestine.**

**a)** Principal component analysis using Bray-Curtis metrics performed based on the taxonomic assignments obtained from the 16S rRNA gene sequencing libraries analysed from colonic stool samples of recolonized mice (red, A samples) at days 13, 20, 30 and 60 days following 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 colours represent different genus taxa, and bar heights signify the relative abundance of each taxon in colonic stool samples from control and recolonized mice (day 13, day 20, day 30 and day 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). **d)** Metabolite analysis was carried out via nuclear magnetic resonance (NMR) on colonic faecal samples from control and recolonized mice and normalized to faecal weight, to determine absolute levels of short-chain fatty acids (SCFAs) in the intestine. Pooled data depicting heat maps made from Z-scores (relative) and summary graphs representing normalised amounts of SCFAs are shown (*P<0.05, ***P<0.001, Kruskal-Wallis test, n=16 (controls) and n=4 (recolonized)).

**Figure 6. The short-chain fatty acid 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

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mice treated with antibiotics that had been supplemented with butyrate, propionate or acetate for 7 days (200mM each). Following *in vitro* stimulation with LPS (1μg/ml), intracellular IL-10 and TNFα 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), n=4 (antibiotics+acetate). **b)** Total colonic LP cells were stimulated *in vitro* with LPS (1μg/ml) in the presence or absence of butyrate (1mM) and mature colonic macrophages identified by flow cytometry and assessed for intracellular TNFα production. Representative flow cytometry plots demonstrating intracellular TNFα 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 100 ng/ml LPS for 18 hours, and TNFα 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 following antibiotic treatment. In one group, antibiotics were supplemented with butyrate (200mM). $CD3^+CD4^+$ T-cell production of IFNγ 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), n=6 (butyrate). All data shown are representative of at least two independent experiments.

**Figure 7. Butyrate causes metabolic reprogramming of intestinal macrophages.**

**a)** RNA sequencing 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 (±200mM butyrate) prior to 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 (KEGG) analysis. **b)** Bone-marrow derived macrophages conditioned with SCFAs

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during development and mRNA for *arg1* analysed 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 oxidative phosphorylation and lipid metabolism as identified by pathway (KEGG) analysis, following RNA sequencing of colonic macrophages from control, recolonized (no butyrate supplementation of antibiotics) or recolonized (butyrate (200mM) supplementation of antibiotics) mice. **d)** Oxygen consumption rate (OCR) of bone-marrow derived macrophages conditioned with SCFAs (1mM) during development, shown at baseline and following 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 following FCCP administration (mitochondrial stress). Results were normalized to protein levels (*P<0.05, **P<0.01, Kruskal-Wallis test, n=4). Lipid metabolism shown as the fold change (reduction) in OCR in macrophages following etomoxir (Eto) 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.

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**Comment [L84]:** The butyrate group is in the middle to highlight all the gene changes induced by butyrate – since there are several genes unique to the butyrate group, the main heatmap looks very confusing if control and recolonized groups are located next to each other.

**Comment [Office85]:** Total protein?

**Comment [L86]:** Yes, although the exact same number of cells were placed in each well, the protein amount was used to make sure the readings can be directly compared.

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(Figure 6b).

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The dysregulated T-cell responses in this study are CCR2-dependent, but the *Ccr2*$^{-/-}$ model does not distinguish between deficiency in monocytes, intermediates, and fully differentiated macrophages that are all CCR2-dependent. The increased expression of IL-6 during recolonization was restricted to Ly6C$^{+}$ intermediates and Ly6C$^{-}$ fully differentiated macrophages, with increased IL-12 expression restricted to macrophages only (Figure 2b). Thus, it seems likely that the CCR2-dependent skewing of T-cell responses may be restricted to the MHC Class II$^{+}$ subsets of intermediates and macrophages rather than monocytes, but this remains to be proven directly. It will be informative in future studies to determine the roles of intermediates versus mature macrophages in the intestine in shaping T-cell responses, with an important discussion point being whether intermediates themselves can be classed as a (more immature) type of macrophage.

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**ACKNOWLEDGMENTS**[Office1]

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**AUTHOR CONTRIBUTIONS**[Office2]

N.S., A.A., M.L., C.A.G., C.L., S.C., G.L.G., T.S., H.W., A.B., V.K., and A.B. carried out experiments, data acquisition and analysis. P.A. taught critical experimental techniques and edited manuscript. J.C. and A.R. provided critical material, edited manuscript. C.A.T. taught critical experimental techniques. P.W. assisted with data interpretation and analysis. D.A.P. provided critical material, edited manuscript. X.L. provided critical expertise and reagents. R.G. and M.A.T. provided critical expertise and reagents, edited manuscript. A.M.M. and L.J.H. provided critical expertise, edited manuscript. S.W.F.M. performed experimental and conceptual design, provided critical expertise, edited manuscript. E.R.M. performed experimental and conceptual design, carried out experiments, data acquisition and analysis, wrote manuscript.

**Data and Materials availability: All data associated with this study are present in the paper or Supplementary Materials.** RNA sequencing data were deposited in the ArrayExpress public database under accession number E-MTAB-7132.

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**Isolation of tissue leukcocytes**

For the isolation of colonic lamina propria cells, colons were removed from mice, cleaned in PBS, chopped into 5mm sections and incubated three times in 2mM EDTA in HBSS to remove the epithelial compartment, then washed and incubated in 1.25mg/ml collagenase D (Sigma), 0.85mg/ml collagenase V (Sigma) and 1mg/ml dispase (Gibco, Invitrogen) in RPMI-1640 (Thermofisher Scientific) for 30-35 minutes in a shaking incubator at 37°C.

**Isolation of mesenteric lymph node leukcocytes**

For the isolation of mesenteric lymph node (MLN) cells, MLNs were removed from mice, chopped finely and incubated in 1mg/ml collagenase D (Sigma) for 35 minutes in a shaking incubator at 37°C.

**Flow cytometric analysis and sorting of cells**

0.5-5 x $10^6$ cells were stained at 4°C in the dark using the antibodies listed in Supplementary Table 1 and analysed using an LSR Fortessa cytometer (BD Biosciences) and FlowJo software (TreeStar). Colonic monocytes and macrophages were sorted using a FACS Aria III and a FACS Aria Fusion, as live gated $CD45^+SiglecF^-Ly6G^-CD11b^+CD64^+$ cells and further subdivided into subsets based on expression of MHC cClass II and Ly6C.[Office3]

**Quantitation of gene expression by real-time reverse transcription PCR**

Total RNA was extracted from monocytes and macrophages FACS-purified from the colons of individual mice using the RNeasy Micro Kit (Qiagen). RNA were reverse transcribed to cDNA using the High Capacity RNA-to-cDNA Kit (ThermoFisher Scientific) and gene expression was assayed using quantitative reverse transcription PCR using PerfeCTa SYBR Green Fastmix (Quanta) on the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) with the primers (Integrated DNA Technologies) detailed in Supplementary table 2. Gene expression levels were normalized to TATA binding protein (TBP). The mean relative gene expression was calculated using the $2^{-d}\Delta^{C(t)}$ method.

**Bone-marrow derived macrophage generation**

Bone marrow progenitors were harvested from femurs and tibias of wild-type adult C57BL/6 mice and cultured for 6 days on petri dishes in the presence of macrophage colony-stimulating factor (M-CSF). Media and M-CSF were changed on day 3 and in some experiments media was supplemented with 1mM SCFA on day 5. Bone-marrow derived macrophages were harvested on day 6 with 2mM EDTA.

**Cytometric bead array**

Intestinal macrophages were isolated by FACS and plated in round-bottomed 96-well tissue culture plates at 50,000 cells per well. The cells were cultured at 37ºC and 5% $CO_2$ in 50µl complete RPMI (10% FCS) in the presence of 100ng/ml LPS (Sigma) ± 500µM butyrate (Sigma). In other experiments, butyrate had been administered to mice with antibiotics *in vivo* as described above, prior to macrophage isolation. After 18 hours, culture supernatants were collected and analysed for the presence of cytokine and chemokines using the Mouse Inflammation CBA kit (BD Biosciences). Data were acquired on an LSR Fortessa (BD Biosciences) and analysed using FCAP Array software (BD Biosciences).

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**LPS stimulation of lamina propria cells *in vitro***

Colonic LP cells were incubated for 4.5 hours with monensin and Brefeldin A[Office4] in the presence or absence of 1µg/ml LPS (Sigma). Monocytes and macrophages were then identified by flow cytometry and assessed for intracellular cytokine production following cell permeabilisation and fixation as described below.

**Assessment of cytokine production by T-cells**

Colonic LP cells were incubated for 4 hours with PMA, Ionomycin, and Golgistop (eBioscience) before being surface stained, fixed, and permeabilised prior to intracellular cytokine staining (eBioscience intracellular staining kit).

**Faecal DNA extractions, quantification and bacterial sequencing**

DNA was extracted from faecal pellets using the FastDNA™ SPIN Kit for Soil (MP Biomedicals) following the manufacturer's instructions but incorporating an extension of the initial bead-beading time to three minutes. The concentration of bacterial DNA

was quantified using Qubit and normalized to 5 ng/ $ml^{-1}$ for all samples. Extracted DNA was used as a template for PCR amplification of the V1/V2 regions of the 16S rRNA gene as detailed in Supplementary Table 3. Amplification conditions of the PCR were: 1 cycle of 94°C 3 min and 25 cycles of 94°C for 45 s, 55°C for 15 s and 72°C for 30 s using a 96 well Thermal Cycler PCR machine. 16S RNA gene libraries were sequenced on the Illumina MiSeq platform with 250 bp paired end reads.

**Bacterial sequence processing**

All raw sequence reads were processed through quality control using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) keeping a minimum quality threshold of 33 for at least 50% of the bases. Reads that passed the threshold were aligned against SILVA database (version: SILVA_119_SSURef_tax_silva)[48] using BLASTN[49] (ncbi-blast-2.2.30+; Max e-value 10e-3) separately for both pairs. After performing the BLASTN alignment, all output files were imported and annotated using the paired-end protocol of MEGAN[50].

**Taxonomic annotation**

Parameter settings of “Min Score = 50”, “Top Percent = 10” were applied to process BLAST files by MEGAN6. Reads which did not have any match to the respective database were placed under a “No hit” node, and reads that were originally assigned to a taxon that did not meet our selected threshold criteria were returned to higher nodes where the threshold was met using the lowest common ancestor (LCA) algorithm. After importing datasets into MEGAN, we obtained MEGAN-own “rma files” for each data set that were mapped onto NCBI taxonomy based on our selected threshold and the files were compared and analysed within MEGAN.

**Metabolite analysis**

All samples were thawed at room temperature and prepared for $^{1}H$ nuclear magnetic resonance (NMR) spectroscopy by mixing 50mg±1mg faeces with 200µl NMR buffer (0.26g $NaH_2PO_4$ and 1.41g $K_2HPO_4$) made up in 100% $D_2O$ (100ml), containing 0.1% NaN3 (100mg) and 1mM sodium 3-(Trimethylsilyl)-propiomate-*d*4 (TSP) as a chemical shift reference and 250µl $D_2O$. All samples were vortexed and then centrifuged at 14,000rpm for 5 min, and the remaining supernatant was removed and

used for semi-quantification of metabolites. The $^{1}$H NMR spectra were recorded at 600MHz on aBruker Avance spectrometer (Bruker BioSpin Gmbh, Rheinstetten, Germany) running Topspin 2.0 software and fitted with a broadband inverse probe. $^{1}$H NMR spectra were acquired with 512 scans, a spectral width of 12300 Hz and an acquisition time of 2.7s. The “noesypr1d” presaturation sequence was used to suppress the residual water signal with a low-power selective irradiation at the water frequency during the recycle delay. Spectra were transformed with a 0.3-Hz line broadening, manually phased, baseline corrected and referenced by setting the TSP methyl signal to 0 ppm. Filtered samples were transferred to NMR tubes with 100µl of NMR buffer and mixed well before reading using the parameters described above. All metabolites were quantified using the software Chenomx NMR suite 7.6$^{TM}$ based on published literature[51-53] and by use of the 2D-NMR methods, COSY, HSQC and HMBC.

### *Trichuris muris* infections

The maintenance, infection and recovery of *Trichuris muris* were as described previously[54]. Briefly, stock infections were maintained in susceptible mouse strains and adult worms harvested at day 42 post infection (p.i.). In all experiments, C57BL/6 mice (control or recolonized (20 or 60 days)) were infected [Office5]with 200 embryonated eggs and worm burdens were assessed on day 21 or day 35 p.i. Proximal colons (2cm section) were harvested in all cases for FACS analysis of adaptive immune responses.

### *Citrobacter rodentium infections*

The nalidixic acid-resistant *Citrobacter rodentium* strain ICC169 (Ghaem-Maghami, Infection and Immunity 2011[Office6]) was grown overnight at 37°C in Luria broth (LB). C57BL/6 mice (control or recolonized (20 or 60 days)) were inoculated with approximately 2x10$^{9}$ bacteria by oral gavage. CFUs were calculated in stool at days 3,5,7 and 10 during the experiment and in caecal tissue at the conclusion of the experiment, by plating mechanically homogenized samples on agar (Lennox formula, Sigma) plates containing 50µg/ml nalidixic acid. Colonization levels were normalized to stool and tissue weight.

