The microbiota regulates neutrophil homeostasis and host resistance to *Escherichia coli* K1 sepsis in neonatal mice

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Neonatal colonization by microbes, which begins immediately after birth, is influenced by gestational age and the mother’s microbiota and is modified by exposure to antibiotics1. In neonates, prolonged duration of antibiotic therapy is associated with increased risk of late-onset sepsis (LOS)2, a disorder controlled by neutrophils3. A role for the microbiota in regulating neutrophil development and susceptibility to sepsis in the neonate remains unclear. We exposed pregnant mouse dams to antibiotics in drinking water to limit transfer of maternal microbes to the neonates. Antibiotic exposure of dams decreased the total number and composition of microbes in the intestine of the neonates. This was associated with decreased numbers of circulating and bone marrow neutrophils and granulocyte/macrophage–restricted progenitor cells in the bone marrow of antibiotic-treated and germ-free neonates. Antibiotic exposure of dams reduced the number of interleukin-17 (IL-17)–producing cells in the intestine and production of granulocyte colony–stimulating factor (G-CSF). Granulocytopenia was associated with impaired host defense and increased susceptibility to *Escherichia coli* K1 and *Klebsiella pneumoniae* sepsis in antibiotic-treated neonates, which could be partially reversed by administration of G-CSF. Transfer of a normal microbiota into antibiotic-treated neonates induced IL-17 production by group 3 innate lymphoid cells (ILCs) in the intestine, increasing plasma G-CSF levels and neutrophil numbers in a Toll-like receptor 4 (TLR4)– and myeloid differentiation factor 88 (MyD88)–dependent manner and restored IL-17–dependent resistance to sepsis. Specific depletion of ILCs prevented IL-17– and G-CSF–dependent granulocytosis and resistance to sepsis. These data support a role for the intestinal microbiota in regulation of granulocytosis, neutrophil homeostasis and host resistance to sepsis in neonates.

Antibiotic exposure near the time of birth reduces the diversity and composition of intestinal microbiota and delays the appearance of beneficial bacteria in children4; such alterations are associated with the development of rheumatoid arthritis, inflammatory bowel disease and obesity5. In neonates, prolonged duration of antibiotic therapy is associated with increased risk of neonatal LOS6. Although a role for the microbiota in neonatal LOS has been proposed6, the mechanisms involved are not understood. The intestinal microbiome undergoes dynamic changes during the neonatal period7, and this change is associated with functional development of the immune system8. To ascertain the role of the microbiota in susceptibility of neonates to LOS, we exposed pregnant dams to ampicillin, gentamicin, vancomycin, metronidazole and neomycin in their drinking water beginning 5 d before delivery. The dams and the neonatal mice continued to receive antibiotics after birth. Thus, neonatal antibiotic exposure refers to antibiotic exposure both in utero and after birth. Antibiotic exposure not only reduced the total number of intestinal microbes but also modified the composition of the intestinal microbiota in neonatal mice (Fig. 1a,b). In untreated mice, Gammaproteobacteria transiently dominated the intestinal microbiota in postnatal day 3 mice. Bacilli and Clostridia were the predominant classes in postnatal day 5–14 mice, similar to patterns seen in human neonates9, whereas Bacteroidia were more prominent by day 14. Perinatal antibiotic exposure not only abolished the appearance of Gammaproteobacteria on day 3 but also prevented colonization by Bacilli on day 14 (Supplementary Fig. 1a,b). These findings were associated with simplification of the intestinal microbiota in antibiotic-exposed neonatal mice (Supplementary Fig. 1c–f), consistent with observations that antibiotics decrease the diversity of the intestinal microbiota in human neonates10. Ampicillin, gentamicin and vancomycin are the most commonly used antibiotics in pregnant mothers and neonates11. Therefore, we confirmed these observations by exposing pregnant dams to the clinically relevant combination of ampicillin, gentamicin and vancomycin in their drinking water. This three-antibiotic regimen similarly reduced the total number of intestinal microbes in neonatal mice (Fig. 1a) and decreased the abundance of Gammaproteobacteria on day 3 and Bacteroidia on day 14, recapitulating the altered microbial composition we observed in neonatal mice exposed to five antibiotics (Supplementary Fig. 1a,b).

