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Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma

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Allergic asthma rates have increased steadily in developed countries, arguing for an environmental aetiology. To assess the influence of gut microbiota on experimental murine allergic asthma, we treated neonatal mice with clinical doses of two widely used antibiotics—streptomycin and vancomycin—and evaluated resulting shifts in resident flora and subsequent susceptibility to allergic asthma. Streptomycin treatment had little effect on the microbiota and on disease, whereas vancomycin reduced microbial diversity, shifted the composition of the bacterial population and enhanced disease severity. Neither antibiotic had a significant effect when administered to adult mice. Consistent with the 'hygiene hypothesis', our data support a neonatal, microbiota-driven, specific increase in susceptibility to experimental murine allergic asthma.

Keywords: antibiotics; asthma; gut; microbiota

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INTRODUCTION

Allergic asthma affects over 100 million people worldwide and its prevalence is increasing, particularly among children in industrialized countries [1]. This growing epidemic has been attributed in part to improved sanitation methods and widespread antibiotic use, which have in turn reduced childhood exposures to microbial antigens critical for proper immune development [2]. Blaser and Falkow [3] have recently revisited the hygiene hypothesis, suggesting that it might not be a decline in childhood infections that is important, rather, it is how modern societal practices are causing the disappearance of ancestral species of our indigenous microbiota that might confer benefits beyond our current understanding. The loss of these ancestral species could be causing a rapid reorganization of the microbial hierarchy in our guts faster than our immune systems can adapt, promoting the emergence of dysregulated immunological disorders like allergic asthma.

Over the last several decades, evidence from human and mouse studies indicate that colonization of the gut early in life has a substantial role in directing immune system development. Disrupting this normal 'immunological program' could increase the likelihood of developing atopic disorders like asthma. Epidemiological data show gut microbiota differs between asthmatic and non-asthmatic infants [4], early life exposure to environmental microorganisms is protective [5] and early life as well as prenatal antibiotic exposures increase the risk of allergic asthma [6–8].

Antibiotic or probiotic administration as a means of microbiota manipulation in experimental models of murine allergic airways disease has provided further insights into what is now referred to as the 'gut–lung axis'. Noverr *et al* [9,10] describe how antibiotic treatment followed by oral administration of *Candida albicans* renders immunocompetent hosts susceptible to allergic airways disease. Similarly, oral administrations of *Mycobacterium vaccae* [11], *Helicobacter pylori* [12] as well as conventional probiotic strains [13,14] have been shown to ameliorate symptoms of allergic airways disease in mice. Several of these studies highlight the necessity of neonatal administration rather than adult, emphasizing that immune modulation is most effective during the developmental period [12,14]. Despite this growing body of evidence that supports a role for the gut microbiota in allergic disease, there is little known about how antibiotic-induced changes in microbial composition affect the immune status of the host and influence disease susceptibility.

The human gut is colonized by $\sim 10^{14}$ bacteria, and contains upwards of 1,000 bacterial species [15]. Specific subsets of the

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microbiota have been shown to differentially regulate immune function. For example, *Bacteroides fragilis* modulates T-helper-type 1/2 (Th1/Th2) balance [16], segmented filamentous bacteria direct Th17 cell differentiation [17] and *Clostridium* species induce regulatory T-cell (Treg) production [18]. Antibiotics have the ability to create global shifts in microbial communities, and are valuable tools for studying how changes in the gut microbiota influence disease states [19].

Here we characterize the effects of two antibiotics on resident murine gut flora and the subsequent susceptibility of these mice to experimental allergic asthma. Although it is known that this model only recapitulates some aspects of the human disease (particularly when mice are only given a single or limited number of antigen challenges) it does recapitulate the airway hyperresponsiveness (AHR) and some of the early phase responses [20]. It has therefore become a very popular model for evaluating susceptibility to Th2-driven allergic airways disease. Our data show that neonatal, but not adult, exposure to certain antibiotics promotes enhanced susceptibility to experimental allergic asthma.

RESULTS

Antibiotic treatment increases asthma severity

To determine whether antibiotic perturbations of intestinal microbiota affect asthma, experimental murine allergic asthma was induced in mice exposed to vancomycin or streptomycin as neonates (including exposure *in utero*) or only as adults. Ovalbumin (OVA)-challenged mice treated early in life with vancomycin, but not streptomycin, resulted in increased total inflammatory cell infiltrates in the bronchoalveolar lavage (BAL) relative to OVA-challenged controls (Fig 1A). Treatment of adult mice with the same antibiotics had no effect on asthma induction (Fig. 1A and supplementary Fig S1 online). Neonatal vancomycin treatment also increased eosinophils in the BAL (Fig 1B). Antigen-specific immunoglobulin-E (IgE) was also significantly higher in the vancomycin-treated animals, consistent with exacerbated disease (Fig 1C). Antibiotic treatment alone (PBS-sensitized and challenged) had no effect on BAL counts (Fig 1A), eosinophil numbers or OVA-specific IgE (data not shown as there was none detected).