**RNA sequencing and analysis**

Strand-specific RNA sequencing libraries were prepared using the Illumina workflow with the TruSeq stranded mRNA sample preparation kit. Paired-end reads (65 x 65 bp) were generated from each sample and 48-192 million reads were obtained from each sample. The fastq files generated by a HiSeq4000 platform (Illumina) were analyzed with FASTQC and any low quality reads and contaminated barcodes were trimmed with Trimmomactic[55]. All libraries were aligned to GRCm38.p4 mouse genome assembly using STAR-2.4.2[56], and only uniquely mapped reads were used for differential gene expression analysis. The mapped reads were counted by genes with *HTseq*[57] against gencode.vM11.annotation.gtf. The differentially expressed genes were identified using DESeq2[58] by pairwise comparisons between the experimental groups. The differentially expressed genes with a p-adjusted value ≤[Office7]0.05 were selected for further validation and analysis. For functional analysis, an R package of topGO and Ingenuity Pathway analysis was used. RNA sequencing data were deposited in the ArrayExpress public database under accession number E-MTAB-7132. [Office8]

**Assessment of cellular metabolism**

For real-time analysis of oxygen consumption rates (OCR), macrophages were analyzed using an XF-96 Extracellular Flux Analyser (Seahorse Bioscience) as described in detail previously[41]. Measurements were taken under basal conditions and following the sequential addition of 1μM oligomycin, 1.5μM fluoro-carbonyl cyanide phenylhydrazone (FCCP), 200μM etomoxir (Eto) and 100nM rotenone plus 1μM antimycin A (R+A; all purchased from Sigma).

**Separation** [Office9]**of colonic lamina propria and muscularis**

Intestinal layers were separated as previously described[59] with some modifications. Briefly, intestines, harvested as described above, were incubated in calcium and magnesium free HBSS with 2% FCS and 1mM DTT in 50mL falcon tubes for 20 minutes at 37$^{\circ 0}$C in a shaking incubator. Tubes containing intestinal samples were vortexed and intestine samples were decanted into sieves before being returned to 50mL tubes containing calcium and magnesium free HBSS with 2% FCS and 1.3mM EDTA and incubated for 45 minutes at 37$^{0}$C in a shaking incubator.

Tubes containing intestinal samples were vortexed and intestine samples were decanted into sieves. After DTT and EDTA incubations, intestinal tissue was separated under a dissecting microscope into muscularis and lamina propria layers. Digestion of these separate layers continued as described above.

**Immunofluorescence microscopy**

Colon sections were drop fixed in 10% sucrose 4% pfa for 2 hours followed by transfer into 30% sucrose PBS for 6 hours. Fixed and dehydrated tissue was embedded in OCT and frozen in isopentane on dry ice. Frozen tissue blocks were stored at -80$^{\circ 0}$C until use. Sections were cut at 8um using a cryostat (Leica CM3050S), mounted on to superfrost slides (Thermo Scientific), air-dried and stored at -80$^{\circ 0}$C until use. On the day of staining, slides were brought to room temperature before staining. Sections were encircled with a pap pen (Daido Sangyo) and blocked with TSA Blocking Reagent (PerkinElmer) for 15 min. Sections were further blocked using avidin and biotin blocking solutions (Biolegend), each for 15 minutes. Sections were washed in TNT [Office10]buffer pH7.5 with 0.03% trition-X between incubations. All incubations were performed in a humidified chamber at room temperature, apart from incubation with the primary antibody, which was performed at 4$^{\circ 0}$C. Purified rat anti- CD68 (clone FA-11, Invitrogen) was diluted in TNT buffer with 1% BSA and applied to sections overnight at 4$^{\circ 0}$C. The following day, after washing with TNT buffer, purified biotin-conjugated donkey anti- rat (Invitrogen) was applied for 2 hours at room temperature. Tyramide labelling was completed using a Molecular Probes TSA kit with HRP-streptavidin and AF555 tryamide according to manufacturer's instructions. Briefly, endogenous peroxidase activity was blocked by incubation in 0.3% $H_2O_2$ for 10 min. Sections were then incubated with streptavidin HRP for 30 minutes, followed by tyramide 555 labelling solution for 5 min. Sections were mounted in ProLong Gold Antifade Mountant with DAPI (Molecular Probes) and allowed to cure overnight at room temperature in the dark.

Images were collected on a Zeiss Axioimager.D2 upright microscope using a 10x objective and captured using a Coolsnap HQ2 camera (Photometrics) through Micromanager software v1.4.23. Images were processed using ImageJ (NIH).

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Figure 1

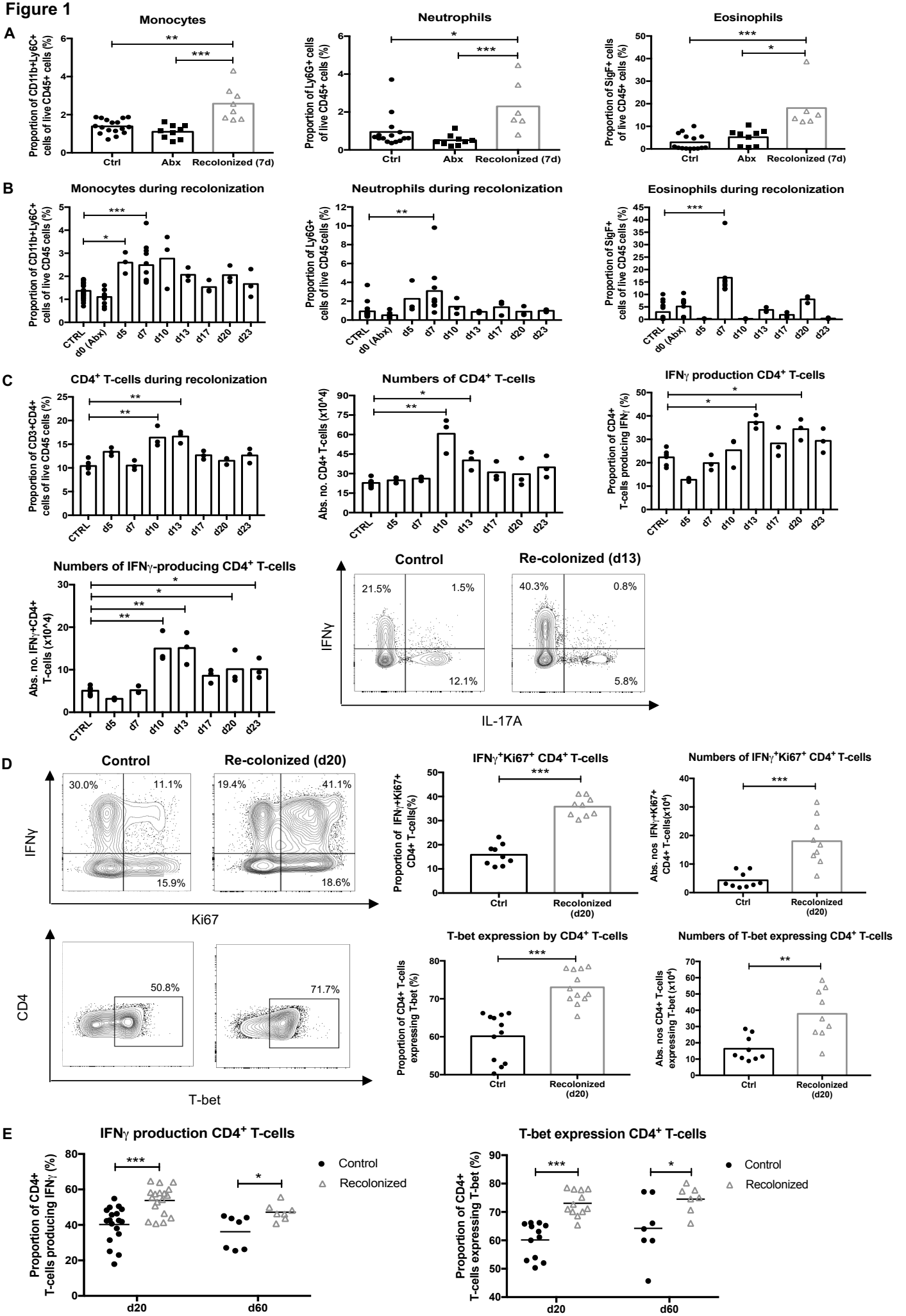

A
Monocytes
Proportion of CD11b+Ly6C+ cells of live CD45+ cells (%)
Ctrl
Abx
Recolonized (7d)
Neutrophils
Proportion of Ly6G+ cells of live CD45+ cells (%)
Eosinophils
Proportion of SigF+ cells of live CD45+ cells (%)
B
Monocytes during recolonization
Proportion of CD11b+Ly6C+ cells of live CD45 cells (%)
CTRL
d0 (Abx)
d5
d7
d10
d13
d17
d20
d23
Neutrophils during recolonization
Proportion of Ly6G+ cells of live CD45 cells (%)
Eosinophils during recolonization
Proportion of SigF+ cells of live CD45 cells (%)
C
CD4+ T-cells during recolonization
Proportion of CD3+CD4+ cells of live CD45 cells (%)
Numbers of CD4+ T-cells
Abs. no. CD4+ T-cells (x10^4)
IFNγ production CD4+ T-cells
Proportion of CD4+ T-cells producing IFNγ (%)
Numbers of IFNγ-producing CD4+ T-cells
Abs. no. IFNγ+CD4+ T-cells (x10^4)
Control
Re-colonized (d13)
21.5%
1.5%
12.1%
40.3%
0.8%
5.8%
IFNγ
IL-17A
D
Control
Re-colonized (d20)
30.0%
11.1%
15.9%
19.4%
41.1%
18.6%
IFNγ
Ki67
IFNγ+Ki67+ CD4+ T-cells
Proportion of IFNγ+Ki67+ CD4+ T-cells(%)
Numbers of IFNγ+Ki67+ CD4+ T-cells
Abs. nos IFNγ+Ki67+ CD4+ T-cells(x10^4)
Recolonized (d20)
50.8%
71.7%
CD4
T-bet
T-bet expression by CD4+ T-cells
Proportion of CD4+ T-cells expressing T-bet (%)
Numbers of T-bet expressing CD4+ T-cells
Abs. nos CD4+ T-cells expressing T-bet (x10^4)
E
IFNγ production CD4+ T-cells
Proportion of CD4+ T-cells producing IFNγ (%)
Control
Recolonized
d20
d60
T-bet expression CD4+ T-cells
Proportion of CD4+ T-cells expressing T-bet (%)