Human neonates demonstrate increased circulating neutrophils 24–72 h after birth, which is temporally associated with exposure to

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and colonization by microbes. We observed a marked increase in the number of circulating neutrophils in neonatal mice (postnatal days 1–3) approaching adult values by postnatal day 14. Perinatal exposure to either the combination of ampicillin, gentamicin, vancomycin, metronidazole and neomycin or the more clinically relevant combination of ampicillin, gentamicin and vancomycin abrogated

![Image 1](https://example.com/image1.png)

**Figure 1** Perinatal antibiotic exposure alters the pattern of microbial colonization in the intestine and attenuates postnatal granulocytosis. (a) 16S ribosomal DNA (rDNA) copy numbers from the intestinal contents of neonatal mice exposed to a combination of three (3 ABX) or five (5 ABX) antibiotics or no antibiotics (No ABX), as determined using real-time PCR. n = 10 for all groups. NS, not significant. (b) Relative abundance of phylum- and class-level commensal bacteria obtained from 16S rDNA pyrosequencing of the intestinal contents of age- and sex-matched neonatal mice exposed to five antibiotics (ABX) or no antibiotics. Each bar represents the pooled intestinal contents from 9 age-defined neonatal mice from 4 different litters. (c,d) Numbers of circulating (c) and bone marrow (d) neutrophils in age- and sex-matched neonatal mice exposed to three or five antibiotics or no antibiotics. n = 12 for all groups. (e) Hematopoietic stem cells (Lin−Sca-1+c-Kit+) and granulocyte/macrophage-restricted progenitor cells (Lin−Sca-1+c-Kit+CD16/32+CD34+) in bone marrow from age- and sex-matched neonatal mice exposed to three antibiotics (ABX) or no antibiotics. Flow cytometry plots are gated on live cells. Representative histograms from 3 separate experiments. n = 12 for all groups. (f) Number of hematopoietic stem cells and lineage-committed progenitor cells in bone marrow from age- and sex-matched neonatal mice exposed to three antibiotics (ABX) or no antibiotics. n = 6 for all groups. (g) Plasma G-CSF levels in age- and sex-matched neonatal mice exposed to three or five antibiotics or no antibiotics. n = 12 for all groups. (h–j) Number of circulating (h) and bone marrow neutrophils (i) and plasma G-CSF levels (j) in age-matched GF and CNV mice and neonatal mice exposed to five antibiotics (ABX). Data are representative of three independent experiments containing 10 mice per group. Results are shown as the means ± s.e.m. Student’s t-test was used to determine statistical significance.
this postnatal granulocytosis in the early neonatal period (Fig. 1c) as compared to age-matched controls. Premature birth and low birth weight strongly correlate with neutropenia in neonates, and therefore we assessed the effect of perinatal antibiotics on gestational age and birth weight. Antibiotic exposure did not significantly change the gestational age, birth weight or postnatal growth in neonatal mice (Supplementary Fig. 1g, h).

The reduction in postnatal granulocytosis in the early neonatal period in antibiotic-exposed mice was accompanied by decreased numbers of bone marrow neutrophils (Ly6G+ cells) (Fig. 1d), decreased numbers of granulocyte/macrophage–restricted progenitor (Lin−Sca-1−c-Kit+CD16/32−CD34+) cells (Fig. 1e,f and Supplementary Fig. 1i) and decreased levels of plasma G-CSF (Fig. 1g), suggesting impaired granulopoiesis in antibiotic-exposed neonatal mice. To determine whether lack of postnatal granulocytosis might reflect antibiotic toxicity, we used germ-free (GF) mice, which have a minimal microbiota. GF mice lack of postnatal granulocytosis might reflect antibiotic toxicity, we used germ-free (GF) mice, which have a minimal microbiota. GF mice used germ-free (GF) mice, which have a minimal microbiota. GF mice

It is unclear whether neutrophils from antibiotic-exposed neonatal mice have distinct phenotypic and functional profiles as compared to those in neonatal mice not exposed to antibiotics (control mice). CXCXR2 and CXCR4 control the release of neutrophils from the bone marrow and their subsequent homing. Surface expression of CXCR2 was lower in neutrophils from antibiotic-exposed neonatal mice, whereas expression of CD11b and CD54, cell surface proteins involved in surface adhesion and transmigration (Supplementary Fig. 1j,k), was unchanged. Phagocytic ability and reactive oxygen species production were not significantly different in neutrophils from antibiotic-exposed neonatal mice as compared to control mice (Supplementary Fig. 1l).