Airway hyperresponsiveness, measured by changes in airway resistance (AR) in response to methacholine (MCh), is another defining feature of asthma. Both OVA-challenged control and streptomycin-treated mice exhibited increased AR over naive animals (Fig 1D); however, those treated with vancomycin showed markedly higher AHR compared to the other groups.

We examined lungs of mice from all treatment groups for histopathology to confirm allergic disease. Pathological scores were consistently higher in the vancomycin-treated animals (Fig 1E). Pathology in streptomycin-treated, OVA-challenged mice was comparable to control OVA-challenged mice. PBS-treated control mice were also compared to PBS-treated vancomycin and streptomycin-treated animals and no differences were detected (supplementary Fig S2 online). In summary, on the basis of an evaluation of BAL infiltrates, OVA-specific IgE, AHR and histopathology, we found that neonatal vancomycin treatment enhances the severity of experimental murine allergic asthma.

Vancomycin treatment profoundly alters gut flora

We next sought to correlate increased sensitivity to asthma to alterations in bacterial communities in the intestine. Total bacteria in stool pellets from antibiotic-treated mice were moderately reduced compared with the controls (Fig 2A). This reduction, however, was less pronounced than the 100-fold reduction previously observed in animals treated with antibiotics that greatly exceed therapeutic levels [21].

Changes in gut community composition after antibiotic treatment were assessed by pyrosequencing the V1–V2 regions of bacterial 16S rRNA genes. A total of 68,014 quality reads were obtained from 69 samples (the data set is available in the Sequence Read Archive (SRA) of NCBI, accession no. SRA050145). Principle coordinate analysis (PCO) showed that antibiotic treatment had the greatest effect on community composition, irrespective of sampling site (ileum/colon) or asthma induction (Fig 2B). In fact, 46.5% of the variability was explained on axis PCO1, emphasizing the large shift in community composition that resulted after vancomycin treatment compared with animals treated with streptomycin. Permutational multivariate analysis of variance indicated that antibiotic treatment significantly affected community composition ($F = 65.1$, $P < 0.0004$), and confirmed that the effect of vancomycin was much greater than that of streptomycin (Bray-Curtis similarity index: control versus streptomycin, 51.66; control versus vancomycin, 1.39). Estimated diversity was similar in control and streptomycin-treated communities, but significantly lower in those treated with vancomycin (Simpson's diversity indices: 0.90, 0.81 and 0.33, respectively). Strikingly, the average number of operational taxonomic units per sample dropped from 54 in the control to 48 in those treated with streptomycin, to only 6 after vancomycin treatment. The 16S rRNA sequences were taxonomically identified at the genus level using the Greengenes database [22] and a naive Bayesian classifier. The 16S rRNA genes and their frequencies classified at the family level (for simplicity) in faeces from control, streptomycin- or vancomycin-treated animals further emphasizes how significantly the bacterial community in the gut changed after antibiotic treatment, particularly in the animals that received vancomycin, irrespective of exposure type (Fig 2C).

Both neonatal and adult antibiotic treatment significantly altered microbial communities in the gut; however, these changes were not identical. The neonatal vancomycin-treated animals possessed a microbiota that was completely different from animals treated with vancomycin only as adults (Bray-Curtis similarity index: 34.7). Interestingly, communities from mice treated with vancomycin only as adults had greater diversity and were less distinct from the other treatments than the communities from mice treated with vancomycin as neonates (Simpson's diversity index: 0.67 and 0.18, respectively). When neonatal and adult antibiotic treatments were compared using PCO, it was clear that the two different exposures produced distinct community structures (supplementary Fig S3 online). Importantly, these data represent two independent experiments, in which cage effects, parent litter and age are all factors that affect microbiota composition, and could contribute to the significant differences observed between even the untreated controls in these experiments.