Figure 2

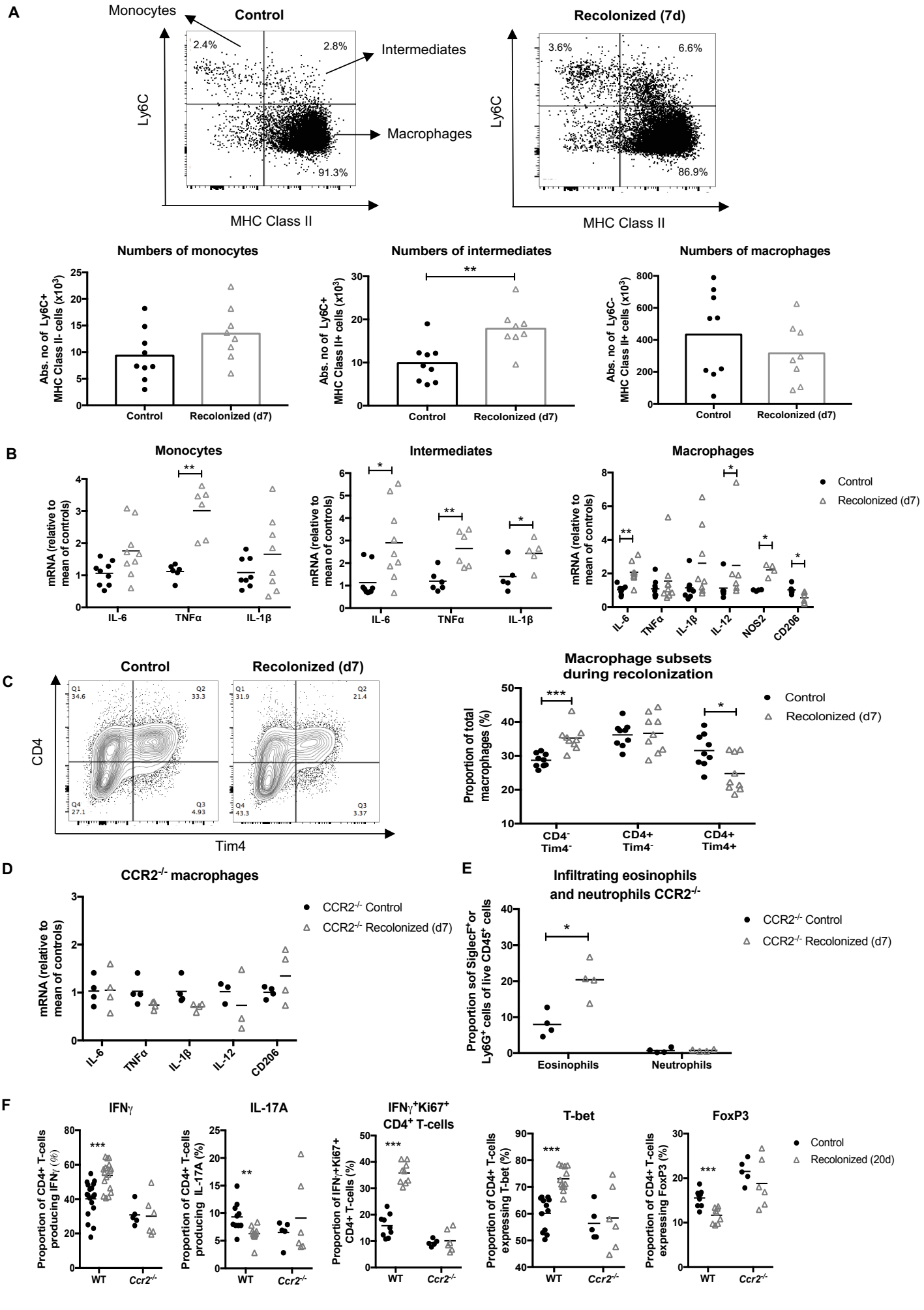

A
Monocytes
Control
Recolonized (7d)
2.4%
2.8%
Intermediates
Macrophages
91.3%
3.6%
6.6%
86.9%
Ly6C
MHC Class II
Numbers of monocytes
Numbers of intermediates
Numbers of macrophages
Abs. no of Ly6C+ MHC Class II- cells (x10³)
Abs. no of Ly6C+ MHC Class II+ cells (x10³)
Abs. no of Ly6C- MHC Class II+ cells (x10³)
Control
Recolonized (d7)
B
Monocytes
Intermediates
Macrophages
mRNA (relative to mean of controls)
IL-6
TNFα
IL-1β
IL-12
NOS2
CD206
C
Control
Recolonized (d7)
Q1 34.6
Q2 33.3
Q4 27.1
Q3 4.93
Q1 31.9
Q2 21.4
Q4 43.3
Q3 3.37
CD4
Tim4
Macrophage subsets during recolonization
Proportion of total macrophages (%)
CD4- Tim4-
CD4+ Tim4-
CD4+ Tim4+
D
CCR2-/- macrophages
CCR2-/- Control
CCR2-/- Recolonized (d7)
E
Infiltrating eosinophils and neutrophils CCR2-/-
Proportion sof SiglecF+or Ly6G+ cells of live CD45+ cells
Eosinophils
Neutrophils
F
IFNγ
IL-17A
IFNγ+Ki67+ CD4+ T-cells
T-bet
FoxP3
Proportion of CD4+ T-cells producing IFNγ (%)
Proportion of CD4+ T-cells producing IL-17A (%)
Proportion of IFNγ+Ki67+ CD4+ T-cells (%)
Proportion of CD4+ T-cells expressing T-bet (%)
Proportion of CD4+ T-cells expressing FoxP3 (%)
WT
Ccr2-/-
Recolonized (20d)

**Figure 3**

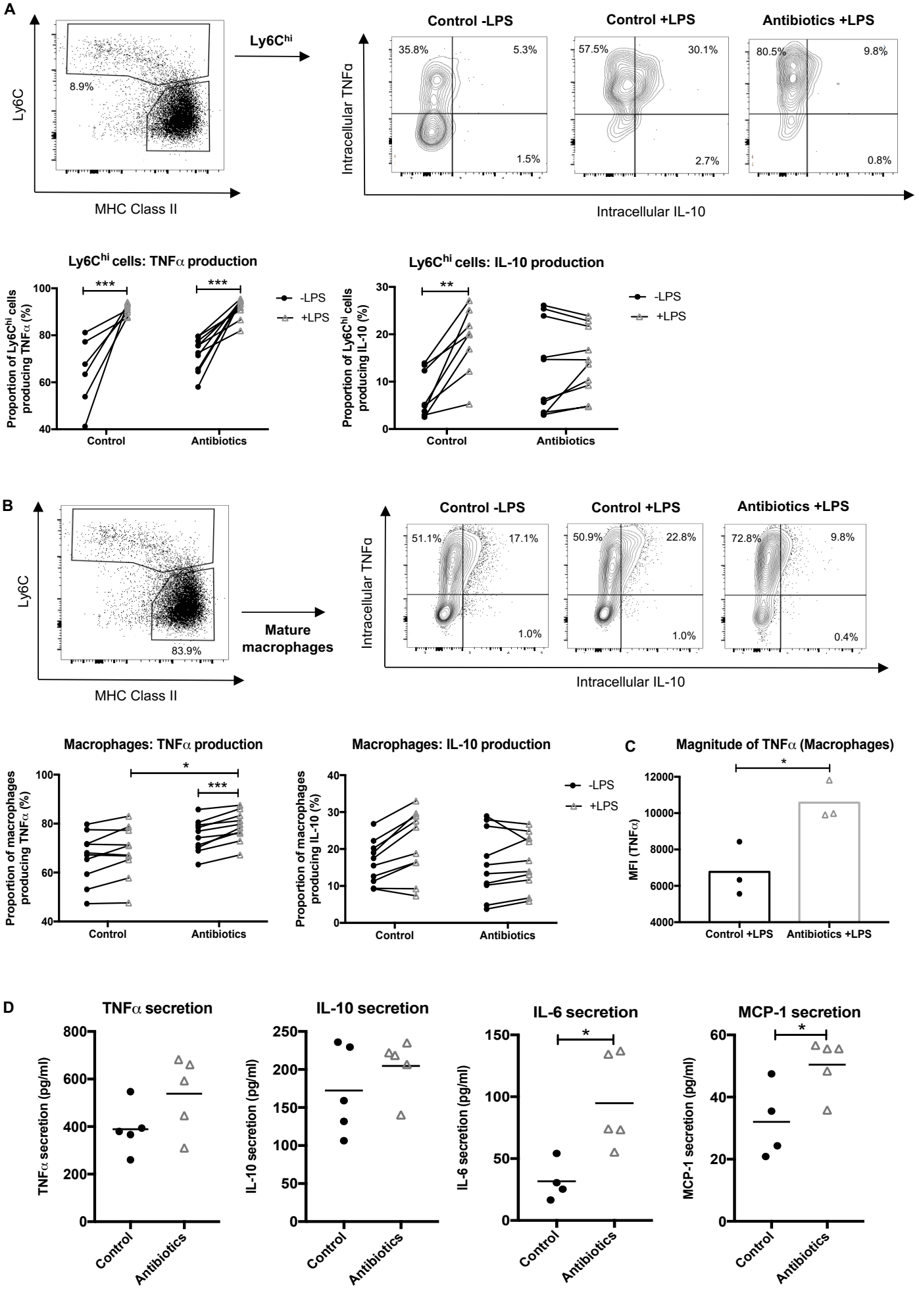

A
Ly6C
MHC Class II
8.9%
Ly6C^hi
Control -LPS
Control +LPS
Antibiotics +LPS
Intracellular TNFα
Intracellular IL-10
35.8%
5.3%
1.5%
57.5%
30.1%
2.7%
80.5%
9.8%
0.8%
Ly6C^hi cells: TNFα production
Proportion of Ly6C^hi cells producing TNFα (%)
***
***
-LPS
+LPS
Control
Antibiotics
Ly6C^hi cells: IL-10 production
Proportion of Ly6C^hi cells producing IL-10 (%)
**
B
Ly6C
MHC Class II
83.9%
Mature macrophages
Control -LPS
Control +LPS
Antibiotics +LPS
Intracellular TNFα
Intracellular IL-10
51.1%
17.1%
1.0%
50.9%
22.8%
1.0%
72.8%
9.8%
0.4%
Macrophages: TNFα production
Proportion of macrophages producing TNFα (%)
*
***
Macrophages: IL-10 production
Proportion of macrophages producing IL-10 (%)
C
Magnitude of TNFα (Macrophages)
MFI (TNFα)
Control +LPS
Antibiotics +LPS
D
TNFα secretion
TNFα secretion (pg/ml)
IL-10 secretion
IL-10 secretion (pg/ml)
IL-6 secretion
IL-6 secretion (pg/ml)
MCP-1 secretion
MCP-1 secretion (pg/ml)

**Figure 4**

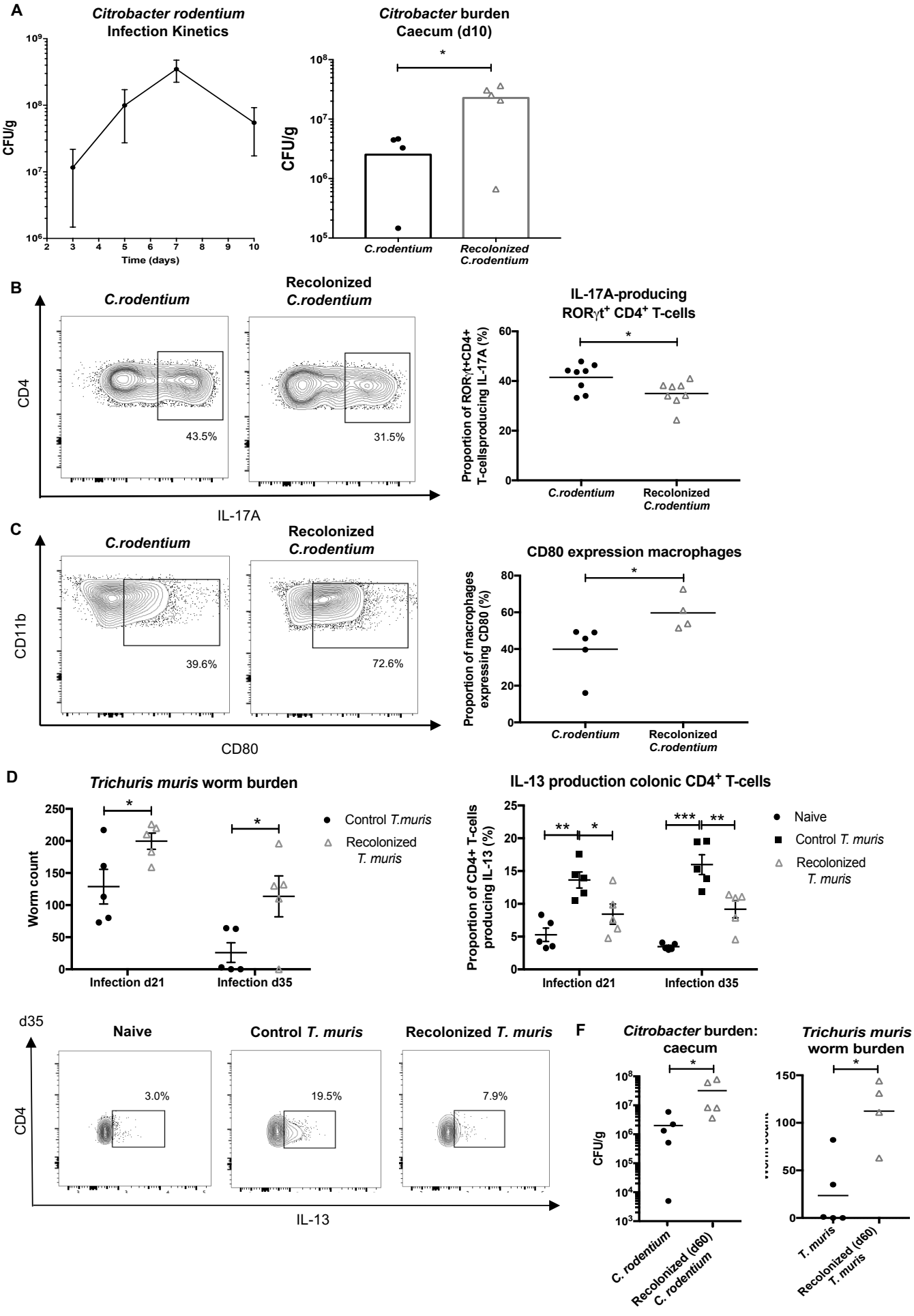
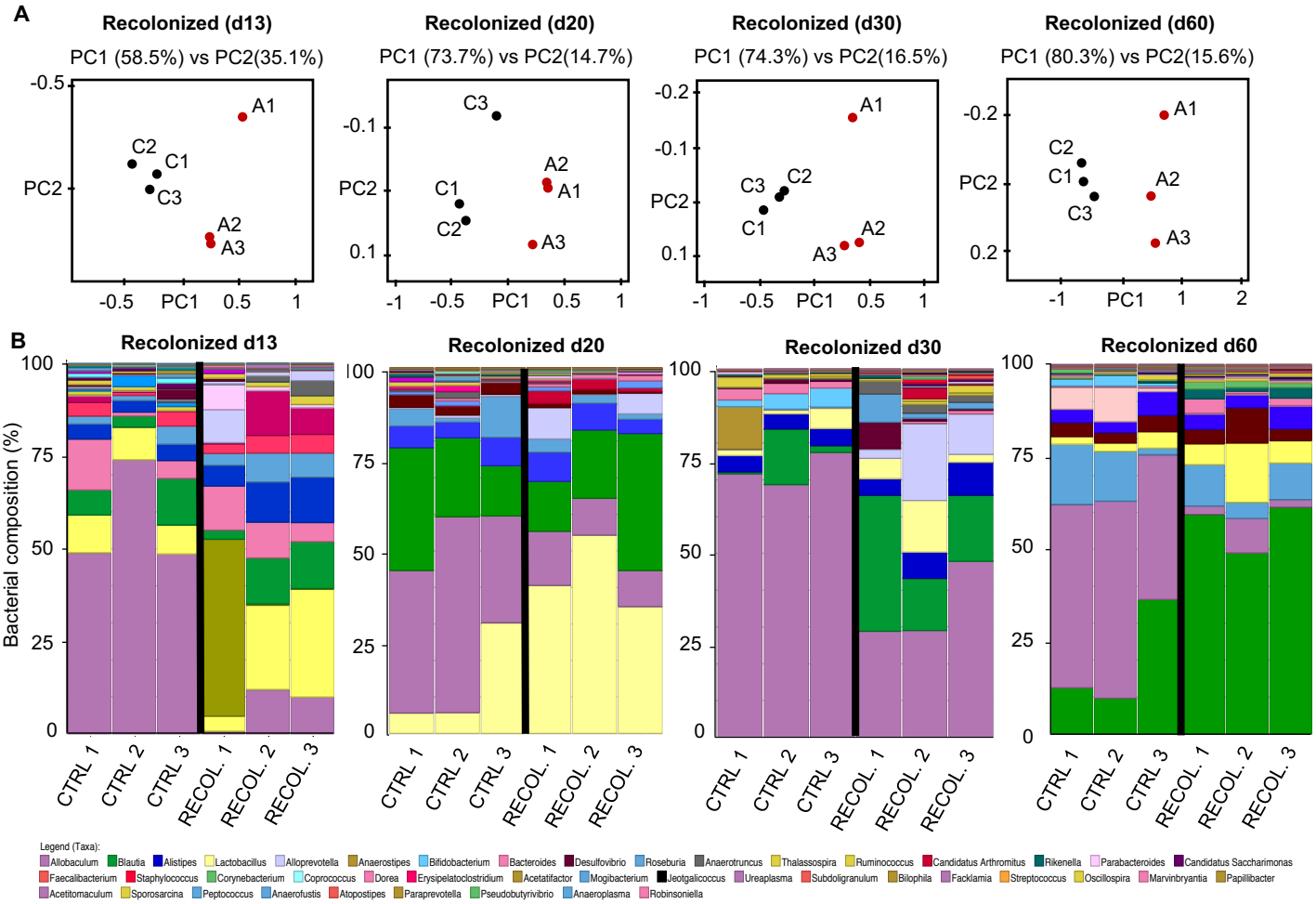
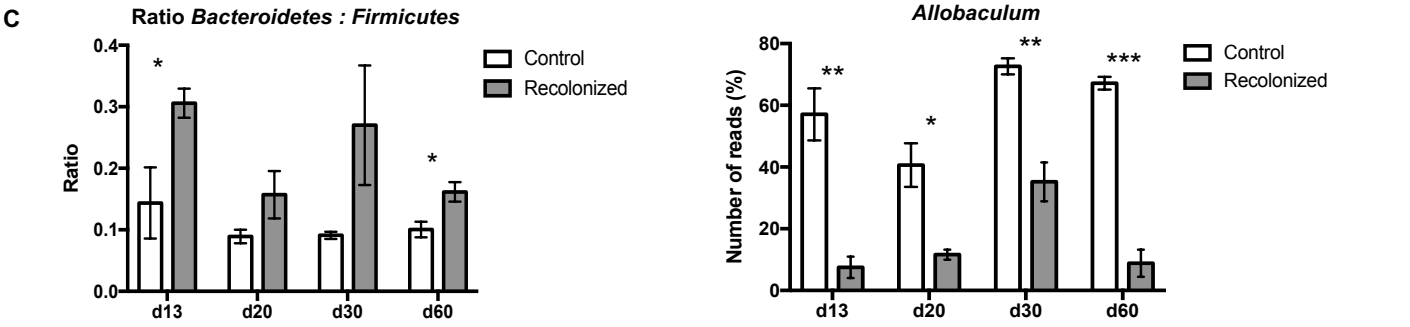

