Regulation of splenic monocyte homeostasis and function by gut microbial products

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Highlights

A 4-day antibiotic treatment eliminates certain bacterial families in the gut

Gut dysbiosis is followed by reduced levels of PRR ligands in the circulation

Reduction of PRR ligands relates to perturbation of splenic Ly6C<sup>high</sup> monocytes

Addition of PRR ligands restores splenic Ly6C<sup>high</sup> monocyte numbers and function

Kolypetri et al., iScience 24, 102356
April 23, 2021 © 2021 The Authors.
https://doi.org/10.1016/j.isci.2021.102356
Regulation of splenic monocyte homeostasis and function by gut microbial products

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SUMMARY
Splenic Ly6C<sup>high</sup> monocytes are innate immune cells involved in the regulation of central nervous system-related diseases. Recent studies have reported the shaping of peripheral immune responses by the gut microbiome via mostly unexplored pathways. In this study, we report that a 4-day antibiotic treatment eliminates certain families of the Bacteroidetes, Firmicutes, Tenericutes, and Actinobacteria phyla in the gut and reduces the levels of multiple pattern recognition receptor (PRR) ligands in the serum. Reduction of PRR ligands was associated with reduced numbers and perturbed function of splenic Ly6C<sup>high</sup> monocytes, which acquired an immature phenotype producing decreased levels of inflammatory cytokines and exhibiting increased phagocytic and anti-microbial abilities. Addition of PRR ligands in antibiotic-treated mice restored the number and functions of splenic Ly6C<sup>high</sup> monocytes. Our data identify circulating PRR ligands as critical regulators of the splenic Ly6C<sup>high</sup> monocyte behavior and suggest possible intervention pathways to manipulate this crucial immune cell subset.

INTRODUCTION
The gut microbiome has recently emerged as a key regulator of the peripheral innate immunity as well as myelopoiesis in the bone marrow (BM) (Belkaid and Harrison, 2017). At steady state, gut microbiome-derived products and metabolites cross the intestinal barrier and enter the bloodstream affecting the differentiation potential of BM precursor cells (Khosravi et al., 2014; Balmer et al., 2014) as well as the function of innate immune cells in peripheral tissues (Gorjifard and Goldszmid, 2016). Microbial peptidoglycan fragments have been reported to be present in the sera and BM of naive mice regulating the anti-microbial ability of BM-derived neutrophils (Clarke et al., 2010). Also, the bacteria-derived lipodipeptide, lipid 654, a Toll-like receptor (TLR) 2 ligand, is present in the serum of healthy individuals, but its levels are significantly decreased in patients with multiple sclerosis (Farrokhi et al., 2013), whereas its administration at low levels in mice attenuates experimental autoimmune encephalomyelitis (EAE) (Anstadt et al., 2016). Recently, the muramyl dipeptide (MDP), a NOD2 ligand, was found to be ubiquitously present in the sera of healthy humans, mice, and monkeys and was reported to participate in the immunoregulation of autoimmune arthritis and EAE (Huang et al., 2019). Another major group of microbial metabolites regulating the peripheral innate immune responses are short-chain fatty acids (SCFAs), products of fermentation of indigestible dietary fibers in the cecum and colon (Kim, 2018). The aforementioned findings demonstrate that in the absence of infection, there is a continuous communication between the gut microbiota and peripheral innate cells mediated by circulating microbial signals. Perturbation of this cross talk affects peripheral autoimmune processes and disease development.

Among peripheral innate immune cells, splenic-resident Ly6C<sup>high/low</sup> monocytes play a prominent immunoregulatory role under inflammatory conditions as they are mobilized and rapidly recruited to injured tissues (Guilliams et al., 2018). During the onset of EAE, splenic Ly6C<sup>high</sup> monocytes acquire an immunosuppressive function (Zhu et al., 2007), which they maintain within the central nervous system (CNS) as they express Nos2 and Arg1, hallmark genes of myeloid-derived suppressor cells (Zhu et al., 2007; Giladi et al., 2020). Their abundance has recently been related to a milder EAE course (Melero-Jerez et al., 2020). Splenic Ly6C<sup>high</sup> monocytes are also involved in the regulation of amyotrophic lateral sclerosis (ALS) because they acquire an M1 signature before disease onset and treatment with an anti-Ly6C monoclonal antibody leads to reduced monocyte recruitment to the spinal cord, diminished neuronal loss, and extended survival in mice (Butovsky et al., 2012). Furthermore, splenic Ly6C<sup>high</sup> monocytes regulate the acute and post-acute
phases of spinal cord injury since prevention of early monocyte infiltration has been associated with improved disease recovery (Blomster et al., 2013). These studies highlight the regulatory function of splenic monocytes during the development of CNS-related diseases and demonstrate the need to further understand their biology.

In this study, we investigated the premise that the gut microbiome generates and secretes pattern recognition receptor (PRR) ligands in the circulation that regulate splenic Ly6Chigh monocyte homeostasis and function at steady state. We performed a 4-day antibiotic treatment to perturb the gut microbiome and simultaneously avoid impairment of hematopoiesis in the BM (Josefsdottir et al., 2017) that is known to affect myeloid cell populations in the spleen (Khosravi et al., 2014). We profiled immediate changes in the levels of several circulating PRR ligands and in the number and function of splenic-resident Ly6Chigh monocytes. Our findings (1) considerably expand the number of known PRR ligands present in the circulation at steady state and (2) demonstrate the role of PRR ligands in modulating the splenic Ly6Chigh monocyte immune profile during steady state.

RESULTS
Families of the Bacteroidetes, Firmicutes, Tenericutes, and Actinobacteria phyla are the most susceptible taxa to our antibiotic treatment

To investigate whether the gut microbiome has a regulatory role on the splenic monocyte homeostasis and function, we initiated our study by treating C57BL/6 mice with a broad-spectrum antibiotic cocktail for 4 days to disturb the gut microbiome. A significant decrease in bacterial abundance was detected in fecal samples 2 and 4 days post antibiotic administration (Figure 1A). Bacterial composition was significantly changed at the levels of alpha (measured by Shannon’s index and observed operational taxonomic units) and beta (measured by unweighted and weighted UniFrac analysis) diversity 2 and 4 days post antibiotic administration (Figures 1B and 1C, and S1). Taxonomic analysis using the linear discriminant analysis effect size (LEfSe) algorithm showed a significant decrease in the relative abundance of Bacteroidetes, Firmicutes, Tenericutes, and Actinobacteria with a concomitant increase in Proteobacteria in the antibiotic-treated group (Figures 1D and 1F). Among the most affected taxa were members of the S24_7 and Rikenellaceae families of the Bacteroidetes phylum because their relative abundance was shifted from 40.71% to 1.1% and 0.49% and from 20.6% to 2.58% and 1.14%, respectively, during treatment (Figures 1E and 1G and Tables S1 and S2). Within the Firmicutes phylum, several members of the Clostridiaceae, Lachnospiraceae, Ruminococcaceae, and Mogibacteriaceae families underwent a significant decrease, whereas a modest reduction was detected for Mollicutes RF39, Tenericutes phylum, and Bifidobacterium, Actinobacteria phylum, in the treated group. Overall, our data describe significant quantitative and qualitative changes in the homeostasis of the gut microbiota after 2 and 4 days of antibiotic treatment and identify certain families of the Bacteroidetes, Firmicutes, Tenericutes, and Actinobacteria phyla as the most susceptible bacterial taxa to our treatment.