Figure 2 Microbiota regulates postnatal granulocytosis and controls host resistance to E. coli. (a) Survival of 5-d-old sex-matched neonatal mice exposed to three or five antibiotics inoculated with E. coli (1 × 10^4 CFU g⁻¹ on day 5) via the intraperitoneal route. n = 10 mice for all groups, Kaplan-Meier log-rank test. (b-d) Circulating neutrophil counts (b,c) or plasma G-CSF levels (d) in 5-d-old sex-matched neonatal mice exposed to three (c,d) or five (b,d) antibiotics or no antibiotics 4 h after inoculation with E. coli. n = 12 for all groups. NS, not significant. Student’s t-test was used to determine statistical significance. (e,f) Survival of 5-d-old sex-matched neonatal mice exposed to five (e) or three (f) antibiotics or no antibiotics and treated with G-CSF after inoculation with E. coli via the intraperitoneal route (1 × 10^4 CFU g⁻¹ on day 5). *Significantly different from neonatal mice not exposed to antibiotics; **significantly different from antibiotic-exposed neonatal mice; n = 12 per group, Kaplan-Meier log-rank test. (g,h) Survival of neonatal mice exposed to five antibiotics (g) or three antibiotics (h) that received intestinal contents from postnatal 3-d-old sex-matched control mice (not exposed to antibiotics) via oral gavage on postnatal day 3 and that were infected with E. coli (1 × 10^4 CFU g⁻¹ on day 5). Survival was assessed 48 h following transfer (postnatal day 5). n = 12 per group, Kaplan-Meier log-rank test. (i,j) Circulating (i) and bone marrow (j) neutrophil counts 48 h following transfer (postnatal day 5) of intestinal contents from 3-d-old control mice (not exposed to antibiotics) via oral gavage on postnatal day 3 to sex-matched neonatal mice exposed to five antibiotics (ABX). Results are shown as the means ± s.e.m. *Significantly different from control (no ABX) neonatal mice, **significantly different from antibiotic-exposed neonatal mice; n = 12 per group, Student’s t-test.
Neutrophils are essential in controlling E. coli serotype K1 infection, a leading cause of LOS and meningitis in human neonates and the leading cause of death in preterm infants. Neutropenia is an important risk factor in fatal neonatal sepsis. To test whether antibiotic exposure modified host defense against infection, we exposed neonatal mice (3, 5, 7, or 14 d old) to the combination of five antibiotics or the more clinically relevant combination of ampicillin, gentamicin and vancomycin through their dams beginning 5 d before birth and continuing until day 14. Antibiotic-exposed mice or age-matched (not exposed to antibiotics) controls were inoculated intraperitoneally with 1 x 10^4 colony-forming units (CFU) per g body weight (CFU g^{-1}) of E. coli K1 or 1 x 10^6 CFU g^{-1} of K. pneumoniae, another important Gram-negative pathogen in neonates. Neonatal mice exposed to the five-antibiotic combination or to the three-antibiotic combination demonstrated increased susceptibility to E. coli K1 as compared to controls (median survival 8 h or 10 h, respectively, versus >72 h for control mice) (Fig. 2a and Supplementary Fig. 2a,b), with increased bacteria in blood, spleen and peritoneal fluid, indicating bacteremia and decreased neutrophil recruitment in the peritoneal fluid as compared to age-matched controls (Supplementary Fig. 2c-e). Similarly, antibiotic-exposed neonatal mice at postnatal day 5 demonstrated increased susceptibility to K. pneumoniae as compared to controls (median survival 14 h versus >72 h) (Supplementary Fig. 2f).

Rapidly responding ‘emergency’ granulocytosis is necessary for sustained output of circulating neutrophils during infection. To determine whether antibiotic exposure alters granulocytosis in response to infection, we measured the number of circulating and bone marrow neutrophils in neonatal mice exposed to either the five- or three-antibiotic protocol and age-matched (no antibiotic-exposed) controls. Treatment in antibiotic-exposed age- and strain-matched controls. Treatment of neonatal mice with an anti–IL-17A blocking antibody resulted in lower circulating neutrophil numbers than those found in neonatal mice treated with isotype control antibody (Supplementary Fig. 3b) and blocked the increase in circulating and bone marrow neutrophils (Fig. 3e,f) and plasma G-CSF (Supplementary Fig. 3c) in antibiotic-exposed neonatal mice after transfer of normal intestinal microbiota. These data confirm that intestinal microbiota regulates postnatal granulocytosis via IL-17A.

IL-17 is produced by various lymphocyte subtypes and by other cells residing in the intestinal lamina propria, suggesting that intestine is a key source of IL-17 production in basal conditions. The number of IL-17-producing cells was lower in the intestines of antibiotic-exposed neonatal mice as compared to age- and sex-matched control mice (Fig. 3g and Supplementary Fig. 3d). The exact identity of the IL-17-producing cells in neonatal intestine is not known. CD4+ T cells are important sources of IL-17 in adult mice, but circulating and bone marrow neutrophil numbers were not diminished in neonatal Rag1−/− mice (Supplementary Fig. 3e,f), which lack mature T and B cells, suggesting that mature T cells are dispensable in regulation of postnatal granulocytosis in neonatal mice. In contrast to IL-17-producing mature T cells, which express T cell receptor β (TCRβ) or γδ (TCRγδ), IL-17–producing innate lymphoid cells can express natural killer cell p46-related protein (NKP46) or RAR-related orphan receptor γ (RORγt). IL-17–producing cells in the lamina propria of neonatal mice were either CD4+ TCRβ−TCRγδ+ (10%) or CD4+ TCRβ−TCRγδ−RORγt NKp46+ (55%) or CD4+ TCRβ+TCRγδ+RORγt NKp46− (35%) (Fig. 3h), suggesting that the majority of the IL-17–producing cells in the neonatal small intestine are group 3 ILCs, which include lymphoid tissue inducer cells and natural cytotoxicity receptor (NCR)-negative ILCs. ILCs are present in increased numbers at mucusal sites and play an important part in maintaining intestinal homeostasis. To confirm the role of ILCs, we administered anti-CD90.2 antibody to neonatal Rag1−/− mice to deplete the ILC populations. The numbers of circulating and bone marrow...
neutrophils were lower in Rag1−/− mice treated with anti-CD90.2 (Thy1.2) antibody as compared to mice treated with isotype control antibody (Fig. 3i,j). These data taken together suggest that the intestinal microbiota controls postnatal granulocytosis by inducing a population of ILCs.