A
Citrobacter rodentium
Infection Kinetics
CFU/g
Time (days)
Citrobacter burden
Caecum (d10)
C.rodentium
Recolonized
C.rodentium
B
C.rodentium
Recolonized
C.rodentium
CD4
IL-17A
43.5%
31.5%
IL-17A-producing
RORγt+ CD4+ T-cells
Proportion of RORγt+CD4+
T-cellsproducing IL-17A (%)
C
CD11b
CD80
39.6%
72.6%
CD80 expression macrophages
Proportion of macrophages
expressing CD80 (%)
D
Trichuris muris worm burden
Worm count
Control T.muris
Recolonized
T. muris
Infection d21
Infection d35
IL-13 production colonic CD4+ T-cells
Proportion of CD4+ T-cells
producing IL-13 (%)
Naive
Control T. muris
Recolonized
T. muris
d35
Naive
Control T. muris
Recolonized T. muris
CD4
IL-13
3.0%
19.5%
7.9%
F
Citrobacter burden:
caecum
CFU/g
C. rodentium
Recolonized (d60)
C. rodentium
Trichuris muris
worm burden
T. muris
Recolonized (d60)
T. muris

Figure 5


A
Recolonized (d13)
PC1 (58.5%) vs PC2(35.1%)
Recolonized (d20)
PC1 (73.7%) vs PC2(14.7%)
Recolonized (d30)
PC1 (74.3%) vs PC2(16.5%)
Recolonized (d60)
PC1 (80.3%) vs PC2(15.6%)
PC1
PC2
A1
A2
A3
C1
C2
C3
B
Recolonized d13
Recolonized d20
Recolonized d30
Recolonized d60
Bacterial composition (%)
CTRL 1
CTRL 2
CTRL 3
RECOL. 1
RECOL. 2
RECOL. 3
Legend (Taxa):
Allobaculum
Blautia
Alistipes
Lactobacillus
Alloprevotella
Anaerostipes
Bifidobacterium
Bacteroides
Desulfovibrio
Roseburia
Anaerotruncus
Thalassospira
Ruminococcus
Candidatus Arthromitus
Rikenella
Parabacteroides
Candidatus Saccharimonas
Faecalibacterium
Staphylococcus
Corynebacterium
Coprococcus
Dorea
Erysipelatoclostridium
Acetatifactor
Mogibacterium
Jeotgalicoccus
Ureaplasma
Subdoligranulum
Bilophila
Facklamia
Streptococcus
Oscillospira
Marvinbryantia
Papillibacter
Acetitomaculum
Sporosarcina
Peptococcus
Anaerofustis
Atopostipes
Paraprevotella
Pseudobutyrivibrio
Anaeroplasma
Robinsoniella


C
Ratio Bacteroidetes : Firmicutes
Ratio
Control
Recolonized
d13
d20
d30
d60
Allobaculum
Number of reads (%)

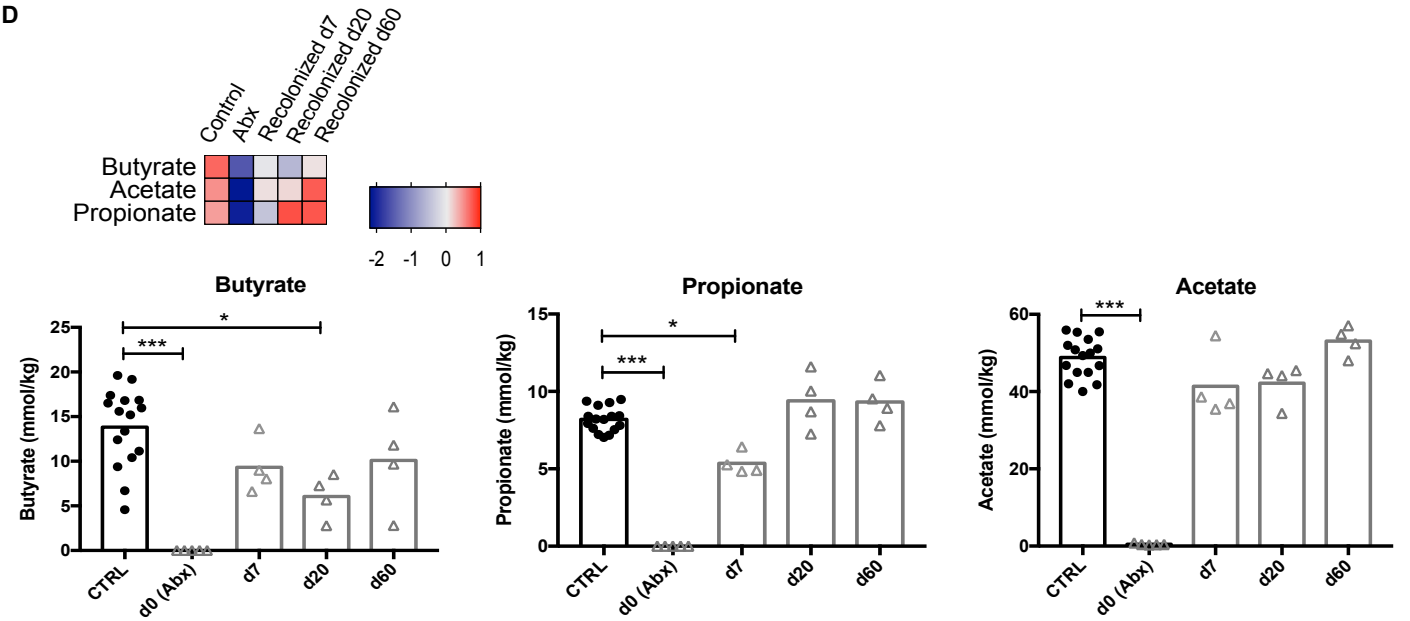

D
Control
Abx
Recolonized d7
Recolonized d20
Recolonized d60
Butyrate
Acetate
Propionate
-2 -1 0 1
Butyrate (mmol/kg)
Propionate (mmol/kg)
Acetate (mmol/kg)
CTRL
d0 (Abx)
d7
d20
d60