Decreased levels of PRR ligands in the circulation and perturbation of the splenic monocyte homeostasis upon dysbiosis of the gut microbiota

We next addressed whether dysbiosis of the gut microbiota affected the levels of microbial products in the circulation of antibiotic-treated animals. Mice were administered drinking water with (n = 7) or without (n = 4) antibiotics, and sera were collected before (day 0) and on days 2 and 4 of treatment. Detection of PRR ligands in the sera of individual mice was tested using the HEK-Blue PRR reporter cell lines. Ligands for TLR2, 3, 4, 5, 7, and 9 and NOD1 and 2 were constitutively present at low levels on day 0 in all mice from both groups (Figures 2A and 2B). By day 4, the levels of all PRR ligands were significantly decreased only in mice receiving antibiotic treatment, suggesting (1) that the gut microbiome is the source producing and secreting PRR ligands into the circulation at steady state and (2) that a brief exposure to antibiotics was sufficient to mediate this reduction in PRR ligand levels.

To examine whether the reduction in the levels of PRR ligands would correlate with changes in the splenic monocyte homeostasis, we analyzed the frequency and absolute numbers of Ly6Chigh monocytes in the spleens of mice treated with or without antibiotics for 2, 4, and 6 days. Both the frequency and absolute numbers of splenic Ly6Chigh monocytes were significantly decreased in the experimental group after 4 and 6 days of antibiotic treatment compared with the untreated mice (Figure 2C). Similar observations were not detected in the BM of the same animals (Figure 2D) suggesting that the short-term antibiotic treatment did not impair hematopoiesis in the BM, known to affect the numbers of splenic monocytes.
16S rRNA Abundance

Shannon's index

Observed OTUs

Unweighted UniFrac

Weighted UniFrac

Antibiotic treatment (days)

Peptostreptococcus

Anaerococcus

Streptococcus

Staphylococcus

Enterococcus

Sutterella

Bifidobacterium
d

Rikenellaceae unclassified

Lachnospiraceae unclassified

Clostridium pflorforme

Anaerostipes

Anaerotruncus

Coprococcus

Roseburia

Ruminococcaceae unclassified

Ruminococcus

Bacteroidales S24_7 unclassified

LDA score (log10)

Erwinia

Leuconostocaceae unclassified

Bacillales unclassified

Lactobacillales unclassified

Microbacteriaceae unclassified

Bacteroides

Agrobacterium

Galliacola

Leuconostocaceae unclassified

Paenibacillus

Geobacter vulcani

Sanguibacter

Corynebacterium

Akkermansia muciniphila

Peptostreptococcus

Anaerococcus

Streptococcus

Staphylococcus

Enterococcus

Sutterella

Streprococcaceae unclassified

Allobaculum

Clostridiales unclassified

Mogibacteriaceae unclassified

Oscillospira

Rikenellaceae unclassified

Lachnospiraceae unclassified

Clostridium pflorforme

Anaerostipes

Anaerotruncus

Coprococcus

Roseburia

Ruminococcus

Bacteroidales S24_7 unclassified

Mollicutes RF39

Leuconostocaceae unclassified

Ruminococcaceae unclassified

Ruminococcus

Bacteroidales S24_7 unclassified

Mollicutes RF39

Leuconostocaceae unclassified

Ruminococcaceae unclassified

Ruminococcus

Bacteroidales S24_7 unclassified

Mollicutes RF39

Leuconostocaceae unclassified

Ruminococcaceae unclassified

Ruminococcus

Bacteroidales S24_7 unclassified

Mollicutes RF39

Leuconostocaceae unclassified

Ruminococcaceae unclassified

Ruminococcus

Bacteroidales S24_7 unclassified

Mollicutes RF39
Phenotypic and functional changes in splenic Ly6Chigh monocytes after depletion of the gut microbiota

Next, we examined the phenotype of splenic monocytes after a 3-day treatment with antibiotics using the Nanostring nCounter platform. RNA profiling of Ly6Chigh monocytes revealed that genes involved in antigen presentation (Ciita, H2-Aa, H2-Ab1, C4d, C4d0, H2-DMa) as well as SOCS3, Stat1, Traf1, and Nkb2 were significantly downregulated following antibiotic treatment (Figure 3A). Significant upregulation was detected for Trem1, a triggering receptor; Cd36, a phagocytic receptor; Camp, cathelicidin anti-microbial peptide (del Fresno et al., 2009); and S100a8 and S100a9, endogenous alarmins that can induce hyporesponsiveness (Austermann et al., 2014). Gene Enrichment Analysis predicted the top 10 biological pathways associated with the differentially expressed (DE) genes such as antigen presentation, interferon and amphoterin signaling, as well as the top 10 putative transcriptional factors regulating their expression (Figure 3B). Changes in expression of Trem1, CD36, S100a8, S100a9, Camp, and Ciita were validated by qPCR (Figure 3C) and were not detected in Ly6Chigh monocytes from antibiotic-treated GF mice (Figure S2). The aforementioned data suggest that splenic Ly6Chigh monocytes acquire an immature phenotype marked by decreased expression of antigen-presenting molecules and increased expression of genes involved in phagocytosis and anti-microbial defense at the RNA level following antibiotic treatment.

At the functional level, splenic Ly6Chigh monocytes from antibiotic-treated mice produced significantly lower levels of TNF-α, IL-6, IL-1α, IL-1β, and IL-10 after lipopolysaccharide (LPS) stimulation compared with control monocytes both at the RNA and protein levels (Figures 3D and 3E). This difference was not due to impaired ex vivo survival of splenic monocytes from antibiotic-treated mice, as previously reported (Hergott et al., 2016) (Figure S4). Also, increased phagocytic ability was detected in Ly6Chigh monocytes from antibiotic-treated mice compared with control mice after intrasplenic injection of fluorescently
Addition of PRR ligands restores splenic Ly6C<sup>high</sup> monocyte numbers and functions in antibiotic-treated mice