IL-23, secreted by macrophages and dendritic cells in the intestine in response to microbial products, has been shown to induce IL-17 production by CD4+ TCRβ+ TCRγδ+ cells and ILCs in mouse models of experimental colitis, but the numbers of circulating and bone marrow neutrophils were not diminished in neonatal Il23−/− mice (Supplementary Fig. 3g), suggesting that IL-23 is dispensable in regulation of postnatal granulocytosis in neonatal mice.

As both commensal and pathogenic bacteria can be recognized by a number of pattern recognition receptors (PRRs)29, we hypothesized that signals derived from the intestinal microbiota could regulate postnatal granulocytosis through signaling via Toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD) family members. We examined the postnatal granulocytic response of mice
deficient in TLR2 (Tlr2<sup>-/-</sup>), TLR4 (Tlr4<sup>-/-</sup>), NOD2 (Nod2<sup>-/-</sup>) and MyD88 (Myd88<sup>-/-</sup>), the latter of which is a crucial adaptor molecule that regulates signaling through multiple TLRs. The number of circulating and bone marrow neutrophils in neonatal Tlr4<sup>-/-</sup> and Myd88<sup>-/-</sup> mice (Fig. 4a–d), but not Tlr2<sup>-/-</sup> or Nod2<sup>-/-</sup> mice (Supplementary Fig. 4a–d), was lower as compared to in age- and strain-matched controls, consistent with previous observations that commensal detection by PRRs and subsequent signaling via MyD88 promotes systemic immune responses. The composition of the intestinal microbiota differs between Tlr4<sup>-/-</sup> and wild-type mice; therefore, to control for potential differences in the microbiota, we blocked TLR4 signaling in wild-type neonatal mice with a neutralizing antibody. Treatment of neonatal mice with anti-TLR4 neutralizing antibody resulted in decreased number of circulating and bone marrow neutrophils compared to neonatal mice treated with isotype control antibody (Supplementary Fig. 4e,f). Nevertheless, Tlr4<sup>-/-</sup> mice, mice treated with anti-TLR4 neutralizing antibody and Myd88<sup>-/-</sup> mice failed to completely recapitulate the phenotype observed in antibiotic-treated or Il17ra<sup>-/-</sup> neonatal mice, suggesting that the TLR4 and MyD88 signaling pathways do not completely account for postnatal granulocytosis and that alternate PRR pathways are involved in regulating postnatal granulocytosis. TIR domain–containing adaptor–inducing interferon-β (TRIF), which relays signals from TLR4 independently of MyD88 (ref. 33), is crucial in neutrophil homeostasis. Therefore, TRIF and members of the NATCH, LRR and PYD domain–containing protein receptor (NLRP) family, such as NLRP6 (ref. 35), which recognize microbial patterns and mediate intestinal homeostasis, could be additional microbial sensors in regulating postnatal granulocytosis. Furthermore, levels of short-chain fatty acids (SCFAs), gut microbiota–derived fermentation products, which have an essential role in intestinal inflammation and host resistance, are decreased in the intestinal contents of antibiotic-exposed mice. Therefore, short-chain fatty acids could play a part in regulating postnatal granulocytosis.

Components of the intestinal microbiota have been shown to differentially regulate adaptive immune function. We tested the effect of lipopolysaccharide (LPS), a ligand of TLR4, which is upstream of MyD88, hypothesizing it could regulate postnatal granulocytosis in neonatal mice. LPS has previously been detected in the intestinal homogenate of newborn mice within 1 h after birth, and fecal LPS levels were shown to be reduced in antibiotic-exposed mice. Administration of LPS (25 ng) by oral gavage was previously shown to increase transcripts of macrophage inflammatory protein 2 in the intestine of neonatal mice delivered by caesarean section to equivalent levels as those seen in vaginally delivered neonatal mice. Therefore, we administered LPS (10 ng by oral gavage) to antibiotic–exposed neonatal mice (postnatal day 3). Twenty-four hours later, we measured levels of IL-17 transcripts in the intestine, plasma G-CSF levels and the numbers of circulating and bone marrow neutrophils in antibiotic-exposed neonatal mice given LPS were higher than in those administered PBS (Fig. 4e–h). Treatment of neonatal mice with anti–IL-17A antibody blocked the increase in circulating and bone marrow neutrophils and plasma G-CSF in antibiotic–exposed neonatal mice after LPS administration (Supplementary Fig. 4g–i). These data suggest that LPS is one of the microbiota–derived signals regulating postnatal granulocytosis in neonates.