Figure 6

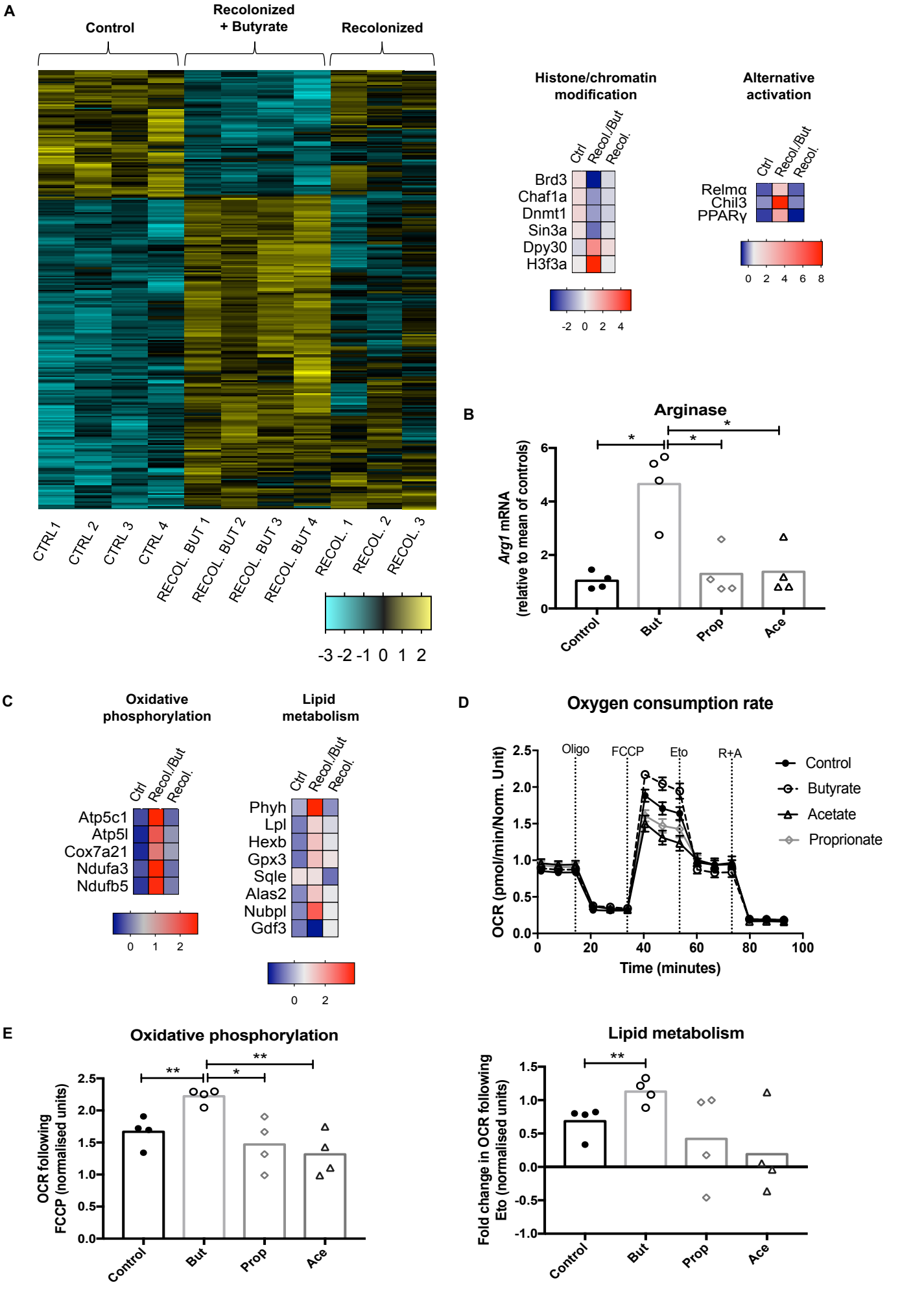
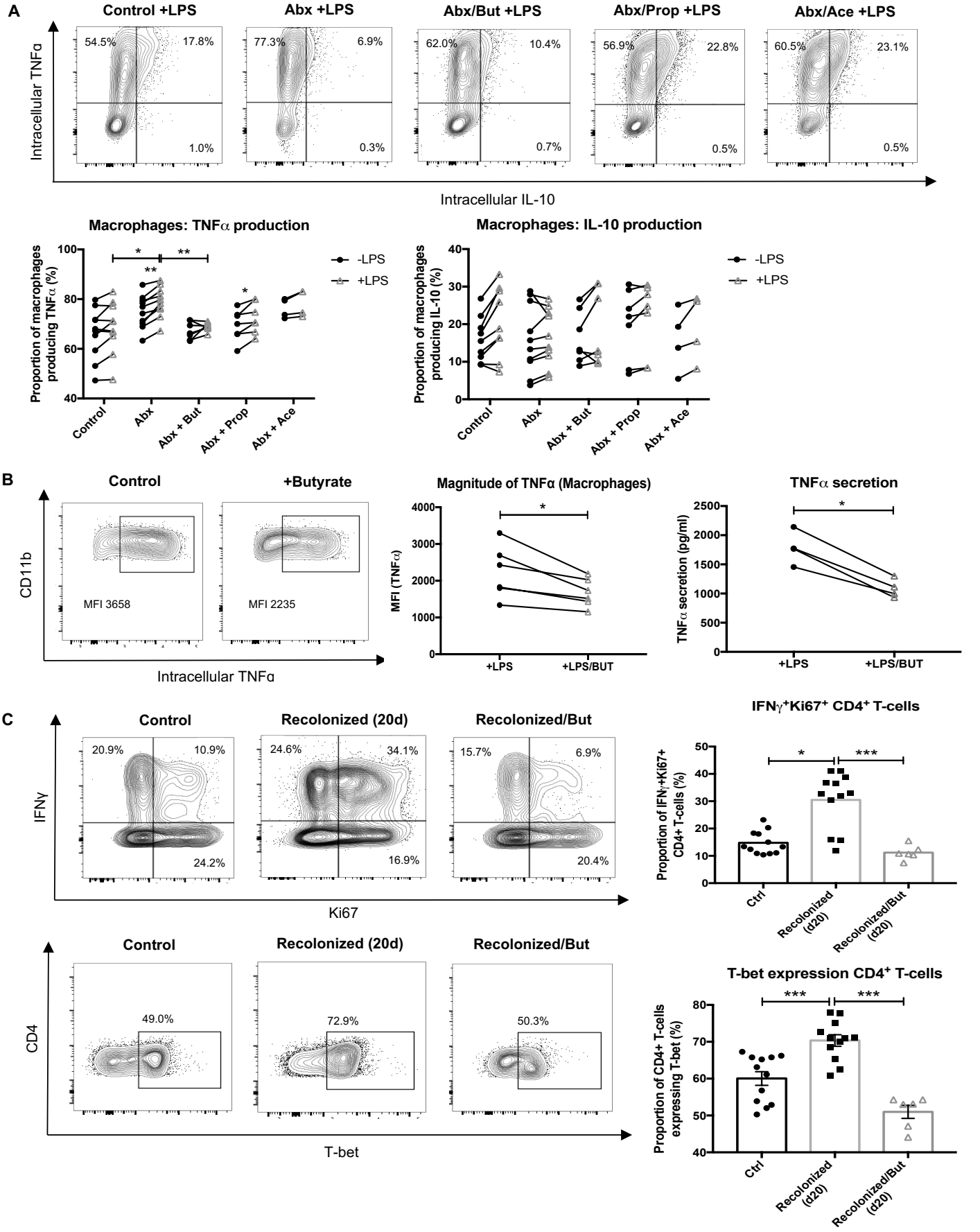
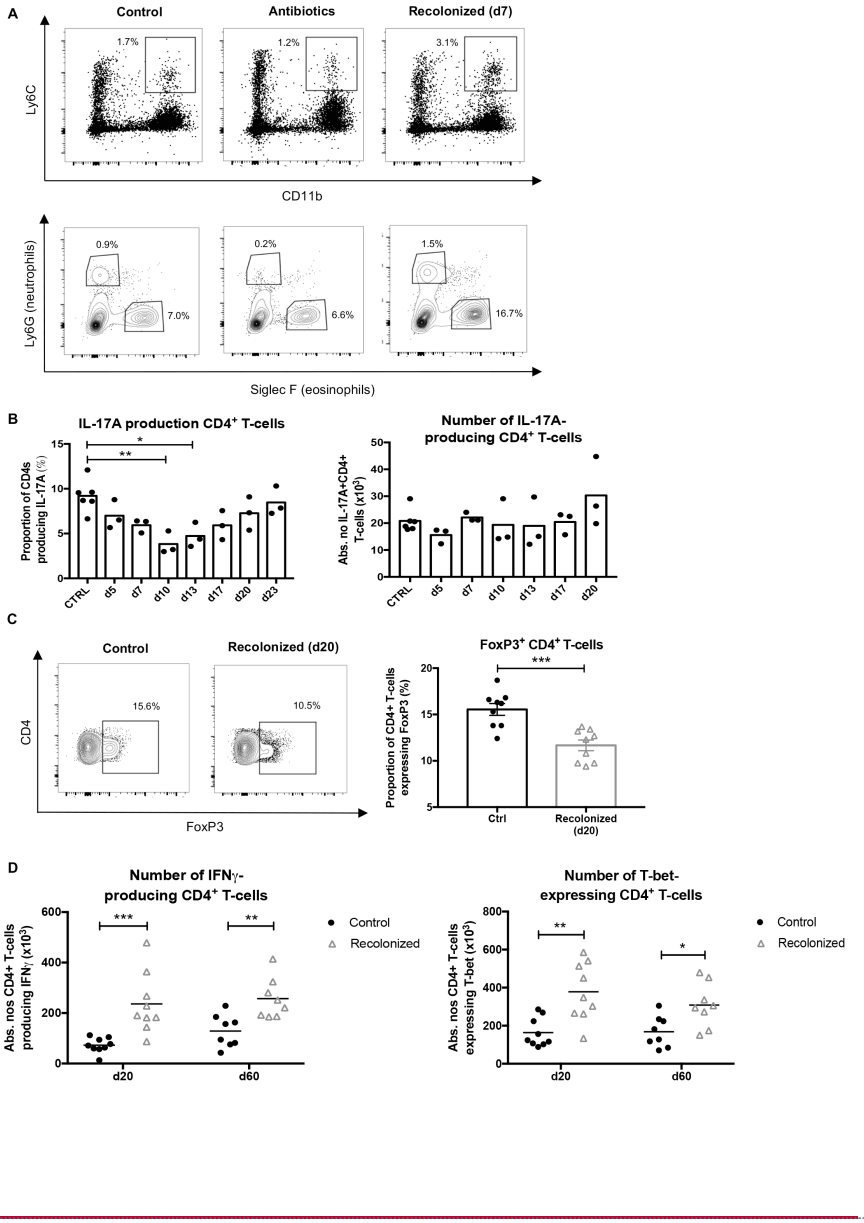

A
Control
Recolonized + Butyrate
Recolonized
CTRL 1
CTRL 2
CTRL 3
CTRL 4
RECOL. BUT 1
RECOL. BUT 2
RECOL. BUT 3
RECOL. BUT 4
RECOL. 1
RECOL. 2
RECOL. 3
-3 -2 -1 0 1 2
Histone/chromatin modification
Ctrl
Recol./But
Recol.
Brd3
Chaf1a
Dnmt1
Sin3a
Dpy30
H3f3a
-2 0 2 4
Alternative activation
Relmα
Chil3
PPARγ
0 2 4 6 8
B
Arginase
Arg1 mRNA (relative to mean of controls)
Control
But
Prop
Ace
C
Oxidative phosphorylation
Atp5c1
Atp5l
Cox7a21
Ndufa3
Ndufb5
0 1 2
Lipid metabolism
Phyh
Lpl
Hexb
Gpx3
Sqle
Alas2
Nubpl
Gdf3
0 2
D
Oxygen consumption rate
OCR (pmol/min/Norm. Unit)
Oligo
FCCP
Eto
R+A
Control
Butyrate
Acetate
Proprionate
Time (minutes)
E
Oxidative phosphorylation
OCR following FCCP (normalised units)
Lipid metabolism
Fold change in OCR following Eto (normalised units)

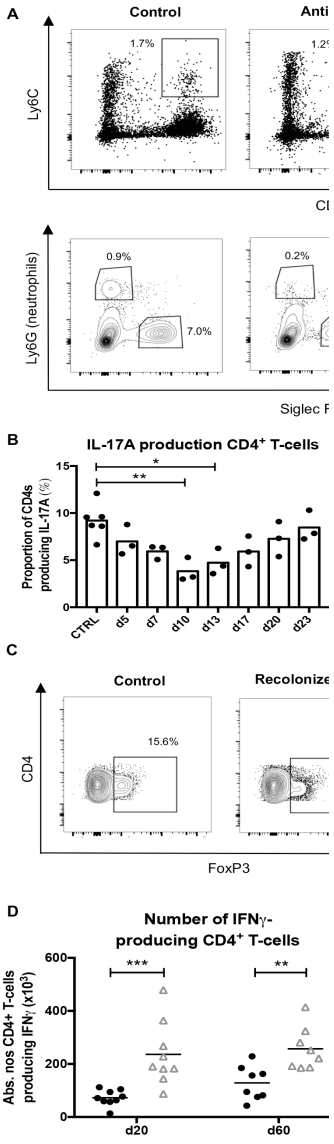
Figure 7


A
Control +LPS
Abx +LPS
Abx/But +LPS
Abx/Prop +LPS
Abx/Ace +LPS
Intracellular TNFα
Intracellular IL-10
54.5%
17.8%
1.0%
77.3%
6.9%
0.3%
62.0%
10.4%
0.7%
56.9%
22.8%
0.5%
60.5%
23.1%
0.5%
Macrophages: TNFα production
Proportion of macrophages producing TNFα (%)
-LPS
+LPS
Control
Abx
Abx + But
Abx + Prop
Abx + Ace
Macrophages: IL-10 production
Proportion of macrophages producing IL-10 (%)
B
Control
+Butyrate
CD11b
MFI 3658
MFI 2235
Intracellular TNFα
Magnitude of TNFα (Macrophages)
MFI (TNFα)
+LPS
+LPS/BUT
TNFα secretion
TNFα secretion (pg/ml)
C
Control
Recolonized (20d)
Recolonized/But
IFNγ
Ki67
20.9%
10.9%
24.2%
24.6%
34.1%
16.9%
15.7%
6.9%
20.4%
IFNγ+Ki67+ CD4+ T-cells
Proportion of IFNγ+Ki67+ CD4+ T-cells (%)
Ctrl
Recolonized (d20)
Recolonized/But (d20)
CD4
T-bet
49.0%
72.9%
50.3%
T-bet expression CD4+ T-cells
Proportion of CD4+ T-cells expressing T-bet (%)

**Supplementary figure 1**




**Supplementary figure 1**

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**Supplementary figure 1. Monocytes, neutrophils, eosinophils, and T-cells in recolonized mice.**

**a)** Representative flow cytometry plots demonstrating $CD11b^+Ly6C^+$ monocytes, $Ly6G^+$ neutrophils, and $SiglecF^+$ eosinophils identified from the colonic lamina propria (LP) of control, antibiotic treated and recolonized mice. **b**) Pooled data depicting proportions and number of $CD3^+CD4^+$ T-cells producing IL-17A following antibiotic treatment and subsequent recolonization at various time points up to 23 days post antibiotics. Dotted line represents mean proportion/numbers of cells in control mice (*P<0.05, **P<0.01, Kruskal-Wallis test, n≥3 for all time points). **c)** Mice were antibiotic treated and subsequently recolonized for 20 days, then $CD3^+CD4^+$ colonic T-cells were identified by flow cytometry and characterized for expression of transcription factor FoxP3. Representative flow cytometry plots and pooled data are depicted (***P<0.001, unpaired Mann-Whitney test, n=9). **d)** Analysis of $CD3^+CD4^+$ T-cells producing IFNγ or expressing transcription factor T-bet in mice that had been recolonized for 20 or 60 days following antibiotic treatment. Data at each time point was compared to age matched controls. Pooled data of absolute numbers are depicted (**P<0.01, ***P<0.01, unpaired Mann-Whitney tests n=8-10).

**Comment [L1]:** I'm not sure why the figures are coming up with gray shading – I've re-embedded some figures and its still white on my laptop and work computer so think perhaps it may be your computer?

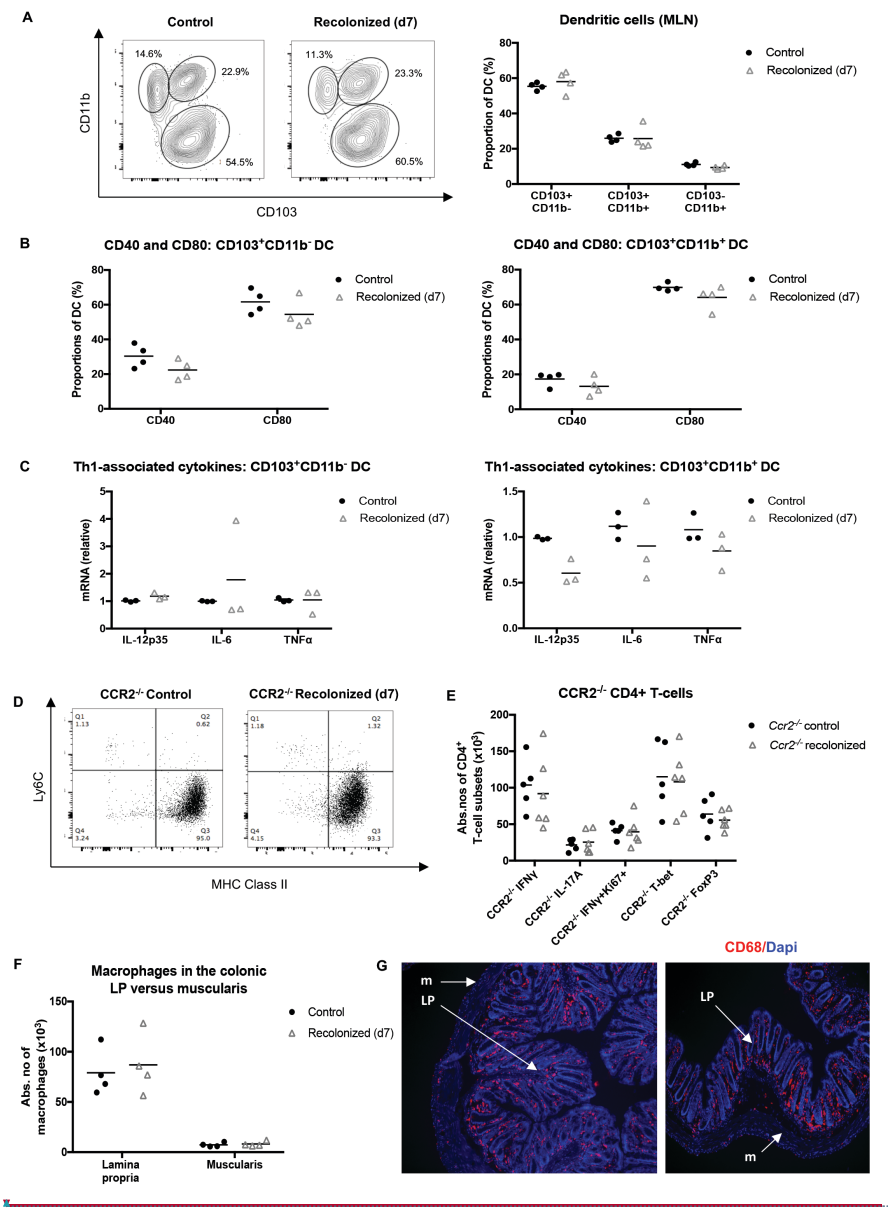
Where possible I have moved the legends onto the same page as the figures now.