Last, we investigated whether addition of certain PRR ligands in the circulation would restore splenic monocyte homeostasis and function in antibiotic-treated mice. As described in transparent methods, after a 3-day treatment with antibiotics, mice were injected intraperitoneally (i.p.) with low levels of (1) a TLR ligand (TLRL) cocktail, (2) ie-DAP (a NOD1 ligand), or (3) MDP (a NOD2 ligand). Injection of ie-DAP, but not of TLRL cocktail or MDP, in antibiotic-treated mice increased the frequency and the absolute numbers of splenic Ly6C<sup>high</sup> cells (Figure 4A). At the functional level, the phagocytic ability of splenic Ly6C<sup>high</sup> cells from antibiotic-treated mice was restored to levels similar to the untreated group when mice were injected with the TLRL cocktail but not with ie-DAP or MDP (Figure 4B). In terms of cytokines, we monitored the production of TNF-α and IL-6 in ex vivo-obtained Ly6C<sup>high</sup> monocytes after LPS stimulation. In antibiotic-treated mice, ie-DAP and MDP, but not TLRL cocktail, produced TNF-α at similar or higher levels compared with untreated mice (Figure 4C). Interestingly, injection of any of the PRR ligands restored the synthesis of IL-6 in cells from the treated group (Figure 4C). Regarding the antimicrobial capacity of splenic Ly6C<sup>high</sup> monocytes, addition of TLRL cocktail and MDP in antibiotic-treated mice brought back to control levels the ability of C. rodentium and E. coli to survive intracellularly (Figure 4D). Taken together, our data suggest that...
Figure 3. Phenotypic and functional changes in splenic Ly6C<sup>high</sup> monocytes after depletion of the gut microbiota
(A) Heatmap depicting hierarchical clustering of significantly upregulated (yellow) and downregulated (blue) genes in splenic Ly6C<sup>high</sup> monocytes from untreated and treated animals for 3 days as calculated by the nSolver software.
(B) The 10 most significant biological networks and 10 top upstream transcriptional regulators of the DE genes of Ly6C<sup>high</sup> monocytes as predicted by the MetaCore software.
(C) Validation of changes in gene expression of Trem-1, S100a8, S100a9, Cd36, Camp, and Ciita by qPCR. Gene expression is presented relative to Gapdh. Data show mean ± SEM, and they are representative of two experiments (n = 10 for antibiotic-treated group, n = 5 for control group).
Figure 3. Continued

(D) Quantitative PCR analysis of the expression of Tnf-α, IL-6, IL-1α, IL-1β, and IL-10 in splenic Ly6CHigh monocytes from antibiotic-treated and untreated mice after ex vivo stimulation with or without LPS. Gene expression has been normalized to Gapdh. Data show mean ± SD, and they are representative of two experiments (n = 10 for antibiotic-treated group, n = 5 for control group).

(E) Detection of Tnf-α, IL-6, IL-1α, IL-1β, and IL-10 in the culture supernatant of splenic Ly6CHigh monocytes from antibiotic-treated and untreated mice after ex vivo stimulation with or without LPS. Data show mean ± SD, and they are representative of two experiments (n = 10 for antibiotic-treated group, n = 5 for control group).

(F) Percentage of splenic Ly6CHigh monocytes from antibiotic-treated and untreated mice containing fluorescently labeled beads, 24 h after intra-splenic bead injection (n = 7 per group). Data show mean ± SD, and they are representative of two experiments.

(G) Gentamicin protection assay on splenic Ly6CHigh monocytes from antibiotic-treated and untreated mice with C. rodentium and E. coli. Values (mean ± SD) show absolute colony-forming unit (CFU) counts after overnight incubation of cell lysates at 37°C and are representative of three experiments (n = 10 for antibiotic-treated group, n = 5 for control group).

Statistical significance was assessed by Student’s t test for pairwise comparisons. p values are shown as *p < 0.05, **p < 0.01.

addition of certain PRR ligands in the circulation of antibiotic-treated mice restores the numbers and normal function of splenic Ly6CHigh monocytes. These findings highlight that (1) perturbation of the splenic Ly6CHigh numbers and function in antibiotic-treated mice is caused by the absence of certain circulating PRR ligands and (2) circulating PRR ligands have diverse and pleiotropic effects on the immune functions of splenic Ly6CHigh monocytes.

**DISCUSSION**

In this study, we examined whether circulating PRR ligands produced constitutively by the gut microbiome regulate the splenic Ly6CHigh monocyte homeostasis and function during steady state. Our study focused on splenic Ly6CHigh monocytes as these immune cells play a regulatory role in the pathogenesis of many diseases such as EAE (Zhu et al., 2007; Melero-Jerez et al., 2020), ALS (Butovsky et al., 2012), stroke (Seifert and Offner, 2018; Pennypacker and Offner, 2015), atherosclerosis (Robbins et al., 2012; Swirski et al., 2016), ischemic myocardial injury (Leuschner et al., 2012; Swirski et al., 2009), lung ischemia-reperfusion injury (Hsiao et al., 2018), colitis (Grisen et al., 2012) and several tumor models (Cortez-Retamozzo et al., 2012; Richards et al., 2013; Wu et al., 2018). We induced dysbiosis of the gut microbiome by administering a broad-spectrum antibiotic cocktail for a very short time period, compared with other studies (Kennedy et al., 2018), to identify the direct role of circulating PRR ligands on the maintenance of monocytes. Our findings delineate a new level of regulation by the microbiome in addition to the regulation of hematopoiesis, previously described in experiments with a 2- to 5-week exposure to antibiotics (Josefsdottir et al., 2017; Khosravi et al., 2014). Similar observations have been reported for neutrophils where the gut microbiome directly affects their function in blood in addition to their production in the BM (Zhang and Frenette, 2019). We conducted our experiments using female mice as the innate immune responses are generally stronger in females than in males (Klein and Flanagan, 2016) and the intestinal microbiota appears consistent throughout the estrous cycle (Wallace et al., 2018).

In our study, we identified the S24_7 and Rikenellaceae families (Bacteroidetes phylum); Clostridiaceae, Lachnospiraceae, Ruminococcaceae, and Mogibacteriaceae families (Firmicutes phylum); Mollicutes RF39 (Tenericutes phylum); and Bifidobacterium (Actinobacteria phylum) as the most susceptible taxa to our antibiotic treatment. This finding, in combination with the parallel reduction of PRR ligands in the circulation of treated animals, demonstrates that these bacterial families may be among the primary sources of PRR ligand production within the gut during steady state. Conversely, our data suggest that all the bacterial taxa that underwent significant expansion during the treatment, including Sutterella, Staphylococcus, and Akkermansia muciniphila, may not contribute significantly to the production of circulating PRR ligands. The simultaneous perturbation of the gut microbiota homeostasis and reduction of PRR ligands from the circulation of antibiotic-treated animals further supports the premise that the gut microbiome constitutively produces and secretes bacterial-derived products in the circulation, as previously shown in antibiotic, streptozotocin-treated mice (Thaiss et al., 2018). To our knowledge, our data report for the first time the simultaneous presence of TLR2, 3, 4, 5, 7, and 9 and NOD1 and NOD2 ligands at low levels in the sera of naive animals, supporting previous studies that have detected NOD1 (Clarke et al., 2010), NOD2 (Huang et al., 2019) and TLR2 (Farrokhi et al., 2013) ligands in the circulation of naive mice.
Reduction of PRR ligands from the circulation of antibiotic-treated mice was associated with perturbation of splenic but not BM-derived Ly6Chigh monocytes. This observation could be explained either by a differential regulation of spleen versus BM-derived monocytes by the gut microbiome or by a prompt replenishment of monocytes by progenitors within the BM of treated animals. Our data propose that the homeostasis of splenic Ly6Chigh monocytes does not depend alone on BM hematopoiesis effects as suggested earlier (Khosravi et al., 2014) but that it can also be impacted directly by signals from commensal bacteria. This apparent discrepancy could be due to the short-term antibiotic treatment we performed (4 days) versus the long-term treatment (4–5 weeks) performed by Khosravi et al.