Antibiotic-specific microbial indicators of asthma

Given that streptomycin-treated animals had comparable disease to controls, bacteria that were depleted or enriched after vancomycin, but not streptomycin, were identified as candidates

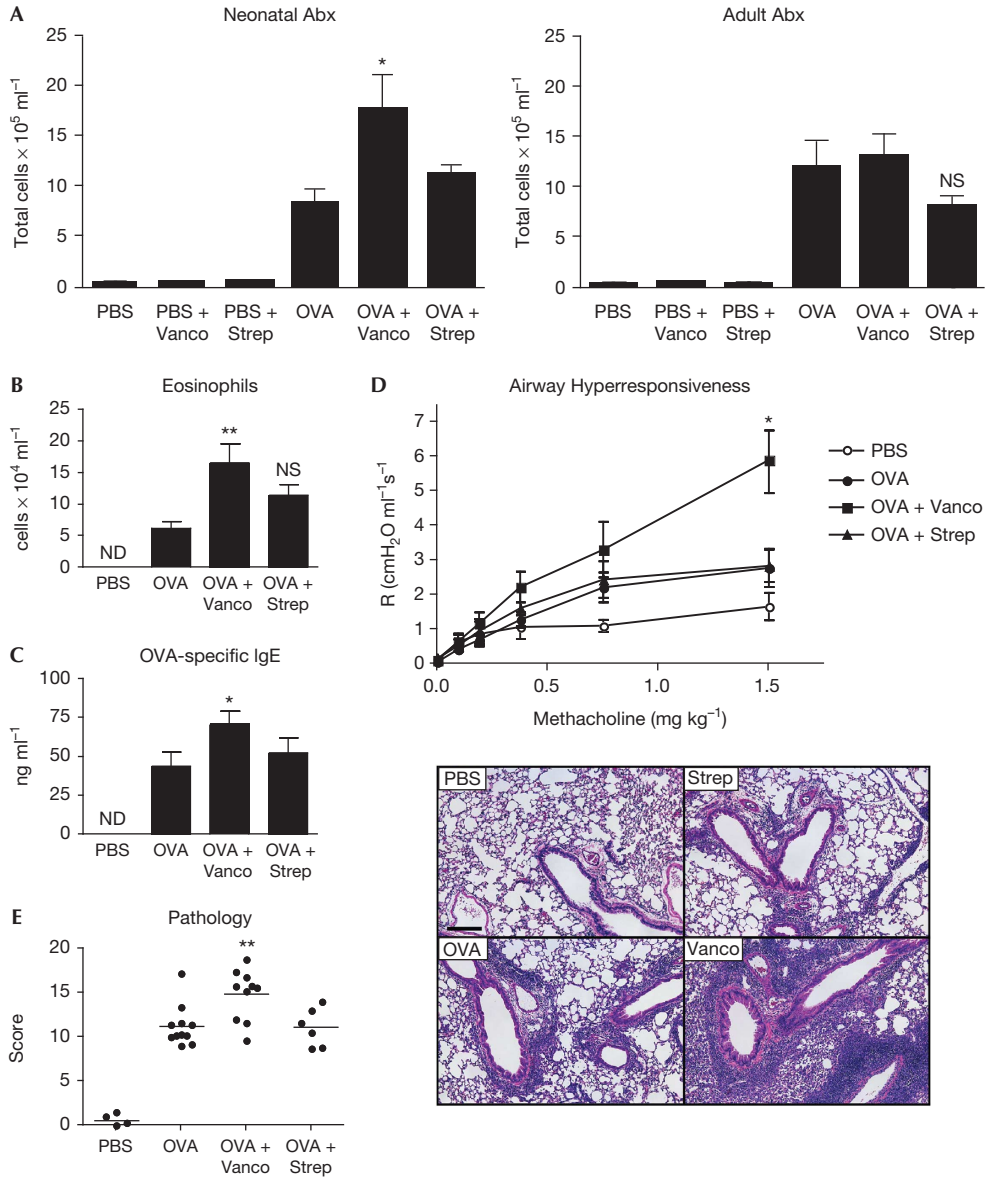

Fig 1 | Neonatal vancomycin treatment exacerbates allergic asthma. (**A**) Total cellular infiltrates from bronchoalveolar lavage (BAL) of control or antibiotic-treated mice challenged with ovalbumin (OVA) or PBS. (**B**) Eosinophil numbers in the BAL quantified by cytospin. (**C**) Serum OVA-specific IgE responses measured by enzyme-linked immunosorbent assay. (**D**) Airway hyperresponsiveness, measured by changes in resistance (R) in response to increasing doses of methacholine administered intravenously. (**E**) Total pathological scores and representative haematoxylin- and eosin-stained lung sections. Scale bar, 300 μm. All assessments were made on day 26. The data are shown as means of 5–7 mice per group ± s.e.m. and represent at least two independent experiments. Statistics shown are based on comparisons to OVA-challenged controls. Antibiotic-treated animals in **B**–**E** were treated neonatally. $*P<0.05$, $**P<0.01$; Abx, antibiotics; ND, none detected; NS, not significant; Strep, streptomycin; Vanco, vancomycin.