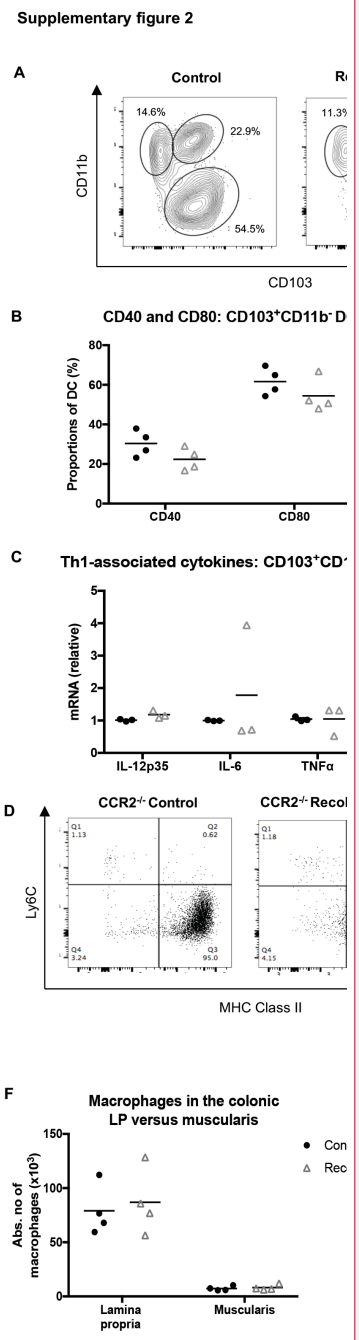
**Comment [Office2]:** The figures in the supp look like they have gray shading in the background. Could be due to the file transfer? Perhaps my screen?

This document will be published exactly as provided. Please superscript anything that you want to be superscripted, including in the figures.

Where possible I suggest cropping the size of the images to allow the legend to be on the same page (e.g. fig S3).

Supplementary figure 2

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**Supplementary figure 2. Characterization of dendritic cells, and distribution of monocytes and macrophages in *Ccr2*$^{-/-}$ mice.**

**a)** Live CD45$^{+}$B220$^{-}$CD64$^{-}$MHC Class II$^{hi}$CD11c$^{+}$ migratory dendritic cells (DCs) were characterized from the mesenteric lymph nodes (MLN) of control mice and mice that had been recolonized for 7 days following antibiotic treatment, and then further subdivided based on expression of CD103 and CD11b, by flow cytometry. Representative flow cytometry plots and pooled data are depicted (unpaired Mann-Whitney tests, n=4). **b)** Expression of co-stimulatory molecules CD40 and CD80 on DCs was assessed by flow cytometry, pooled data are depicted (unpaired Mann-Whitney tests, n=4). **c)** DC subsets from the MLN of control mice, and mice that had been recolonized for 7 days were sorted by flow cytometry and mRNA abundance for *il12p35, il6,* and *tnfα* were analyzed by qRT-PCR. Results are mean expression normalized to *TATA binding protein (tbp)* and shown relative to mean of control mice in all cases (n=3). **d)** *Ccr2*$^{-/-}$ mice were antibiotic treated and subsequently recolonized for 7 days. Monocytes and macrophages were characterized from live CD45$^{+}$SiglecF$^{-}$Ly6G$^{-}$CD11b$^{+}$CD64$^{+}$ LP cells based on expression of Ly6C (monocytes and immature macrophages) and MHC Class II (immature and mature macrophages). Representative flow cytometry plots are depicted and representative of four individual experiments with similar results. **e)** Analysis of CD3$^{+}$CD4$^{+}$ T-cells producing IFNγ, IL-17A, or expressing Ki67, T-bet, or FoxP3 in mice that had been recolonized for 20 days following antibiotic treatment of *Ccr2*$^{-/-}$ mice. Pooled data of absolute numbers are depicted (unpaired Mann-Whitney tests, n=5-6). **f)** Lamina propria and muscularis compartments of the colon were manually separated and absolute numbers of mature macrophages (CD45$^{+}$ live, Siglec F$^{-}$ Ly6G$^{-}$, CD11b$^{+}$, CD64$^{+}$, Ly6C$^{-}$, MHC Class II$^{+}$ cells) were identified in the separate compartments by flow cytometry. Pooled data depicting absolute numbers (unpaired Mann-Whitney tests, n=4). **g)** Fluorescence microscopy images of demonstrating CD68 staining (red) of colonic intestinal sections. (DAPI nuclear staining in blue). On images m = muscularis and LP = lamina propria.

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**Comment [Office4]:** Should this be a gene?

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**Comment [Office5]:** What is on the left and what is on the right?

**Comment [L6]:** These are two example images showing the same thing

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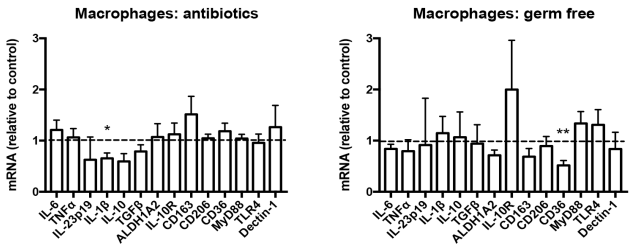
**Comment [L7]:** The figures have all been amended now to say d7 throughout

**Comment [Office8]:** Some keys say "7d" and some say "d7." Up to you if you want to go through and make it consistent.

Supplementary figure 3




**Supplementary figure 3. Gene expression profiles of mature colonic macrophages from antibiotic-treated and germ free mice.**

Mature colonic macrophages (Live CD45$^+$SiglecF$^-$Ly6G$^-$CD11b$^+$CD64$^+$Ly6C$^-$MHC Class II$^+$ LP cells) from antibiotic-treated (7 days) and germ free mice were sorted by flow cytometry and mRNA for *il6, tnfα, il23p19, il1β, il10, tgfβ, aldh1a2, il10r, cd163, cd206, myd88, tlr4* and *dectin-1* analyzed by qRT-PCR. Results are mean expression normalized to *TATA binding protein (tbp)* and shown relative to untreated (control) mice in all cases (dotted line). (*P<0.05, **P<0.01, Two-way ANOVA, n=4-9 (antibiotics) and n=3-7 (germ-free)).

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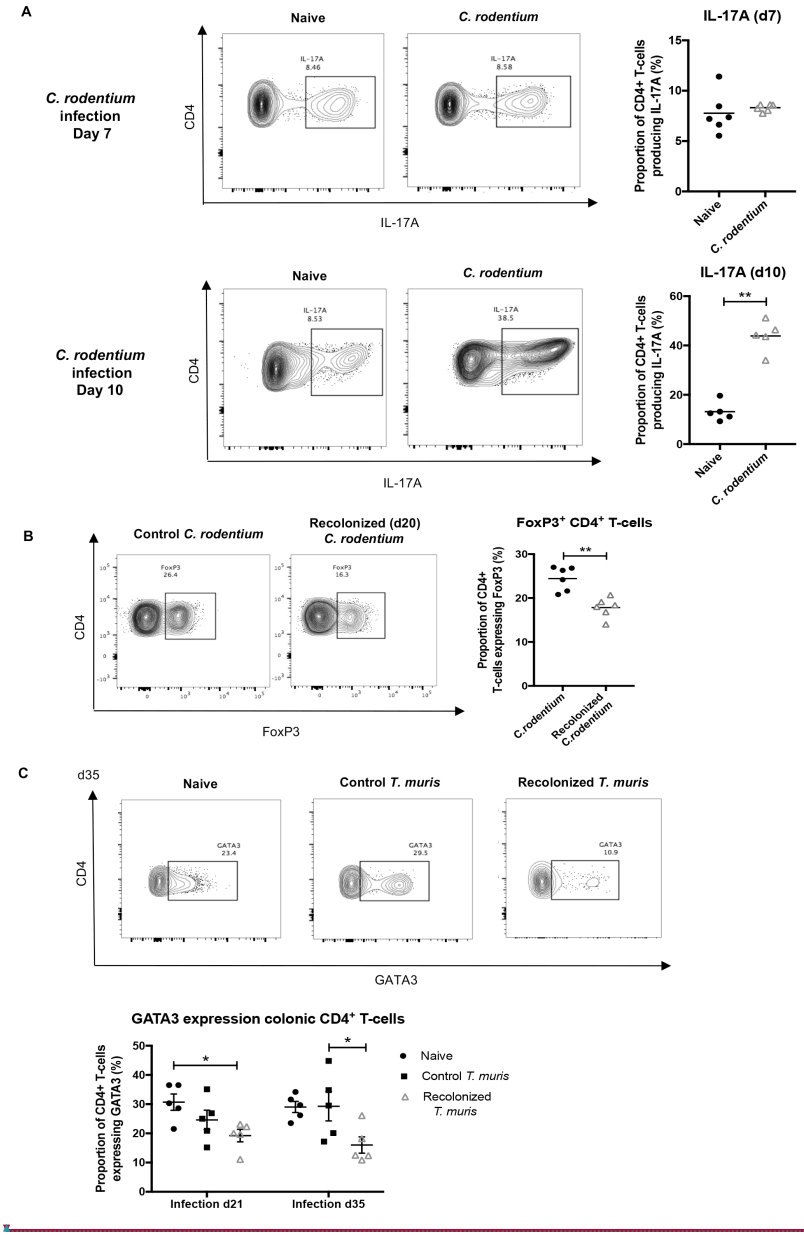
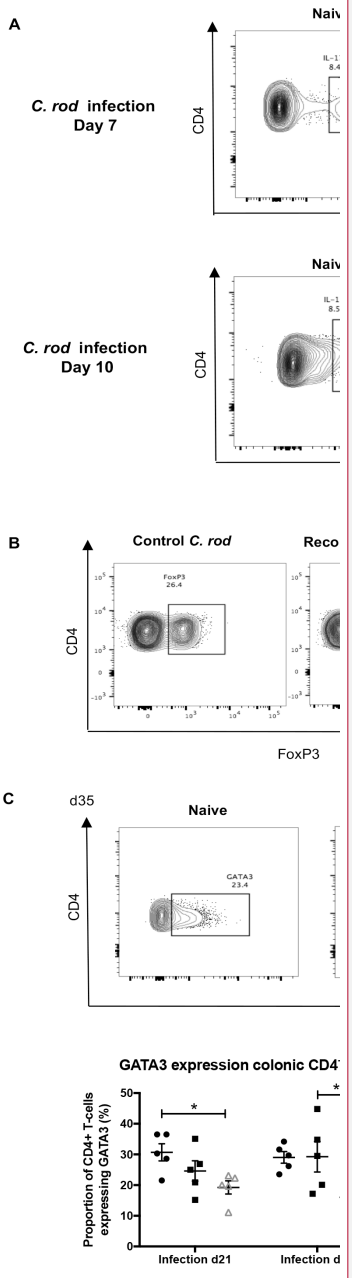
Supplementary figure 4




Supplementary figure 4

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**Supplementary figure 4. Sustained immune dysfunction in recolonized mice repolarizes immune responses to infection**

**a)** Mice were infected with *Citrobacter rodentium* and T-cell responses in the colon analyzed at 7 and 10 days post infection. Live $CD45^+CD3^+CD4^+$ colonic T-cells were identified by flow cytometry and characterized for production of the cytokine IL-17A. Representative flow cytometry plots and pooled data are depicted (**P<0.01, non-paired Mann-Whitney test, n=5-6). **b)** Mice were treated with antibiotics and subsequently recolonized for 20 days prior to infection with *Citrobacter rodentium.* Live $CD45^+CD3^+CD4^+$ colonic T-cells were identified by flow cytometry and characterized for expression of the transcription factor FoxP3. Representative flow cytometry plots and pooled data are depicted (**P<0.01, non-paired Mann-Whitney test, n=6). **c)** Mice were treated with antibiotics and subsequently recolonized for 20 days prior to infection with the intestinal helminth *Trichuris muris,* alongside non-antibiotic treated mice. Live $CD45^+CD3^+CD4^+$ colonic T-cells were identified by flow cytometry and characterized for expression of the transcription factor GATA3. Representative flow cytometry plots and pooled data are depicted (*P<0.05, Kruskal-Wallis test, n=5).