Disruption of the monocyte homeostasis is microbiome-mediated because we did not observe similar changes in the numbers of splenic Ly6Chigh monocytes in antibiotic-treated GF animals. Perturbation was also related to increased apoptotic rate within the spleen further supporting that deprivation of gut microbiota-derived signals has an immediate impact on the homeostasis of splenic monocytes. We did not detect increased differentiation to DC/macrophages or increased trafficking of monocytes from the spleen to the bloodstream or to the gut of antibiotic-treated mice in response to increased bacterial cell death.

Splenic Ly6Chigh monocytes underwent transcriptional and functional changes after 3 days of antibiotic administration. Molecules involved in antigen presentation pathways (Ciita, H2-Aa, H2-Ab1, Cd74, Cd40, H2-DMa) as well as Stat1, Traf1, and Nkb2 were significantly downregulated, whereas Trem1, a...
proinflammatory factor; CD36, a scavenger receptor; and Camp, an antimicrobial peptide, were significantly upregulated. Functionally, Ly6C<sup>high</sup> monocytes from antibiotic-treated mice produced lower levels of proinflammatory cytokines and they had acquired increased phagocytic and antimicrobial abilities, hallmarks of endotoxin-tolerant monocytes (del Fresno et al., 2009). It could be that in the absence of circulating tonic signals, S100a8 and S100a9, two calcium-binding proteins, act as endogenous alarmins inducing a state of hyporesponsiveness to monocytes, similar to exogenous LPS (Austermann et al., 2014).

As dysbiosis of the gut microbiome can destabilize the whole metabolome signature in the circulation (Vennocchi et al., 2016), we addressed whether perturbation of the splenic Ly6C<sup>high</sup> monocyte numbers and function in antibiotic-treated mice is specifically regulated by the levels of circulating PRR ligands. We report that the NOD1 signaling pathway regulates the lifespan of splenic Ly6C<sup>high</sup> monocytes confirming findings from a previous study (Hergott et al., 2016). The presence of TLRL increased the phagocytic properties of Ly6C<sup>high</sup> cells, as has previously been described in cell lines in vitro (Doyle et al., 2004). Further studies need to address which TLRL, individually or in combination, has this regulatory role. Interestingly, our observation that the antimicrobial capacity of Ly6C<sup>high</sup> monocytes is regulated by TLRL and MDP is in agreement with previous studies suggesting enhancement of the antimicrobial capacity of phagocytes by TLRs (Brightbill et al., 1999) and MDP (O’Reilly and Zak, 1992). In terms of cytokine production, the levels of TNF-α were restored after injection of ie-DAP and MDP in LPS-stimulated Ly6C<sup>high</sup> monocytes, whereas any PRR ligand affected the production of IL-6. Our data demonstrate that individual circulating PRR ligands have pleiotropic effects on the immune functions of splenic Ly6C<sup>high</sup> monocytes. Further studies will be performed to dissect the underlying mechanisms of these complex interactions. Also, in our study, we have not addressed the impact of the gut dysbiosis on the levels of other microbial metabolites, such as SCFAs, β-glucans, and bile acids, known to affect the biology of monocytes (Fiorucci et al., 2018; van de Wouw et al., 2019).

Taken together, our findings suggest that the ecology of the intestinal microbiota can control the homeostasis and function of splenic Ly6C<sup>high</sup> monocytes during steady state via regulation of the levels of circulating PRR ligands. Identification of the PRR ligand-monocyte crosstalk provides an opportunity to develop intervention strategies to modulate splenic monocytes, an important immune cell subset in the pathogenesis of cardiovascular and CNS-related diseases.

Limitations of the study
In this study we demonstrate that circulating PRR ligands produced by the gut microbiome regulate the number and immune functions of splenic Ly6C<sup>high</sup> monocytes. Whether circulating PRR ligands act directly upon splenic Ly6C<sup>high</sup> monocytes or indirectly via other immune cells in vivo can be further investigated in future studies.

Resource availability
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Materials availability
This study did not generate new unique reagents.

Data and code availability
The raw data from the 16S rRNA sequencing library and the Nanostring data have been uploaded in Mendeley (https://doi.org/10.17632/tjd8jznzskr.1).

All datasets supporting the current study are available from the corresponding author on request.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.
SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102356.

ACKNOWLEDGMENTS

We thank Vladimir Yeliseyev for technical assistance with GF mouse experiments, Joseph Rone for technical assistance with bacterial cultures, Kit Fuhrman and Clement David for assistance with analysis of Nanostring data, Biopolymers Facility at Harvard Medical School for sequencing data, and Deneen E. Kozoriz and Rajesh K. Krishnan for sorting. The research was supported by NIH grant RO1# NS087226 to H.W.

AUTHOR CONTRIBUTIONS

Conceptualization, P.K. and H.L.W.; investigation, P.K., A.S., and V.W.; methodology, P.K., S.L., L.M.C., M.F., R.R., and D.G.; resources: S.L.; formal analysis, P.K., L.M.C., and V.W.; writing – original draft, P.K. and H.L.W.; writing – review & editing, P.K. and H.L.W.; funding acquisition, H.L.W.; supervision, H.L.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: December 30, 2020
Revised: February 17, 2021
Accepted: March 22, 2021
Published: April 23, 2021

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Supplemental information

Regulation of splenic monocyte homeostasis
and function by gut microbial products

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Figure S1. Alterations in the composition of the gut microbiome after a short-term antibiotic treatment. Related to Figure 1.
Principal coordinates analysis (PCoA) of the A) unweighted and B) weighted Unifrac distances of the 16S rRNA sequencing data as visualized by Emperor. The two groups are represented by colored circles (untreated mice) and squares (antibiotic-treated mice). Timepoints are shown in red (day 0), blue (day 2), and orange (day 4) color. Differences between the antibiotic-treated and untreated group were statistical significant as calculated by the permanova paired wise test.
Figure S2. Perturbation of splenic Ly6C\textsuperscript{high} monocyte homeostasis in antibiotic-treated mice is microbiome-mediated. Related to Figure 2 and 3.

SPF and GF mice were treated with or without the antibiotic cocktail for 4 days. On day 4, the frequency and absolute numbers of Ly6C\textsuperscript{high} monocytes were estimated by flow cytometry. A) Frequency and B) absolute number per mg of tissue of splenic Ly6C\textsuperscript{high} monocytes from antibiotic-treated and untreated SPF and GF mice. Data show the mean ± s.dev. (n=7-8 mice per group). Statistical significance was assessed by two-tailed student’s t test. * p < 0.05, ** p <0.01, *** p < 0.001; C) Volcano plot showing fold changes in gene expression in splenic Ly6C\textsuperscript{high} monocytes from antibiotic-treated vs untreated GF mice using the nCounter Nanostring Immunology panel. Each dot presents one gene while the color of the dot shows the significance in the change of gene expression. A fold change ≥ 1.5 (upregulation) and ≤ -1.5 (downregulation) was considered significant whereas p values were calculated by the nSolver analysis software. Blue dots represent genes with no statistically significant change in their expression whereas red dots show genes which expression levels were significantly affected by the antibiotic treatment.
Figure S3. Perturbation of the splenic Ly6C<sup>high</sup> monocyte homeostasis after gut dysbiosis is not related to increased differentiation to DC/macrophages or increased exit from the tissue. Related to Figure 2.