In conclusion, we identified a role for the microbiota in regulating postnatal granulocytosis and resistance to sepsis in neonates. Components of the microbiota signal, through a TLR4– and MyD88–dependent mechanism, induce IL-17A production by group 3 ILCs, increasing plasma G-CSF levels and the numbers of circulating and bone marrow neutrophils. A better understanding of how the intestinal microbiota affects postnatal development and function of neutrophils could lead to therapeutically relevant strategies to...
restore granulocytosis. These results could form the basis for future clinical studies for microbiota manipulation and transplantation to ameliorate antibiotic-induced microbiota dysbiosis and improve neonatal mortality.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.S.D., J.N.W. and G.S.W. conceived of the study. H.S.D. and G.S.W. designed the experiments. P.M.O. and J.K.K. provided reagents. H.S.D., O.R.M., Y.L., N.D., I.M. and C.E.O. carried out experiments. H.S.D. and G.S.W. analyzed the data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Penders, J. et al. Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 118, 511–521 (2006).
2. Kuppala, V.S., Meinzen-Derr, J., Morrow, A.L. & Schibler, K.R. Prolonged initial experiments. P.M.O. and J.K.K. provided reagents. H.S.D., O.R.M., Y.L., N.D., I.M. and C.E.O. carried out experiments. H.S.D. and G.S.W. analyzed the data and wrote the manuscript.

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3. Sarkar, S., Bhagat, I., Hieber, S. & Donn, S.M. Can neutrophil responses in very late onset sepsis in preterm infants. Pediatr. Res.

4. Henao-Mejia, J. et al. Human microbiome in health and disease. Trends Mol. Med. 12, 21–28 (2006).

5. Kawasaki, K. et al. Development of the intestinal bacterial composition in very late onset sepsis in preterm infants. Pediatr. Res.

6. Nakayama, Y. et al. Neonatal neutropenia in low birthweight premature infants. Am. J. Perinatol. 12, 34–38 (1995).

7. Martin, C. et al. Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following sepsis. Immunity 19, 583–593 (2003).

8. Pluschke, G. & Pelkonen, S. Host factors in the resistance of newborn mice to E. coli infection. Microb. Pathog. 4, 93–102 (1988).

9. Cohen-Wolkowiez, M. et al. Early and late onset sepsis in late preterm infants. Pediatr. Infect. Dis. J. 28, 1052–1056 (2009).

10. Liesche, G.J. et al. Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. Blood 84, 1737–1746 (1994).

11. Sekirov, I. et al. Antibiotic-induced perturbations of the intestinal microbiota alter host susceptibility to enteric infection. Infect. Immun. 76, 4726–4736 (2008).

12. Clarke, T.B. et al. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat. Med. 16, 228–231 (2010).

13. Schwarzenberger, P. et al. Requirement of endogenous stem cell factor and granulocyte colony-stimulating factor for IL-17-mediated granulopoiesis. J. Immunol. 164, 4783–4789 (2000).

14. Ye, P. et al. Requirement of interleukin 17 receptor signaling for lung CXCL chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. J. Exp. Med. 194, 519–527 (2001).

15. Ivanov, I.I. et al. The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 126, 1121–1133 (2006).

16. Ivanov, I.I. et al. Specific microbiota direct the differentiation of IL-17–producing T helper cells in the mucosa of the small intestine. Cell Host Microbe 4, 337–349 (2008).

17. Denning, T.L., Wang, Y.C., Patel, S.R., Williams, I.R. & Pulemark, B. Laminapropia macrophages and dendritic cells differentially induce regulatory and inflammatory 17–producing T cell responses. Nat. Immunol. 8, 1086–1094 (2007).

18. Mombaerts, P. et al. RAG1–deficient mice have no mature B and T lymphocytes. Cell 68, 867–877 (1992).

19. Spits, H. et al. Innate lymphoid cells—a proposal for uniform nomenclature. Nat. Rev. Immunol. 13, 145–149 (2013).

20. Sawa, S. et al. Lineage relationship analysis of RORγt+ innate lymphoid cells. Science 330, 665–669 (2010).

21. Buonocore, S. et al. Innate lymphoid cells drive interleukin-23–dependent innate intestinal pathology. Nature 484, 1371–1375 (2010).

22. Medzhitov, R. TLR-mediated innate immune recognition. Semin. Immunol. 19, 1–2 (2007).

23. Schnare, M. et al. Toll-like receptors control activation of adaptive immune responses. Nat. Immunol. 2, 947–950 (2001).

24. Ichinohe, T. et al. Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc. Natl. Acad. Sci. USA 108, 5354–5359 (2011).