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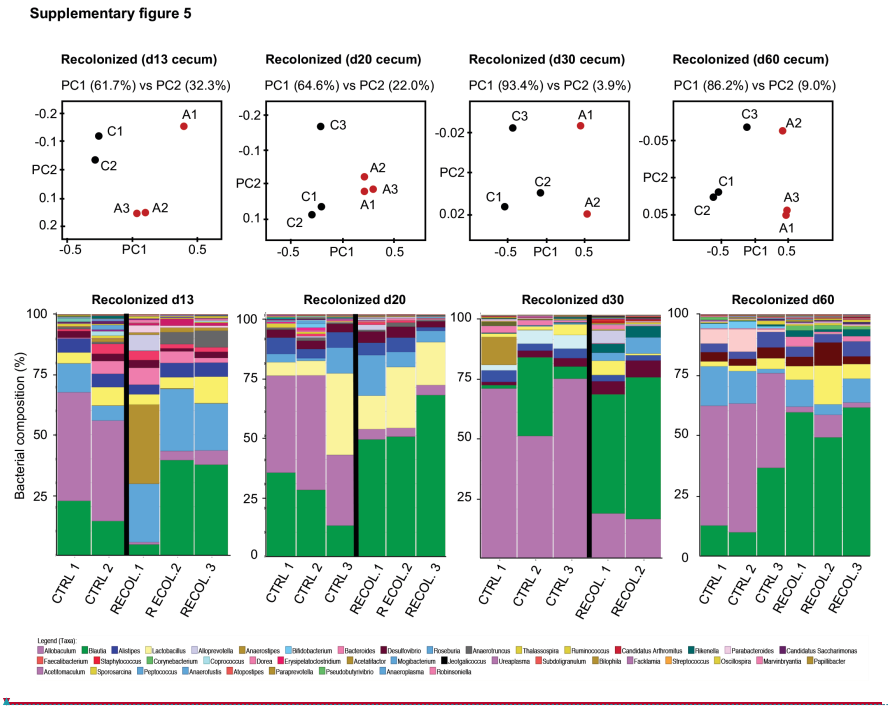


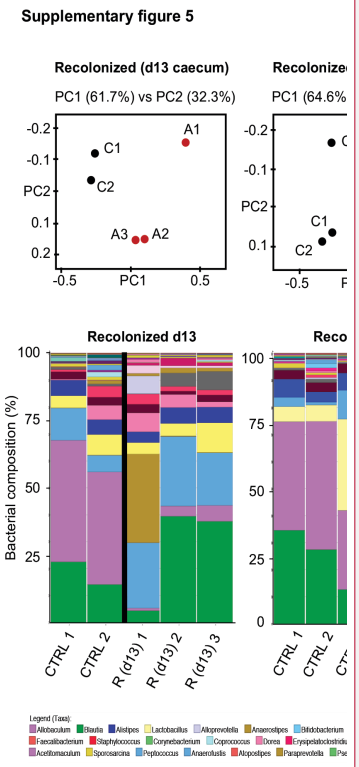


**Supplementary figure 5. Recolonization of antibiotic-treated mice causes long-term disruption of the intestinal microbiota**

**a)** Principal coordinate analysis using Bray-Curtis metrics were performed on the taxonomic assignments from cecal stool samples from recolonized mice (red, A samples) at days 13, 20, 30 and 60 days following antibiotic treatment and age-matched controls (dark blue, C samples). Results are shown as three individual experiments. **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 (day 13, day 20, day 30 and day 60).

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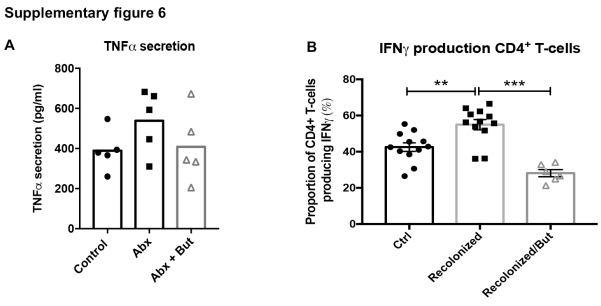


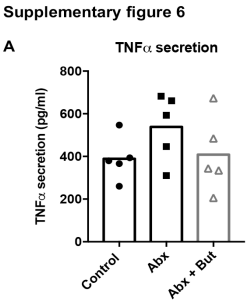


**Supplementary figure 6. The short-chain fatty acid butyrate alleviates antibiotic-associated immune dysfunction.**

**a)** FACS sorted colonic macrophages (live $CD45^+$ Siglec $F^-$ $Ly6G^-$ $CD11b^+$ $CD64^+$ $Ly6C^-$ MHC Class $II^+$ cells) from control or recolonized mice (7 days, ±200mM butyrate) were cultured for 18 hours with 100 ng/ml LPS and secreted protein was measured in supernatants by cytometric bead array (Kruskal-Wallis test, n=5). **b)** Live $CD45^+CD3^+CD4^+$ colonic T-cells were characterized from control mice and mice that had been recolonized for 20 days following antibiotic treatment. In some cases, antibiotics were supplemented with butyrate (200mM). $CD3^+CD4^+$ T-cell production of IFNγ was determined by flow cytometry. Representative flow cytometry plots and pooled data are depicted (**P<0.01, ***P<0.001, Kruskal-Wallis test, n=12 (control and recolonized), n=6 (butyrate). All data shown are representative of at least two independent experiments.

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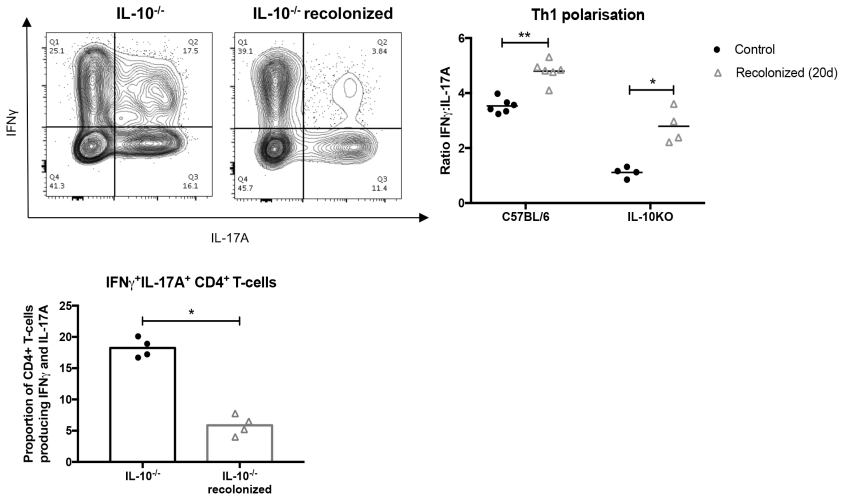
Supplementary figure 7







**Supplementary figure 7. Th1 polarization in recolonized *il-10*$^{-/-}$ mice.**

Representative flow cytometry plots demonstrating IFNγ and IL-17A production from $CD3^{+}CD4^{+}$ T-cells identified from the colonic lamina propria of control and recolonized mice, following stimulation with PMA and Ionomycin. Pooled data depicting ratio of IFNγ to IL-17A producing T-cells following antibiotic treated and subsequent recolonization (20d) and proportions of $CD4^{+}$ T-cells co-producing IFNγ and IL-17A (*P<0.05, **P<0.01, unpaired Mann-Whitney tests, n=4-6 for all time points).

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## Supplementary materials and methods

### Isolation of tissue leukocytes

For the isolation of colonic lamina propria cells, colons were removed from mice, cleaned in PBS, chopped into 5mm sections and incubated three times in 2mM EDTA in HBSS to remove the epithelial compartment, then washed and incubated in 1.25mg/ml collagenase D (Sigma), 0.85mg/ml collagenase V (Sigma) and 1mg/ml dispase (Gibco, Invitrogen) in RPMI-1640 (Thermofisher Scientific) for 30-35 minutes in a shaking incubator at 37°C.

### Isolation of mesenteric lymph node leukocytes

For the isolation of mesenteric lymph node (MLN) cells, MLNs were removed from mice, chopped finely and incubated in 1mg/ml collagenase D (Sigma) for 35 minutes in a shaking incubator at 37°C.

### Separation of colonic lamina propria and muscularis

Intestinal layers were separated as previously described *(60)* with some modifications. Briefly, intestines, harvested as described above, were incubated in calcium and magnesium free HBSS with 2% FCS and 1mM DTT in 50mL falcon tubes for 20 minutes at 37°C in a shaking incubator. Tubes containing intestinal samples were vortexed and intestine samples were decanted into sieves before being returned to 50mL tubes containing calcium and magnesium free HBSS with 2% FCS and 1.3mM EDTA and incubated for 45 minutes at $37^0$C in a shaking incubator. Tubes containing intestinal samples were vortexed and intestine samples were decanted into sieves. After DTT and EDTA incubations, intestinal tissue was separated under a dissecting microscope into muscularis and lamina propria layers. Digestion of these separate layers continued as described above.

### Flow cytometric analysis and sorting of cells

0.5-5 x $10^6$ cells were stained at 4°C in the dark using the antibodies listed in Supplementary Table 1 and analysed using an LSR Fortessa cytometer (BD Biosciences) and FlowJo software (TreeStar). Colonic monocytes and macrophages were sorted using a FACS Aria III and a FACS Aria Fusion, as live gated

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$CD45^+SiglecF^-Ly6G^-CD11b^+CD64^+$ cells to >98% purity and further subdivided into subsets based on expression of MHC class II and Ly6C.

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**Quantitation of gene expression by real-time reverse transcription PCR**

Total RNA was extracted from monocytes and macrophages FACS-purified from the colons of individual mice using the RNeasy Micro Kit (Qiagen). RNA were reverse transcribed to cDNA using the High Capacity RNA-to-cDNA Kit (ThermoFisher Scientific) and gene expression was assayed using quantitative reverse transcription PCR using PerfeCTa SYBR Green Fastmix (Quanta) on the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) with the primers (Integrated DNA Technologies) detailed in Supplementary table 2. Gene expression levels were normalized to TATA binding protein (TBP). The mean relative gene expression was calculated using the $2^{-d}\Delta^{C(t)}$ method.

**Bone-marrow derived macrophage generation**

Bone marrow progenitors were harvested from femurs and tibias of wild-type adult C57BL/6 mice and cultured for 6 days on petri dishes in the presence of macrophage colony-stimulating factor (M-CSF). Media and M-CSF were changed on day 3 and in some experiments media was supplemented with 1mM SCFA on day 5. Bone-marrow derived macrophages were harvested on day 6 with 2mM EDTA.

**Cytometric bead array**

Intestinal macrophages were isolated by FACS and plated in round-bottomed 96-well tissue culture plates at 50,000 cells per well. The cells were cultured at 37ºC and 5% $CO_2$ in 50µl complete RPMI (10% FCS) in the presence of 100ng/ml LPS (Sigma) ± 500µM butyrate (Sigma). In other experiments, butyrate had been administered to mice with antibiotics *in vivo* as described above, prior to macrophage isolation. After 18 hours, culture supernatants were collected and analysed for the presence of cytokine and chemokines using the Mouse Inflammation CBA kit (BD Biosciences). Data were acquired on an LSR Fortessa (BD Biosciences) and analysed using FCAP Array software (BD Biosciences).

**LPS stimulation of lamina propria cells *in vitro***

Colonic LP cells were incubated for 4.5 hours with monensin and Brefeldin A (Sigma) in the presence or absence of 1μg/ml LPS (Sigma). Monocytes and macrophages were then identified by flow cytometry and assessed for intracellular cytokine production following cell permeabilisation and fixation as described below.

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**Assessment of cytokine production by T-cells**

Colonic LP cells were incubated for 4 hours with PMA, Ionomycin, and Golgistop (eBioscience) before being surface stained, fixed, and permeabilised prior to intracellular cytokine staining (eBioscience intracellular staining kit).

**Faecal DNA extractions, quantification and bacterial sequencing**

DNA was extracted from faecal pellets using the FastDNA SPIN Kit for Soil (MP Biomedicals) following the manufacturer's instructions but incorporating an extension of the initial bead-beading time to three minutes. The concentration of bacterial DNA was quantified using Qubit and normalized to 5 ng/ml for all samples. Extracted DNA was used as a template for PCR amplification of the V1/V2 regions of the 16S rRNA gene as detailed in Supplementary Table 3. Amplification conditions of the PCR were: 1 cycle of 94°C 3 min and 25 cycles of 94°C for 45 s, 55°C for 15 s and 72°C for 30 s using a 96 well Thermal Cycler PCR machine. 16S RNA gene libraries were sequenced on the Illumina MiSeq platform with 250 bp paired end reads.

**Bacterial sequence processing**

All raw sequence reads were processed through quality control using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) keeping a minimum quality threshold of 33 for at least 50% of the bases. Reads that passed the threshold were aligned against SILVA database (version: SILVA_119_SSURef_tax_silva) *(48)* using BLASTN *(49)* (ncbi-blast-2.2.30+; Max e-value 10e-3) separately for both pairs. After performing the BLASTN alignment, all output files were imported and annotated using the paired-end protocol of MEGAN *(50)*.

**Taxonomic annotation**

Parameter settings of "Min Score = 50", "Top Percent = 10" were applied to process BLAST files by MEGAN6. Reads which did not have any match to the respective

database were placed under a “No hit” node, and reads that were originally assigned to a taxon that did not meet our selected threshold criteria were returned to higher nodes where the threshold was met using the lowest common ancestor (LCA) algorithm. After importing datasets into MEGAN, we obtained MEGAN-own “rma files” for each data set that were mapped onto NCBI taxonomy based on our selected threshold and the files were compared and analysed within MEGAN.