A) Frequency and absolute number of DC/macrophages in splenic cell suspensions of mice treated with or without antibiotics for 3 days. Data show mean ± s.dev. and they are representative of two experiments (n=6 per group). Statistical significance was assessed by Student’s t-test. P values are shown as *p<0.05, **p<0.01, ***p<0.001; B) Experimental design of intrasplenic transfer of CD45.1<sup>+</sup> splenic Ly6C<sup>high</sup> monocytes to CD45.2<sup>+</sup> C57BL/6 mice, pretreated with or without antibiotics for 3 days. Data show frequency of CD45.1<sup>+</sup> cells out of LIN<sup>−</sup> in splenic cell suspensions and blood six hours after cell transfer. Data show mean ± s.dev. and they are representative of two experiments (n=6 per group).
Figure S4. Short antibiotic treatment does not impair splenic Ly6C\textsuperscript{high} monocyte survival ex vivo. Related to Figure 3.

FACS-sorted splenic Ly6C\textsuperscript{high} monocytes from mice treated with or without antibiotics for 3 days were stimulated ex vivo with 100 ng/ml LPS. Twenty-four hours later, cells were stained with Annexin V and viability dye eFluor780 to enumerate the frequency of cell death by flow cytometry. Data show the frequency of viable (black bars), early apoptotic (white bars), late apoptotic (blue bars) and total apoptotic cells (red bars) in LPS-stimulated splenic Ly6C\textsuperscript{high} from antibiotic-treated and control animals. Data represent the mean ± s.dev. of technical duplicate wells.
### Supplemental Table 1. Relative abundance of taxa significantly reduced during antibiotic treatment. Related to Figure 1

| Phylum      | Class        | Order      | Family    | Genus           | D0       | D2       | D4       |
|-------------|--------------|------------|-----------|-----------------|----------|----------|----------|
| Bacteroidetes | Bacteroidia  | Bacteroidales | S24_7     | unclassified    | 40.7%*   | 1.01%    | 0.49%    |
| Bacteroidetes | Bacteroidia  | Bacteroidales | Rikenellaceae | unclassified    | 20.6%    | 2.58%    | 1.14%    |
| Firmicutes   | Clostridia   | Clostridiales | Clostridiaceae | unclassified    | 6.5%     | 8.34%    | 3.09%    |
| Firmicutes   | Clostridia   | Clostridiales | Lachnospiraceae | unclassified    | 3.1%     | 1.26%    | 0.33%    |
| Firmicutes   | Clostridia   | Clostridiales | Ruminococcaceae | Oscillospira    | 1.8%     | 1.58%    | 0.62%    |
| Tenericutes  | Mollicutes   | RF39       | unclassified | unclassified    | 1.5%     | 0.44%    | 0.29%    |
| Firmicutes   | Clostridia   | Clostridiales | Lachnospiraceae | Coprococcus    | 0.9%     | 0.21%    | 0.03%    |
| Firmicutes   | Clostridia   | Clostridiales | Ruminococcaceae | Ruminococcus   | 0.5%     | 0.16%    | 0.03%    |
| Firmicutes   | Clostridia   | Clostridiales | Lachnospiraceae | Roseburia      | 0.4%     | 0.00%    | 0.00%    |
| Firmicutes   | Clostridia   | Clostridiales | Ruminococcaceae | unclassified   | 0.3%     | 0.07%    | 0.03%    |
| Firmicutes   | Clostridia   | Clostridiales | Lachnospiraceae | Clostridium piliforme | 0.2%     | 0.00%    | 0.00%    |
| Firmicutes   | Clostridia   | Clostridiales | Ruminococcaceae | Anaerotruncus | 0.1%     | 0.03%    | 0.00%    |
| Firmicutes   | Clostridia   | Clostridiales | Mogibacteriaceae | unclassified   | 0.1%     | 0.03%    | 0.01%    |
| Firmicutes   | Erysipelotrichi | Erysipelotrichales | Erysipelotrichaceae | Allobaculum   | 0.1%     | 0.04%    | 0.01%    |
| Firmicutes   | Clostridia   | Clostridiales | Lachnospiraceae | Dorea          | 0.04%    | 0.01%    | 0.02%    |
| Firmicutes   | Clostridia   | Clostridiales | Lachnospiraceae | Anaerostipes   | 0.04%    | 0.00%    | 0.00%    |
| Actinobacteria | Actinobacteria | Bifidobacteriales | Bifidobacteriaceae | Bifidobacterium | 0.36%    | 0.07%    | 0.22%    |

*Data show mean relative abundance of taxa averaged from eight mice.*
Supplemental Table 2. Relative abundance of taxa significantly increased during antibiotic treatment. Related to Figure 1.

| Phylum              | Class                  | Order         | Family            | Genus                | D0  | D2   | D4   |
|---------------------|------------------------|---------------|-------------------|----------------------|-----|------|------|
| Proteobacteria      | Betaproteobacteria     | Burkholderiales | Alcaligenaceae    | Sutterella           | 0.83%<sup>a</sup> | 23.3% | 36.7% |
| Firmicutes          | Bacilli                | Bacillales    | Bacillaceae       | Staphylococcus       | 0   | 15.4%| 17.2%|
| Verrucomicrobia     | Verrucomicrobiae       | Verrucomicrobiales | Verrucomicrobiaceae | Akkermansia municiphila | 0.35% | 5.57% | 5.59% |
| Firmicutes          | Bacilli                | Lactobacillales | Streptococcaceae  | Streptococcus        | 0   | 0.26%| 2.42%|
| Firmicutes          | Bacilli                | Lactobacillales | Enterococcaceae   | Enterococcus         | 0   | 0.8% | 1.72%|
| Firmicutes          | Clostridia             | Clostridiales | Lachnospiraceae   | Ruminococcus gnavus  | 0.66% | 0.85%| 1.64%|
| Firmicutes          | Bacilli                | Lactobacillales | Leuconostocaceae  | unclassified         | 0   | 0.04%| 0.8% |
| Firmicutes          | Clostridia             | Clostridiales | Peptostreptococcaceae | Peptostreptococcus | 0   | 0.05%| 0.54%|
| Firmicutes          | Bacilli                | Lactobacillales | unclassified      | unclassified         | 0   | 0   | 0.48%|
| Firmicutes          | Bacilli                | Bacillales    | Bacillaceae       | Geobacillus vulcani  | 0   | 0.03%| 0.4% |
| Firmicutes          | Erysipelotrichi        | Erysipelotrichales | Erysipelotrichaceae | Coprobacillus       | 0.03% | 0.1% | 0.39%|
| Firmicutes          | Clostridia             | Clostridiales | Tissierellaceae   | Gallicola            | 0   | 0   | 0.26%|
| Firmicutes          | Clostridia             | Clostridiales | Tissierellaceae   | Anaerococcus         | 0   | 0.03%| 0.24%|
| Actinobacteria      | Actinomycetales        | Sanguibacteraceae | Sanguibacter      | Sanguibacter         | 0   | 0   | 0.13%|
| Actinobacteria      | Actinomycetales        | Corynebacteriae | Corynebacterium   | Corynebacterium      | 0   | 0   | 0.09%|
| Firmicutes          | Bacilli                | Bacillales    | Paenibacillaceae  | Paenibacillus        | 0   | 0   | 0.09%|
| Proteobacteria      | Alphaproteobacteria    | Rhizobiales   | Rhizobiacese      | Agrobacterium        | 0   | 0   | 0.09%|
| Firmicutes          | Clostridia             | Clostridiales | Clostridiae       | Clostridium          | 0.1% | 0.11%| 0.06%|
| Firmicutes          | Bacilli                | Bacillales    | unclassified      | unclassified         | 0   | 0   | 0.04%|
| Proteobacteria      | Gammaproteobacteria    | Enterobacteriales | Enterobacteriaceae | Erwinia              | 0   | 0   | 0.01%|