that might affect disease susceptibility (Table 1). This list highlights bacterial taxa that were significantly depleted or enriched in the vancomycin-treated animals based on the point biserial correlation coefficient (supplementary methods online). For sequence information on these indicators refer to supplementary methods, Data Set S1 online.

Antibiotics alter CD4$^+$CD25$^+$Foxp3$^+$ Tregs in the colon

Several members of the indigenous gut microbiota have been implicated in the induction of Treg cells in the gut [18,23]. To provide insight into how vancomycin-treated microbiota might predispose mice to exacerbated experimental asthma, we compared levels of CD4$^+$CD25$^+$Foxp3$^+$ T cells (a differentiation antigen expression phenotype typical of Treg cells) in the colon of untreated, vancomycin- and streptomycin-treated animals. We found colonic CD4$^+$CD25$^+$Foxp3$^+$ T cells to be strikingly reduced in the vancomycin-treated animals (Fig 3A), while their frequency in streptomycin-treated animals were comparable to controls. This reduction in phenotypic Tregs was not detected in the lungs of vancomycin-treated animals (Fig 3B). There is

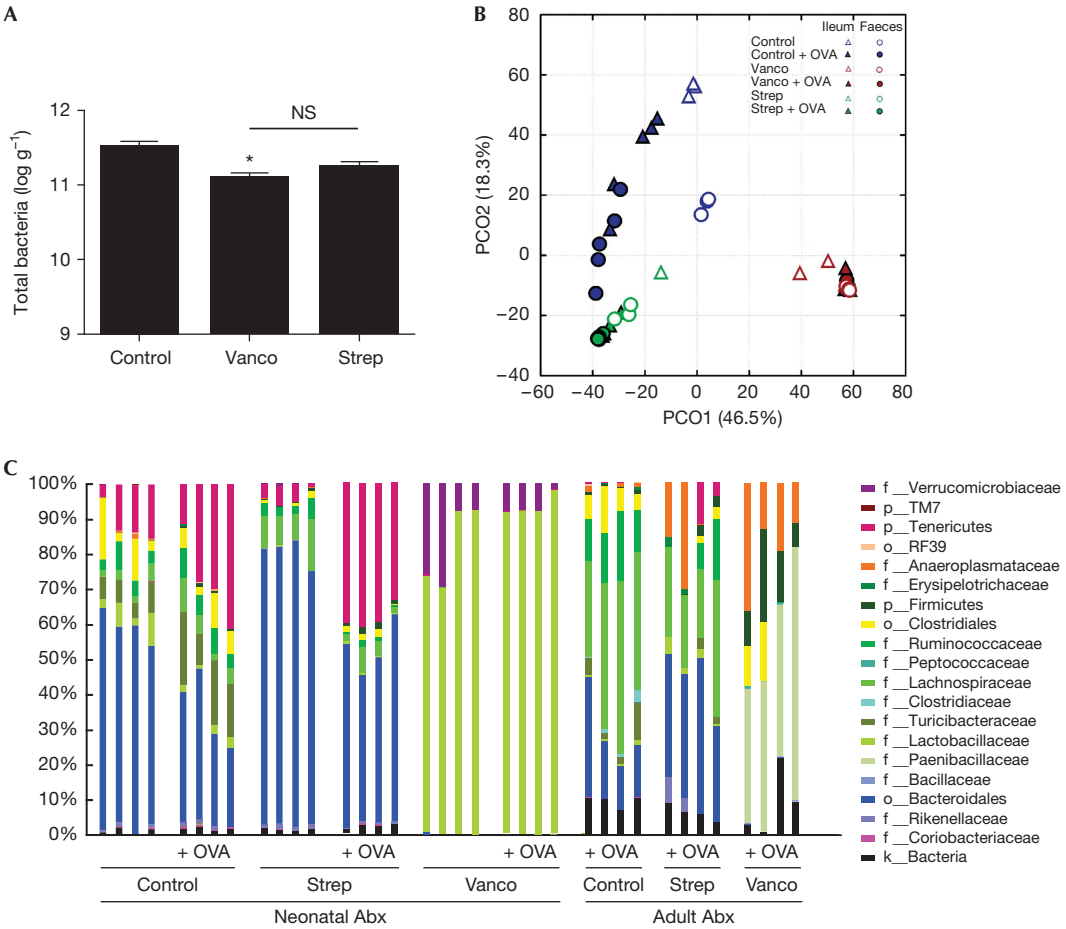

Fig 2 | Antibiotic treatment alters gut bacterial communities. (**A**) Total bacteria in faeces of control or antibiotic-treated mice quantitated by SYBR green. These data are shown as means of 3–5 mice per group ± s.e.m. and represent two independent experiments. *$P<0.05$ relative to control; NS, not significant. (**B**) Bacterial communities from ileum and faeces of naive or ovalbumin (OVA)-challenged control and antibiotic-treated mice were compared using principal coordinate analysis (PCO). (**C**) Family level phylogenetic classification of 16S rRNA gene frequencies in faeces collected from naive or OVA-challenged control and antibiotic-treated animals. Those indicated with a classification level other than family level (f) could only be identified confidently to the level indicated. Classification scheme: k, kingdom; p, phylum; c, class; o, order; f, family. Each bar represents one mouse. Rare taxa were removed from the legend, but still included in the graph. Antibiotic-treated animals in **A** and **B** were treated neonatally. Abx, antibiotics; Strep, streptomycin; Vanco, vancomycin.

increasing evidence to suggest that mucosal sites function together as a system-wide organ [24]. The gut–lung axis, a proposed mechanism of probiotic action, is an extension of this idea. Probiotic studies indicate that tolerogenic responses from the gut via gut-associated lymphoid tissue spread to the airways only in response to immune challenge and inflammation [25]. The data shown represent Treg populations from naive, unchallenged mice. Hence, further studies are required to determine whether a key orchestrator of tolerogenic mucosal immune responses is altered in vancomycin-treated animals.