25. Ubeda, C. et al. Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice. J. Exp. Med. 209, 1445–1456 (2012).

26. Yamamoto, M. et al. Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. Science 301, 640–643 (2003).

27. Bugil, S. et al. Steady-state neutrophil homeostasis is dependent on TLR4/TRIF signaling. Blood 121, 723–733 (2013).

28. Elinav, E. et al. Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. Nat. Rev. Cancer 13, 759–771 (2013).

29. Henao-Mejia, J. et al. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature 482, 179–185 (2012).

30. Cho, I. et al. Antibiotics in early life alter the murine colon microbiome and adiposity. Nature 488, 621–626 (2012).

31. Mazmanian, S.K., Liu, C.H., Tzianabos, A.O. & Kasper, D.L. An immunomodulatory role for specific microbiota in the development of intestinal innate immune responses. Immunology 144, 1445–1456 (2012).

32. Elinav, E. et al. Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. Nat. Rev. Cancer 13, 759–771 (2013).

33. Henao-Mejia, J. et al. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature 482, 179–185 (2012).

34. Cho, I. et al. Antibiotics in early life alter the murine colon microbiome and adiposity. Nature 488, 621–626 (2012).

35. Mazmanian, S.K., Liu, C.H., Tzianabos, A.O. & Kasper, D.L. An immunomodulatory role for specific microbiota in the development of intestinal innate immune responses. Immunology 144, 1445–1456 (2012).

36. Henao-Mejia, J. et al. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature 482, 179–185 (2012).

37. Cho, I. et al. Antibiotics in early life alter the murine colon microbiome and adiposity. Nature 488, 621–626 (2012).

38. Mazmanian, S.K., Liu, C.H., Tzianabos, A.O. & Kasper, D.L. An immunomodulatory role for specific microbiota in the development of intestinal innate immune responses. Immunology 144, 1445–1456 (2012).

39. Lotz, M. et al. Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells. J. Exp. Med. 203, 973–984 (2006).

40. Daemen, S., Goris, H., de Boer, F., Halie, M.R. & van der Waa, J. Recovery of murine myelopoiesis after cytostatic reduction by Ara-C. Effect of bactracin-induced changes in the intestinal microflora and influence of timing. Leuk. Res. 15, 1013–1018 (1991).
ONLINE METHODS
Animals and neonatal antibiotic-exposure. We bred C57/BL6 mice deficient in TLR2, TLR4, Rag1, IL-17, IL-23 (subunit p19), MyD88 or Nod2 and appropriate wild-type controls at the Children’s Hospital of Philadelphia animal facility. We maintained the germ-free C57/BL6 neonatal mice (gift from D. Artis, University of Pennsylvania) in plastic isolator cages with autoclaved feed and water in the University of Pennsylvania Germ-Free Core facility. We treated pregnant wild-type or gene-targeted female mice with sterile drinking water mixed with 5 different antibiotics (ampicillin, gentamicin, metronidazole, vancomycin and neomycin) (all 1 mg ml⁻¹ from Sigma-Aldrich), or 3 different antibiotics (ampicillin, gentamicin and vancomycin) (all 1 mg ml⁻¹) starting from embryonic day 15. After birth, neonatal mice from multiple litters were pooled and randomly distributed to control for founder effect and to minimize in-cage variations. The dams and the neonatal mice continued to receive antibiotic-containing drinking water for the duration of the experiment. Thus neonatal antibiotic exposure refers to antibiotic exposure both in utero and after birth. We used neonatal C57/BL6, Rag1⁻/⁻, Tlr2⁻/−, Tlr4⁻/−, Nod2⁻/⁻, Il17ra⁻/−, Myd88⁻/⁻, Il23r⁻/⁻ and germ-free C57/BL6 mice between ages 1 and 21 d and appropriate, age-, gender- and genetic strain–matched controls in subsequent experiments to account for any variations in data. The Children’s Hospital of Philadelphia Institutional Animal Care and Use Committee (IACUC) approved all protocols.

Neonatal late-onset sepsis. We grew E. coli serotype K1 or K. pneumoniae (American Type Culture Collection, #3816) (37 °C, 200 r.p.m.) in Luria Bertani (LB) broth containing 100 µg ml⁻¹ rifampin (Sigma) or tryptic soy (TS) broth, respectively, to log-phase growth. To mimic E. coli or K. pneumoniae late-onset neonatal sepsis, we inoculated neonatal mice (postnatal day 3–14) with either E. coli (1 × 10⁶ CFU g⁻¹) or K. pneumoniae (1 × 10⁶ CFU g⁻¹) via intraperitoneal (i.p.) injection¹ and killed them 72 h later or earlier if moribund. To assess bacterial burden, we homogenized the spleen and liver in sterile PBS. We plated serial dilutions of spleen or liver homogenates or blood or peritoneal fluid in LB agar plates with rifampin (100 µg ml⁻¹) and incubated (37 °C, overnight) to count the number of CFU of E. coli.