*Data show mean relative abundance of taxa averaged from eight mice.*
## TRANSPARENT METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti-mouse CD3-APC  | Biolegend | 100236; RRID: AB_2561456 |
| Anti-mouse CD3-PerCP-Cy5.5 | Biolegend | 100218; RRID: AB_1595492 |
| Anti-mouse B220-APC | Biolegend | 103212; RRID: AB_312997 |
| Anti-mouse NK1.1-APC | Biolegend | 108710; RRID: AB_313397 |
| Anti-mouse CD49b-APC | Biolegend | 108910; RRID: AB_313417 |
| Anti-mouse LY6G-APC | Biolegend | 127614; RRID: AB_2227348 |
| Anti-mouse Siglec-F-APC | Miltenyi Biotec | 130-102-241; RRID:AB_2653452 |
| Anti-mouse CD11c-BV421 | Biolegend | 117330; RRID: AB_11219593 |
| Anti-mouse CD11c-BV605 | Biolegend | 117334; RRID: AB_2562415 |
| Anti-mouse F4/80-BV421 | Biolegend | 123132; RRID: AB_11203717 |
| Anti-mouse I-A<sup>b</sup>-BV421 | BD Biosciences | 562928; RRID: AB_2737897 |
| Anti-mouse I-A<sup>b</sup>-APC/Fire™ 750 | Biolegend | 116424; RRID: AB_2721487 |
| Anti-mouse CD11b-PeCy7 | Biolegend | 101216; RRID: AB_312799 |
| Anti-mouse Ly6C-Alexa488 | Biolegend | 128022; RRID: AB_10639728 |
| Anti-mouse Ly6C-Brilliant Violent 785 | Biolegend | 128041; RRID: AB_2565852 |
| Anti-mouse CD115-PE | Biolegend | 128008; RRID: AB_1186132 |
| Anti-mouse CD45-PE | Biolegend | 135506; RRID: AB_1937253 |
| Anti-mouse CD45.1-BV711 | Biolegend | 110739; RRID: AB_1937253 |
| Anti-mouse CD45-BUV395 | BD Biosciences | 564279; RRID: AB_2651134 |
| Fixable Viability Dye eFluor™ 506 | ThermoFisher Scientific | 65-0866-18 |
| Fixable Viability Dye eFluor™ 660 | ThermoFisher Scientific | 65-0864-18 |
| Fixable Viability Dye eFluor™ 780 | ThermoFisher Scientific | 65-0865-18 |
| **Bacterial Strains** |        |            |
| *Citrobacter rodentium* | ATCC | 51459 |
| *Escherichia coli, strain K12* | The Coli Genetic Stock Center at Yale | CGSC#7296 |
| **Cell lines** |        |            |
| HEK-Blue mTLR2 | Invivogen | hkb-mtlr2 |
| HEK-Blue mTLR3 | Invivogen | hkb-mtlr3 |
| HEK-Blue mTLR4 | Invivogen | hkb-mtlr4 |
| HEK-Blue mTLR5 | Invivogen | hkb-mtlr5 |
| HEK-Blue mTLR7 | Invivogen | hkb-mtlr7 |
| HEK-Blue mTLR9 | Invivogen | hkb-mtlr9 |
| HEK-Blue mNOD1 | Invivogen | hkb-mnod1 |
| HEK-Blue mNOD2 | Invivogen | hkb-mnod2 |
| HEK-Blue Null1 | Invivogen | hkb-null1 |
| HEK-Blue Null1-k | Invivogen | hkb-null1k |
| HEK-Blue Null1-v | Invivogen | hkb-null1v |
| Chemicals, peptides and enzymes                  |                 |                 |
|-------------------------------------------------|-----------------|-----------------|
| Histopaque-1077                                  | Sigma           | 10771           |
| Ampicillin trihydrate                            | Sigma           | A6140           |
| Buprenorphine SR                                 | Wildlife Pharmaceuticals | [link](https://wildpharm.com/medications/labanimals/item/3-buprenorphine-sr-1ml.html) |
| Meloxicam SR                                     | Wildlife Pharmaceuticals | [link](https://wildpharm.com/meloxicamsr5mllab.html) |
| Metronidazole                                    | Sigma           | M1547           |
| Neomycin trisulfate salt hydrate                 | Sigma           | N1876           |
| Vancomycin HCL                                   | Research Products International | V06500-5.0 |
| Gentamicin reagent solution                      | ThermoFisher Scientific | 15750-060 |

| Commercial assays                                 |                 |                 |
|-------------------------------------------------|-----------------|-----------------|
| eBioscience™ Annexin V Apoptosis Detection Kit APC | ThermoFisher Scientific | 88-8007-74 |
| FAM-FLIVO® In vivo Polycaspase Assay             | ImmunoChemistry Technologies, LLC | 981 |
| Legendplex Mouse Inflammation Panel             | BioLegend       | 740446          |
| Mouse TNF-α Duoset ELISA kit                    | R&D Systems     | DY410-05        |
| Mouse IL-6 Duoset ELISA kit                     | R&D Systems     | DY406-05        |
| Mouse IL-10 Duoset ELISA kit                    | R&D Systems     | DY417-05        |
| Mouse IL-1α/IL-1F1 Duoset ELISA kit             | R&D Systems     | DY400-05        |
| Mouse IL-1β/IL-1F2 Duoset ELISA kit             | R&D Systems     | DY401-05        |

| PRR ligands                                      |                 |                 |
|-------------------------------------------------|-----------------|-----------------|
| Pam2CSK4                                         | Invivogen       | trlr-pm2s-1     |
| Poly (I:C) HMW                                    | Invivogen       | trlr-pic        |
| LPS, *E. coli* O55:B5                            | Sigma           | L4524           |
| FLA-ST                                           | Invivogen       | trlr-stfla      |
| CL264                                            | Invivogen       | trlr-c264e-5    |
| ODN1826                                          | Invivogen       | trlr-1826-1     |
| iE-DAP                                           | Invivogen       | trlr-dap        |
| MDP                                              | Invivogen       | trlr-mdp        |