DISCUSSION

Over the last several decades, substantial effort has been focused on identifying bacterial populations that correlate with the development of allergy-related disorders [4,25]. The results differ significantly depending on the study, which likely stems from differences in sample populations and methods of microbiota analysis. Nevertheless, most of these studies point towards the first 6 months of life as a 'critical window', during which the infant gut microbiota might influence key immunological events that alter allergic sensitization [4]. In the current study we have addressed the same question using a different approach: by creating defined shifts in the microbiota with antibiotics early in life, we have identified members of the microbiota that differ between more and less susceptible individuals.

In this study, animals treated neonatally with vancomycin showed increases in all major indicators of experimental allergic asthma. Correspondingly, vancomycin treatment markedly shifted the composition of the gut microbiota, compared with both streptomycin-treated and control populations. Adult vancomycin treatment also altered the microbiota, however, to a different extent, which could be why differences in disease susceptibility ensued. Because our neonatal antibiotic model encompassed several critical time points (that is, prenatal, perinatal, weaning), it

Table 1 | Bacterial taxa that correlate with exacerbated asthma

Phylum	Terminal classification*	Rank‡	Greengenes nearest match§	Relative abundance			*P*-value
				Control	Vanco‖	Strep	
Actinobacteria	Adlercreutzia	g	OTU 916	0.38	0.00	0.23	0.0047
Bacteroidetes	Alistipes	g	OTU 1053	0.74	0.00	0.84	0.000006
Bacteroidetes	Bacteroidales	o	OTU 991	42.15	0.17	63.63	0.000003
Firmicutes	Lachnospiraceae	f	Unclassified	0.91	0.01	1.08	0.0037
Firmicutes	Lachnospiraceae	f	OTU 2087	3.87	0.02	5.18	0.0024
Firmicutes	Ruminococcaceae	f	Unclassified	0.28	0.01	0.80	0.0062
Firmicutes	Clostridiales	o	OTU 1995	6.44	0.00	0.93	0.022
Tenericutes	Tenericutes	p	Unclassified	19.05	0.06	21.64	0.0019
Firmicutes	Lactobacillus	g	Unclassified	0.76	3.40	0.54	0.000002
Firmicutes	Lactobacillus	g	OTU 1865	3.85	84.21	0.00	0.000002
Verrucomicrobia	Akkermansia muciniphila	s	OTU 4410	0.01	11.96	0.11	0.000002

OTU, operational taxonomic unit; Strep, streptomycin; Vanco, vancomycin. Data shown represent neonatal antibiotic-treated mice. *The closest-related taxon at which the corresponding sequence could be confidently classified; ‡Taxonomic rank: p, phylum; o, order; f, family; g, genus; s, species; §Sequences were assigned to an OTU based on their relatedness to sequences in the Greengenes database; ‖Reductions are in red, enrichments in green.