Transfer of intestinal microbiota to the antibiotic–exposed neonatal mice. We collected intestinal contents from non–antibiotic-exposed neonatal mice at various ages (postnatal day 1, 3 or 5 or 7, 10 or 21 d) and weighed and suspended them in sterile water. We transferred intestinal contents (200 µg in 50 µl PBS) from postnatal day 1 or 3 or 5 or 7, 10- or 21-d-old neonatal mice or vehicle (50 µl PBS) to age- and sex-matched antibiotic-exposed neonatal mice by a single oral gavage via fine polyethylene tubing. We pooled the neonatal mice in each experimental group and randomly redistributed them to minimize in-cage variations. The dams and neonatal mice continued to receive antibiotic-containing drinking water and after 48 h were examined to determine whether the antibiotic–exposed neonatal mice or plasma G-CSF or inoculated via i.p. route with E. coli.

Treatment with monoclonal depleting or neutralizing monoclonal antibodies, G-CSF and TLR agonist. We injected neonatal mice with anti-Ly6G antibody (clone RB6-865, BD Biosciences or clone IA-1A-8, Bioxcel) or anti-IL-17A antibody (clone 50104, R&D), anti-TLR4 antibody (clone MTS510, eBioscience) or anti–CD90.2 antibody (clone 30H12, Bioxcel) or anti-IgG2A (clone 54447, R&D) (all 5 µg per g body weight) via i.p. route daily on postnatal days 0–3. We injected anti-bacterial-exposed neonatal mice with recombinant mouse G-CSF (10 µg per g body weight) (cat. #1A-17, R&D) via i.p. route on postnatal day 3. We treated antibiotic-exposed neonatal mice (postnatal day 3) with LPS (Invitrogen) (10 ng in 50 µl PBS) or vehicle (50 µl endotoxin-free PBS) by oral gavage via fine polyethylene tubing and after 48 h inoculated them via i.p. route with E. coli.

Bone marrow isolation and flow cytometry. For characterization of bone marrow neutrophils, we pooled the femurs from 2–3 neonatal mice, flushed them with 5% FCS in DMEM and incubated them (4 °C, 5 min) with ACK lysis buffer. We then incubated (4 °C, 30 min) the cells (2 × 10⁶) with Live/Dead Blue viability dye (Invitrogen) and then stained them with allophycocyanin (APC)-conjugated anti–mouse Ly6G antibody (clone RB6-8C5, R&D), fluorescein isothiocyanate (FITC)-conjugated anti–mouse CXC4R4 antibody (clone 12G5, Biolegend), phycocerythrin (PE)-conjugated anti–mouse CXC2R2 antibody (clone SE8, Biolegend), PE/Cy7-conjugated anti–mouse CD45 antibody (clone 30F-11, Biolegend), washed them (2×) and resuspended them in flow cytometry buffer (PBS, 0.5% FCS and 0.1% sodium azide). All antibodies were diluted 1:100. To determine the hematopoietic progenitor cells in the bone marrow, we incubated the cells (2 × 10⁶) with Live/Dead Blue viability dye and then stained them with FITC-conjugated anti–mouse lineage marker antibodies (CD3, CD4, CD8a, TCRβ, CD45R, CD11c, CD11b, CD19, TER119, Ly6G/Ly6C, NK1.1 and FcR1) (Biolegend, cat. 133301, 1:100), Alexa Fluor 647–conjugated anti–mouse CD34 antibody (clone RAM34, BD Biosciences), PE/Cy7-conjugated anti–mouse c-Kit antibody (clone 2B8, Biolegend), brilliant violet (BV) 711–conjugated anti–mouse Sca-1 (clone D7, Biolegend) and PE-conjugated anti–mouse–CD16/CD32 (clone 93, Biolegend), washed them (2×) and resuspended them in flow cytometry buffer. All antibodies were diluted 1:100. We used isotype-matched antibodies as negative controls. We collected the data with an LSR II flow cytometer (BD Biosciences) and analyzed the data with FlowJo (Treestar).