| Software                                         |                 |                 |
|-------------------------------------------------|-----------------|-----------------|
| FlowJo v.10.5.3.                                 | FlowJo, LLC     | https://www.flowjo.com |
| BioRender                                        | Biorender.com   |                 |
| GraphPad Prism v.8.2.0.                          | Prism-graphpad.com | https://www.graphpad.com/scientific-software/prism/ |
| Metacore                                         | Clarivate Analytics | https://portalгенего.com/ |
| LefSE                                            |                 | https://huttenhower.sph.harvard.edu/galaxy/ |
## Taqman Probes

|                | Manufacturer        | Catalogue Number          |
|----------------|----------------------|---------------------------|
| Mouse IL-1α    | Applied Biosystems   | Mm00439620_m1             |
| Mouse IL-1β    | Applied Biosystems   | Mm00434228_m1             |
| Mouse IL-6     | Applied Biosystems   | Mm00446190_m1             |
| Mouse IL-10    | Applied Biosystems   | Mm01288386_m1             |
| Mouse TNF-α    | Applied Biosystems   | Mm00443258_m1             |
| Mouse Trem1    | Applied Biosystems   | Mm01278455_m1             |
| Mouse S100a8   | Applied Biosystems   | Mm00496696_g1             |
| Mouse S100a9   | Applied Biosystems   | Mm00656925_m1             |
| Mouse CD36     | Applied Biosystems   | Mm00432403_m1             |
| Mouse Camp     | Applied Biosystems   | Mm00438285_m1             |
| Mouse Ciita    | Applied Biosystems   | Mm00482914_m1             |
| Mouse Gapdh    | Applied Biosystems   | Mm99999915_g1             |

## Others

| Product Description                              | Manufacturer     | Catalogue Number |
|-------------------------------------------------|------------------|------------------|
| Fluoresbrite® Yellow Green Microspheres, 0.5 micrometer | Polysciences, Inc | 17152-10        |

## Deposited data

- Mendeley data: [http://dx.doi.org/10.17632/tjdwzskr.1](http://dx.doi.org/10.17632/tjdwzskr.1)

## Animals

Female C57BL/6 (stock number 000664) and B6 CD45.1 (stock number 002014) mice were purchased from The Jackson Laboratory and were used at 6-8 weeks of age. All animals were kept in a pathogen-free facility at Brigham and Women’s Hospital (BWH) in accordance with the animal protocol guidelines prescribed by the Institutional Animal Care and Use Committee of BWH. Germ-free C57BL/6 mice were bred and raised in gnotobiotic isolators at Massachusetts Host-Microbiome Center at BWH. Mice were housed in at least two cages per group to minimize cage-related effects.

## Antibiotic treatment

SPF and GF mice were treated with a broad-spectrum antibiotic cocktail consisting of vancomycin (0.5 g/L), ampicillin (1 g/L), neomycin (1 g/L) and metronizadole (1g/L) dissolved in drinking water. SPF mice received the antibiotic-containing water ad libitum for
2-6 days in all experiments. The antibiotic cocktail was administered by oral gavage for 4 days only in GF and control SPF animals to avoid signs of dehydration seen in GF mice treated with antibiotic-containing water ad libitum. All antibiotics were purchased from Sigma except vancomycin (Research Products International).

**Preparation of single-cell suspensions from blood, spleen and bone marrow.**

Blood was isolated from the facial vein of mice using a 5 mm goldenrod lancet (VWR) and collected in BD Microtainer tubes with lithium heparin (Becton Dickinson). Heparinized-blood was loaded onto Histopaque-1077 (Sigma) and centrifuged for 20 minutes at 900 x g, at 25° C. The interphase containing blood mononuclear cells was collected and stained for monocyte subsets. Single-cell suspensions from spleens were prepared by mechanical disruption using a 70-μm-cell strainer/nylon mesh (Fisher Scientific). Perfusion was not performed before isolation of spleens as the number of contaminating blood monocytes is negligible (Swirski et al., 2009). Bone marrow cells were isolated from both femurs and tibias of animals after centrifugation of bones at 14,000 rpm for 15 seconds. Red blood cells were eliminated from splenic and bone marrow cell suspensions using the ACK lysing buffer (Lonza). Viability and total cell numbers were determined upon staining with Trypan Blue (GE Healthcare Life Sciences) using a hemocytometer.

**Flow cytometry and cell-sorting**

Single-cell suspensions from blood, spleen and bone marrow were stained with an anti-CD16/32 antibody (BioXCell) to block Fc receptors for 15 minutes on ice. Afterwards, the antibodies described in Key Resources Table were used to stain cell suspensions. Live
monocytes were identified as CD11b<sup>high</sup> LIN (eFluor660, CD3, B220, NK1.1, CD49b, Ly6G, Siglec-F<sup>-</sup>) (F4/80, CD11c, I-Ab<sup>low</sup>) and Ly6C<sup>high</sup>. Macrophages/dendritic cells were identified as CD11b<sup>high</sup> LIN (eFluor660, CD3, B220, NK1.1, CD49b, Ly6G, Siglec-F<sup>-</sup>) (F4/80, CD11c, I-Ab<sup>high</sup>) and Ly6C<sup>low</sup>. Monocytes/macrophages/dendritic cell numbers were calculated by multiplying the number of total cells with the percent of the respective gate. To calculate the absolute cell number within the splenic tissue, total monocyte number was normalized to weight (mg) of spleen. Data were acquired on a BD LSRII and BD LSRFortessa (BD Biosciences) and analyzed with FlowJo v.10.5.3. (FlowJo, LLC). For cell sorting, splenic and bone marrow suspensions were pre-enriched in CD11b<sup>+</sup> cells using the CD11b Microbeads (Miltenyi Biotec) and then stained with the antibodies for sorting. Cells were sorted in a BD FACSARia II (BD Biosciences) using a 100 μm nozzle at 20 psi.

**Quantitative real-time PCR (Q-PCR)**

FACS-sorted splenic Ly6C<sup>high</sup> monocytes were lysed in RLT buffer and RNA was extracted using the RNeasy® Micro Kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized by the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). qPCR was performed using Taqman Universal PCR Master Mix and Taqman Gene Expression Assays (Applied Biosystems) in technical duplicate wells. All primers and probes were from Applied Biosystems as described in Key Resources Table. All assays were performed using a ViiA7 system (Applied Biosystems). Results were normalized to the housekeeping gene Gapdh. The relative expression of each target gene was expressed as (2<sup>-ΔCt</sup>) (Livak et al., 2001), where ΔCt is the difference between the mean Ct of the target gene and the mean Ct of Gapdh. Gene expression of Gapdh was stable between the
antibiotic-treated and control groups.

**nCounter gene expression and analysis**

FACS-sorted splenic Ly6C<sub>high</sub> monocytes from experimental and control groups were lysed in RLT buffer and cell lysates were hybridized with reporter and capture probes for nCounter Gene Expression code sets (Mouse Immunology Panel), according to the manufacturer's instructions (Nanostring Technologies). Analysis of expression of 561 genes was performed using the nSolver analysis software, version 4.0.62 (Nanostring Technologies). Data were normalized to spiked positive controls and housekeeping genes. Transcript counts less than 50 were excluded from the analysis as they were considered as background.