**Metabolite analysis**

All samples were thawed at room temperature and prepared for $^{1}$H nuclear magnetic resonance (NMR) spectroscopy by mixing 50mg±1mg faeces with 200µl NMR buffer (0.26g $NaH_2PO_4$ and 1.41g $K_2HPO_4$) made up in 100% $D_2O$ (100ml), containing 0.1% NaN3 (100mg) and 1mM sodium 3-(Trimethylsilyl)-propiomate-*d*4 (TSP) as a chemical shift reference and 250µl $D_2O$. All samples were vortexed and then centrifuged at 14,000rpm for 5 min, and the remaining supernatant was removed and used for semi-quantification of metabolites. The $^{1}$H NMR spectra were recorded at 600MHz on aBruker Avance spectrometer (Bruker BioSpin Gmbh, Rheinstetten, Germany) running Topspin 2.0 software and fitted with a broadband inverse probe. $^{1}$H NMR spectra were acquired with 512 scans, a spectral width of 12300 Hz and an acquisition time of 2.7s. The “noesypr1d” presaturation sequence was used to suppress the residual water signal with a low-power selective irradiation at the water frequency during the recycle delay. Spectra were transformed with a 0.3-Hz line broadening, manually phased, baseline corrected and referenced by setting the TSP methyl signal to 0 ppm. Filtered samples were transferred to NMR tubes with 100µl of NMR buffer and mixed well before reading using the parameters described above. All metabolites were quantified using the software Chenomx NMR suite 7.6 based on published literature *(51-53)* and by use of the 2D-NMR methods, COSY, HSQC and HMBC.

***Trichuris muris* infections**

The maintenance, infection and recovery of *Trichuris muris* were as described previously *(54)*. Briefly, stock infections were maintained in susceptible mouse strains and adult worms harvested at day 42 post infection (p.i.). In all experiments, C57BL/6 mice (control or recolonized (20 or 60 days)) were infected by oral gavage

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with 200 embryonated eggs and worm burdens were assessed on day 21 or day 35 p.i. Proximal colons (2cm section) were harvested in all cases for FACS analysis of adaptive immune responses.

***Citrobacter rodentium infections***

The nalidixic acid-resistant *Citrobacter rodentium* strain ICC169 *(55)* was grown overnight at 37°C in Luria broth (LB). C57BL/6 mice (control or recolonized (20 or 60 days)) were inoculated with approximately $2x10^9$ bacteria by oral gavage. CFUs were calculated in stool at days 3,5,7 and 10 during the experiment and in caecal tissue at the conclusion of the experiment, by plating mechanically homogenized samples on agar (Lennox formula, Sigma) plates containing 50µg/ml nalidixic acid. Colonization levels were normalized to stool and tissue weight.

**RNA sequencing and analysis**

Strand-specific RNA sequencing libraries were prepared using the Illumina workflow with the TruSeq stranded mRNA sample preparation kit. Paired-end reads (65 x 65 bp) were generated from each sample and 48-192 million reads were obtained from each sample. The fastq files generated by a HiSeq4000 platform (Illumina) were analyzed with FASTQC and any low quality reads and contaminated barcodes were trimmed with Trimmomactic *(56)*. All libraries were aligned to GRCm38.p4 mouse genome assembly using STAR-2.4.2 *(57)*, and only uniquely mapped reads were used for differential gene expression analysis. The mapped reads were counted by genes with *HTseq (58)* against gencode.vM11.annotation.gtf. The differentially expressed genes were identified using DESeq2 *(59)* by pairwise comparisons between the experimental groups. The differentially expressed genes with a p-adjusted value <0.05 were selected for further validation and analysis. For functional analysis, an R package of topGO and Ingenuity Pathway analysis was used. RNA sequencing data were deposited in the ArrayExpress public database under accession number E-MTAB-7132.

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**Assessment of cellular metabolism**

For real-time analysis of oxygen consumption rates (OCR), macrophages were analyzed using an XF-96 Extracellular Flux Analyser (Seahorse Bioscience) as

described in detail previously *(41)*. Measurements were taken under basal conditions and following the sequential addition of 1μM oligomycin, 1.5μM fluoro-carbonyl cyanide phenylhydrazone (FCCP), 200μM etomoxir (Eto) and 100nM rotenone plus 1μM antimycin A (R+A; all purchased from Sigma).

**Immunofluorescence microscopy**

Colon sections were drop fixed in 10% sucrose 4% pfa for 2 hours followed by transfer into 30% sucrose PBS for 6 hours. Fixed and dehydrated tissue was embedded in OCT and frozen in isopentane on dry ice. Frozen tissue blocks were stored at -80°C until use. Sections were cut at 8um using a cryostat (Leica CM3050S), mounted on to superfrost slides (Thermo Scientific), air-dried and stored at -80°C until use. On the day of staining, slides were brought to room temperature before staining. Sections were encircled with a pap pen (Daido Sangyo) and blocked with TSA Blocking Reagent (PerkinElmer) for 15 min. Sections were further blocked using avidin and biotin blocking solutions (Biolegend), each for 15 minutes. Sections were washed in Tris NaCl Tween (TNT) buffer pH7.5 with 0.03% trition-X between incubations. All incubations were performed in a humidified chamber at room temperature, apart from incubation with the primary antibody, which was performed at 4°C. Purified rat anti-CD68 (clone FA-11, Invitrogen) was diluted in TNT buffer with 1% BSA and applied to sections overnight at 4°C. The following day, after washing with TNT buffer, purified biotin-conjugated donkey anti-rat (Invitrogen) was applied for 2 hours at room temperature. Tyramide labelling was completed using a Molecular Probes TSA kit with HRP-streptavidin and AF555 tryamide according to manufacturer's instructions. Briefly, endogenous peroxidase activity was blocked by incubation in 0.3% $H_2O_2$ for 10 min. Sections were then incubated with streptavidin HRP for 30 minutes, followed by tyramide 555 labelling solution for 5 min. Sections were mounted in ProLong Gold Antifade Mountant with DAPI (Molecular Probes) and allowed to cure overnight at room temperature in the dark.

Images were collected on a Zeiss Axioimager.D2 upright microscope using a 10x objective and captured using a Coolsnap HQ2 camera (Photometrics) through Micromanager software v1.4.23. Images were processed using ImageJ (NIH).

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**Supplementary table 1: Antibodies**

| Antibody | Clone | Source |
|---|---|---|
| CD45 | 30-F11 | Biolegend |
| CD11b | M1/70 | Biolegend |
| CD64 | X54-5/7.1 | Biolegend |
| Siglec F | E50-2440 | BD Biosciences |
| Ly6G | IA8 | Biolegend |
| MHC Class II | M5/114.15.2 | eBioscience |
| Ly6C | HK1.4 | Biolegend |
| CD103 | 2E 7 | Biolegend |
| B220 | RA3-6B2 | Biolegend |
| Tim4 | 54(RMT4-54) | eBioscience |
| IL-10 | JES5-16E3 | eBioscience |
| TNFalpha | MP6-XT22 | Biolegend |
| CD3 | 17A2 | Biolegend |
| CD4 | GK1.5 | Biolegend |
| Ki67 | B56 | BD Biosciences |
| IFNgamma | XMG1.2 | BD Biosciences |
| IL-17A | TC11-18H10.1 | Biolegend |
| Live/dead dye | | eBioscience |

**Supplementary table 1. Antibodies.**

Description of antibodies used in flow cytometry experiments, including clone and source.

**Supplementary table 2: qPCR primer sequences**

| Gene | Sense | Antisense |
|---|---|---|
| TATA BP | 5' CTC AGT TAC AGG TGG CAG CA 3' | 5' GCC CAA GTA GCA GCA CAG A 3' |
| IL-6 | 5' TTC CAT CCA GTT GCC TTC TT 3' | 5' ATT TCC ACG ATT TCC CAG AG 3' |
| IL-12 | 5' GAC CAT CAC TGT CAA AGA GTT 3' | 5' AGG AAA GTC TTG TTT TTG AAA T 3' |
| TNFalpha | 5' CAC CAC CAT CAA GGA CTC AA 3' | 5' GAG GCA ACC TGA CCA CTC TC 3' |
| IL-1beta | 5' CGC TCA GGG TCA CAA GAA AC 3' | 5' GAG GCA AGG AGG AAA ACA CA 3' |
| IL-23p19 | 5' AGC GGG ACA TAT GAA TCT ACT 3' | 5' GTC CTA GTA GGG AGG TGT GAA 3' |
| ALDH1A2 | 5' TGT AAT CCA GCC ACA GGA GAG CAA 3' | 5' ACG TCC TCT TTC AGA CGC ATC CAT 3' |
| TGFbeta | 5' TAA AGA GGT CAC CCG CGT GCT AAT 3' | 5' ACT GCT TCC CGA ATG TCT GAC GTA 3' |
| IL-10 | 5' CAA CAT ACT GCT AAC CGA CTC CT 3' | 5' TGG GGC ATC ACT TCT ACC A 3' |
| IL-10R | 5' CTT CAG TTC TCA GGA CGC CGC C 3' | 5' CGT ATG CAA TGA ATT CTA GGG T 3' |
| CD163 | 5' CCT TGG AAA CAG AGA CAG GC 3' | 5' TCC ACA CGT CCA GAA CAG TC 3' |
| CD206 | 5' TGT GGT GAG CTG AAA GGT GA 3' | 5' CAG GTG TGG GCT CAG GTA GT 3' |
| CD36 | 5' ATT GTA CCT GGG AGT TGG CG 3' | 5' CAG CCA GGA CTG CAC CAA TA 3' |
| MyD88 | 5' CAC CTG TGT CTG GTC CAT TG 3' | 5' CTG TTG GAC ACC TGG AGA CA 3' |
| TLR4 | 5' GGC AAC TTG GAC CTG AGG AG 3' | 5' CAT GGG CTC TCG GTC CAT AG 3' |
| TGFBR1 | 5' TTG CAC TTA TGC TGA TGG TCT 3' | 5' AGT GAT GGA TCC TCT TCA TTT 3' |

**Supplementary table 2. qPCR primer sequences for macrophage gene expression.**

Description of primer sequences used to determine expression of particular genes by intestinal macrophages in qRT-PCR experiments.

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**Supplementary table 3: Primer sequences for amplifying V1+V2 region of 16S rRNA gene using MiSeq Illumina.**

| 9 FW primers 12 RV primers | Total Primer sequences |
|---|---|
| V1FW_SD501 | AATGATACGGCGACCACCGAGATCTACACAAGCAGCATATGGTAATTGTAGMGTTYGATYMTGGCT |
| V1FW_SD502 | AATGATACGGCGACCACCGAGATCTACACACGCGTGATATGGTAATTGTAGMGTTYGATYMTGGCT |
| V1FW_SD503 | AATGATACGGCGACCACCGAGATCTACACCGATCTACTATGGTAATTGTAGMGTTYGATYMTGGCT |
| V1FW_SD504 | AATGATACGGCGACCACCGAGATCTACACTGCGTCACTATGGTAATTGTAGMGTTYGATYMTGGCT |
| V1FW_SD505 | AATGATACGGCGACCACCGAGATCTACACGTCTAGTGTATGGTAATTGTAGMGTTYGATYMTGGCT |
| V1FW_SD506 | AATGATACGGCGACCACCGAGATCTACACCTAGTATGTATGGTAATTGTAGMGTTYGATYMTGGCT |
| V1FW_SD507 | AATGATACGGCGACCACCGAGATCTACACGATAGCGTTATGGTAATTGTAGMGTTYGATYMTGGCT |
| V1FW_SD508 | AATGATACGGCGACCACCGAGATCTACACTCTACACTTATGGTAATTGTAGMGTTYGATYMTGGCT |
| V1FW_SA501 | AATGATACGGCGACCACCGAGATCTACACATCGTACGTATGGTAATTGTAGMGTTYGATYMTGGCT |
| V2RV_SD701 | CAAGCAGAAGACGGCATACGAGATACCTAGTAAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT |
| V2RV_SD702 | CAAGCAGAAGACGGCATACGAGATACGTACGTAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT |
| V2RV_SD703 | CAAGCAGAAGACGGCATACGAGATATATCGCGAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT |
| V2RV_SD704 | CAAGCAGAAGACGGCATACGAGATCACGATAGAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT |
| V2RV_SD705 | CAAGCAGAAGACGGCATACGAGATCGTATCGCAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT |
| V2RV_SD706 | CAAGCAGAAGACGGCATACGAGATCTGCGACTAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT |
| V2RV_SD707 | CAAGCAGAAGACGGCATACGAGATGCTGTAACAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT |
| V2RV_SD708 | CAAGCAGAAGACGGCATACGAGATGGACGTTAAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT |
| V2RV_SD709 | CAAGCAGAAGACGGCATACGAGATGGTCGTAGAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT |
| V2RV_SD710 | CAAGCAGAAGACGGCATACGAGATTAAGTCTCAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT |
| V2RV_SD711 | CAAGCAGAAGACGGCATACGAGATTACACAGTAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT |
| V2RV_SD712 | CAAGCAGAAGACGGCATACGAGATTTGACGCAAGTCAGTCAGCCGCTGCCTCCCGTAGGAGT |

**Supplementary table 3. Primer sequences for 16S bacterial sequencing.**

List of primer sequences for amplifying V1+V2 region of 16S rRNA gene using MiSeq Illumina in experiments to determine the composition of bacterial communities from colonic and cecal stool samples.

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