Isolation of lamina propria lymphocytes and detection of IL-17–producing innate lymphoid cells. We pooled and cut the freshly resected terminal ilea from 3–4 neonatal mice into 2- to 5-mm pieces and incubated (37 °C, 15 min) them in extraction buffer (HBSS, 15 mM HEPES and 1 mM EDTA) to remove the epithelial cells. We then incubated (37 °C, 30 min) the cut tissues with shaking (150 r.p.m.) in digestion buffer (RPMI 1640 with 10% FBS, 15 mM HEPES, 1% penicillin/streptomycin (wt/vol) and 300 U ml⁻¹ collagenase VIII). We isolated the lamina propria lymphocytes from the resultant single-cell suspension by discontinuous Percoll (GE Healthcare) gradient at 40–80% interface and incubated (37 °C, 5 h, 5% CO₂) the cells (4 × 10⁶) in culture medium containing RPMI 1640 with 10% FCS, 1× nonessential amino acids, 10 mM HEPES, 2 mM l-glutamine (all from Invitrogen) and 1% penicillin/streptomycin with 1:1,000 Golgi Stop (554724, BD Biosciences), 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml calcium ionophore A23187 (both from Sigma-Aldrich). We washed and incubated (4 °C, 10 min) the cells (1 × 10⁶) with anti-mouse CD16/CD32 (eBioscience) and then re-incubated (4 °C, 30 min) with FITC-conjugated anti–mouse TCRβ antibody (Clone JK16-133.18, eBiosciences), PE/Cy7-conjugated anti–mouse TCRβ antibody (Clone GL3, Biolegend), APC-conjugated anti–mouse CD4 antibody (Clone RM4-5, Biolegend) and BV-conjugated anti–mouse NKp46 antibody (Clone 29A1.4, eBiosciences) (all diluted 1:100). For intracellular staining for IFN-γ, we washed and fixed (4 °C, 60 min) the surface-stained cells in 1× Cytofix/Cytoperm buffer (BD Biosciences) and permeabilized them (4 °C, overnight) using 1× Permeabilization Buffer (BD Biosciences) according to manufacturer instructions. We stained the cells intracellularly with PE-conjugated anti–mouse IL-17 antibody (clone TC11-18H10.1, Biolegend) or PerCP-conjugated anti–mouse RORγt antibody (clone B2D, eBiosciences) (all diluted 1:50) and then washed (2×) and resuspended them in flow cytometry buffer. We collected the data with LSR II (BD Biosciences) and analyzed the data with FlowJo (Treestar).

Analysis of microbiota and quantification of bacterial 16S ribosomal DNA. We collected intestinal contents from control (non–antibiotic exposed) neonatal mice or antibiotic exposed neonatal mice at various ages (1–21 d) and snap froze them (~80 °C). As intestinal contents were not available in adequate amounts for individual sampling from neonatal mice, we pooled intestinal contents from 6–8 neonatal mice from 3 separate litters per treatment group so as to minimize cage effects. We extracted DNA from intestinal contents using QIAamp DNA Stool Mini Kit (Qiagen), quantified 16S rDNA by RT-PCR using degenerate or class-specific bacterial 16S rDNA primers and probes for Gammadaptobacteria or Bacilli. For 16S rDNA sequencing, we amplified the V2 region of microbial 16S rDNA by high-fidelity PCR with barcoded 8F and 338R universal primers with A and B sequencing adapters, respectively, and bidifo primers (Roche) and sequenced them with Genome Sequencer GS-FLX Titanium system (Roche) at the University of North Carolina Microbiome core facility (Chapel Hill, NC). We decoded and processed the...
sequences using the QIIME software package (Version 1.7) and custom R package code\textsuperscript{44}. We used phylogenetic diversity (PD) to compute and visualize \( \alpha \) diversity and unweighted and weighted Unifrac for \( \beta \) diversity. We tested the observed differences in Unifrac distances between antibiotic-treated groups and across different ages for significance using a \( t \) test, and we corrected the reported \( P \) values for multiple comparisons using a Monte Carlo permutation procedure with 10,000 iterations.

We determined the number of circulating and bone marrow neutrophils, calculated the phagocytosis index and determined the ROS production by the bone marrow neutrophils as described before\textsuperscript{45}. We isolated the RNA and determined the transcript levels of IL-17 in the neonatal small intestine and the lungs as described before\textsuperscript{45}. We determined the levels of plasma G-CSF as described before\textsuperscript{45}.

**Statistical analyses.** To determine group sizes necessary for adequate statistical power, power analysis was performed using preliminary data sets. The investigators were blinded to group allocation during collection and analysis of the data, and all inclusion/exclusion criteria were preestablished. All data meet the assumptions of the statistical tests used. We compared differences between groups by the unpaired Student’s \( t \)-test or Student Neumann Keul’s test (GraphPad Prism 4). We considered \( P \) values <0.05 significant. We used the Kaplan-Meier log-rank test to compare survival between groups.

41. Hill, D.A. et al. Metagenomic analyses reveal antibiotic-induced temporal and spatial changes in intestinal microbiota with associated alterations in immune cell homeostasis. *Mucosal Immunol.* **3**, 148–158 (2010).
42. Rudi, K., Tannaes, T. & Vatn, M. Temporal and spatial diversity of the tap water microbiota in a Norwegian hospital. *Appl. Environ. Microbiol.* **75**, 7855–7857 (2009).
43. Oliwa-Stasiak, K., Kolaj-Robin, O. & Adley, C.C. Development of real-time PCR assays for detection and quantification of *Bacillus cereus* group species: differentiation of *B. weihenstephanensis* and rhizoid *B. pseudomycoides* isolates from milk. *Appl. Environ. Microbiol.* **77**, 80–88 (2011).
44. Caporaso, J.G. et al. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **7**, 335–336 (2010).
45. Mei, J. et al. *Ccr2* and *Cxc5* regulate the IL-17/G-CSF axis and neutrophil homeostasis in mice. *J. Clin. Invest.* **122**, 974–986 (2012).