**Metacore analysis**

Data were analyzed using the Metacore software. Differentially expressed genes (with corresponding fold-changes and p values) were uploaded and analyzed by the Enrichment Analysis Tool as well as the Interactome Tool to generate biological networks and predict the top ten transcription factors involved in the regulation of the differentially expressed genes.

**Adoptive transfer of splenic monocytes**

Splenic Ly6C<sub>high</sub> monocytes were transferred to recipient mice intrasplenically. Recipient CD45.2 mice were administered water with or without antibiotics for 3 days. Before cell transfer, recipient mice were s.c. injected with 0.6 mg/kg buprenorphine SR and 4 mg/kg
meloxicam SR solutions to minimize post-operative pain (both from Wildlife Pharmaceuticals) and shaved on the middle left side of the abdomen. Then, a skin incision between the last rib and hip joint was made and \(5 \times 10^4\) FACS-sorted splenic Ly6C\(^\text{high}\) monocytes from CD45.1 mice, resuspended in 20 μl sterile PBS, were injected in the subcapsular layer of the spleens of CD45.2 mice. Next, the tissue was sutured using 4.0 chromic gut absorbable sutures. Six hours after the transfer, cell suspensions from blood and spleens of the recipient mice were prepared, stained for the presence of CD45.1\(^+\) monocytes and analyzed by flow cytometry.

**In vivo phagocytosis**

Mice were treated with or without antibiotics for three days. On day three, animals underwent the surgical process described above. Once, spleen was exteriorized, we injected 20 μl of Fluoresbrite Yellow Green Microspheres (0.5 μm; Polysciences) per mouse. Next, the tissue was sutured using 4.0 chromic gut absorbable sutures. Twenty-four hours later, splenic single-cell suspensions were prepared and the frequency of fluorescently labeled splenic Ly6C\(^\text{high}\) monocytes was quantified by flow cytometry.

**Detection of cell death ex vivo**

FACS-sorted splenic Ly6C\(^\text{high}\) monocytes were stimulated with 100 ng/ml LPS for 24 hours. Then, cells were stained with the eBioscience Annexin V apoptosis detection kit and eBioscience Fixable viability dye eFluor780 (all from ThermoFisher Scientific), according to the manufacturer’s instructions. Early apoptotic cells were considered as Annexin V\(^+\) eFluor780\(^-\) whereas late apoptotic cells were Annexin V\(^+\) eFluor780\(^+\). Necrotic cells,
Annexin V<sup>-</sup> eFluor780<sup>+</sup>, were not detected. For determining the apoptotic rate of monocytes from antibiotic-treated mice, animals were administered water with or without antibiotics for 3 days. On day 3, splenic cell suspensions were incubated with FAM-FLIVO for 60 minutes at 37° C for 60 minutes. Cells were washed twice, fixed and analyzed by flow cytometry.

**Ligand injections**

Naïve mice were administered water with or without antibiotics for three days. One and two days after initiation of the antibiotic treatment, mice were injected i.p. with ie-DAP (25 μg/mouse) or MDP (25 μg/mouse). Another group of mice received a TLR ligand cocktail consisting of Pam2CSK4 (5 μg/mouse), LPS (5 μg/mouse), CL264 (5 μg/mouse), ODN1826 (5 μg/mouse) and FLAST (1 μg/mouse) only once, one day after initiation of the antibiotics.

**Gentamicin Protection assay**

FACS-sorted splenic Ly6C<sup>high</sup> monocytes were incubated for 2 hours with *C. rodentium* or *E. coli*, K12 strain, at a multiplicity of infection (MOI) of 20. Next, cells were washed to remove extracellular bacteria and further treated with gentamicin for 1 hour. Then, supernatant was collected to ensure the absence of live extracellular bacteria within the wells and cells were lysed in 1% triton buffer. Serial dilutions of the cell lysates and supernatants were plated on agar plates in triplicates and the number of colonies was counted after overnight incubation at 37° C. Results are presented as absolute CFU counts.
Detection of cytokines in the supernatant

FACS-sorted splenic Ly6C<sup>high</sup> monocytes from antibiotic-treated and untreated mice were stimulated with or without 100 ng/ml LPS (from E. coli O55:B5, Sigma) for 24 hours. Cell-free culture supernatants were examined for the presence of cytokines using the Legendplex Mouse Inflammation Panel (Biolegend). Quantification of TNF-α, IL-6, IL-1α, IL-1β and IL-10 in samples was also assessed by ELISA kits (DuoSet Kits, R&D Systems), according to the manufacturer's instructions.

Detection of bacterial products in murine sera

C57BL/6 mice were treated with or without antibiotics for 4 days and sera were isolated from both groups before as well as 2 and 4 days after initiation of the treatment. To assess the presence of bacterial products, sera from all time points were incubated with the HEK-Blue TLR2, TLR3, TLR4, TLR5, TLR7, TLR9, NOD1, NOD2 as well as the control cell lines which lack the corresponding PRR (all from Invivogen). Briefly, HEK293 cells stably co-express murine TLRs and an NF-κB/AP-1 inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene. In the presence of TLR agonists, activation of NF-κB/AP-1 leads to SEAP secretion in the cell supernatant. Addition of a specific SEAP color substrate in the wells produces a purple/blue color that was measured at 620 nm using a microplate reader. The HEK-Blue cell lines were incubated with HEK-Blue detection medium for sixteen hours before measuring the O.D., according to the manufacturer's instructions.
**Microbial Quantification by qPCR**

Bacterial DNA was extracted from fecal samples using the DNeasy Powerlyzer PowerSoil kit (Qiagen), according to the manufacturer’s instructions. qPCR analysis was performed in duplicate wells by Taqman amplification reactions consisting of genomic DNA, Taqman® Universal PCR Master Mix (Applied Biosystems) and the following primer pair: Forward primer: TCCTACGGGAGGCAGCAGT, Reverse primer: GGACTACCAGGGTATCTAATCCTGTT, Probe (FAM): CGTATTACCGCGGCTGCTGGCAC (Nadkarni et al., 2002). Relative expression of the 16S gene was calculated based on the expression of the gene before treatment in each mouse. Relative expression was normalized to mg of fecal sample.

**16s rRNA sequencing and taxonomic microbiota analysis**

Fecal DNA was isolated as described above. Amplicons spanning variable region 4 (V4) of the 16S rRNA gene were generated with barcode containing primers and sequenced on Illumina MiSeq. Data processing was performed using an established protocol (Caporaso et al., 2010) using the Qiime software (version 2018.11). Shannon’s index and observed OTUs were used to measure alpha diversity whereas beta diversity was expressed as weighted and unweighted UniFrac distance. A Principal coordinates analysis (PCoA) plot was generated to visualize the similarity of the community members. The LefSe algorithm (Segata et al., 2011) was used to identify differentially abundant features that are statistically different among different groups.
QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical data analysis was performed with Prism software version 8.0 (GraphPad Software). For comparisons of two groups, two-tailed Student’s t-test was used. Comparisons of multiple groups were made using one-way ANOVA, as described in the figure legends. Data represent mean ± standard error of the mean (SEM) or mean ± standard deviation (s.dev.), as indicated in the figure legend. P < 0.05 was considered significant.

SUPPLEMENTAL REFERENCES

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