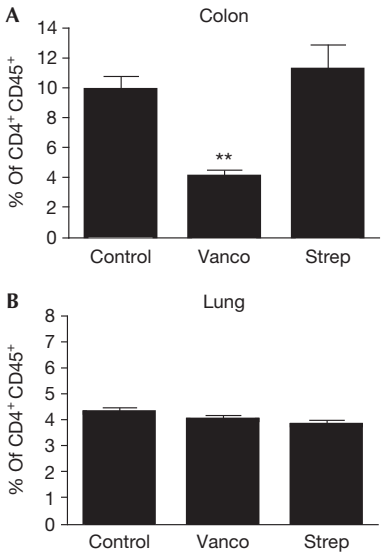

Fig 3 | Vancomycin treatment reduces $CD4^+CD25^+Foxp3^+$ regulatory T-cell accumulation in the colon but not in the lung. At 7 weeks of age, the percentage of $CD25^+Foxp3^+$ cells within the $CD45^+CD4^+$ cell population in the colon (**A**) or lung (**B**) of naive mice treated with neonatal antibiotics was analysed. The data shown are means of three mice per group ± s.e.m. and represent three independent experiments. Vanco, vancomycin; Strep, streptomycin. $^{**}P < 0.01$.

will be important to look at how antibiotic treatment at each time point individually affects disease outcome.

Microbial diversity was also reduced after vancomycin, but not streptomycin treatment, despite having similar numbers of total bacteria. There is evidence that reduced diversity in the infant gut microbiota correlates with increased risk of allergic disorders [26]. On the basis of the extent of microbial reorganization in the gut after neonatal antibiotic treatment, several bacterial populations were positively and negatively associated with disease severity.

Vancomycin-shifted microbiota could alter host immune development in several ways such as inducing Th2 hyperreactivity or reducing key tolergenic regulatory mechanisms. Consistent with our report, Atarashi *et al* [18] observed that vancomycin treatment reduces colonic $CD4^+Foxp3^+$ Tregs. They suggest that this observation is due to a reduction in *Clostridium* species (clusters IV and XIVa) that they identify as potent inducers of Tregs. We also found that depletion of *Clostridiales* with vancomycin correlated with a reduction in cells expressing a $CD4^+CD25^+Foxp3^+$ Treg phenotype; however, vancomycin treatment also reduced other bacterial phyla. Unlike the colon, we were not able to detect significant changes in $CD4^+CD25^+Foxp3^+$ Treg numbers in the lung. Perhaps a more detailed look at Treg populations in the lung and mediastinal lymph nodes over the course of disease induction, measuring Treg suppressive capacity or analysing other regulatory cell populations (for example, interleukin 10 producing Tr1 cells), may provide new insights.

Surprisingly, *Bacteroidetes* were almost entirely depleted after vancomycin treatment, despite not being directly targeted by the antibiotic; they were replaced by an overgrowth of *Lactobacilli*. Several *Bacteroides* strains have been implicated in Treg differentiation [18,23] and their depletion might also explain the altered susceptibility to experimental allergic asthma. Epidemiological data indicate that antibiotic use in early infancy as well as infants born by caesarean section show similar decreases in *Bacteroides* species [27], both of which are factors associated with increased childhood asthma [8,28]. *Lactobacilli* were negatively correlated with Treg abundance in this study, despite their previous associations with Treg induction in probiotic studies [29]. These regulatory effects are likely

strain-dependent, and perhaps there are other species or strains of *Lactobacilli* that have reciprocal effects that remain uncharacterized. Interestingly, *Lactobacilli* actually only represent a small proportion of total bacteria in the healthy mammalian intestine [21] despite their potential benefits, which suggests that an overgrowth of particular *Lactobacilli* species might do more harm than good. Colonizing gnotobiotic animals with subsets of the bacteria discussed here might help identify whether they have specific roles in the development of experimental allergic asthma.

In our study, two events were required to significantly affect the outcome of experimental asthma: (1) global restructuring of the intestinal microbiota with vancomycin and (2) altering the microbiota during infancy, a time point when key immunological thresholds are established. There is substantial evidence that microbial signals are required for lymphoid tissue development and maintenance in the gut (reviewed in Hill and Artis [30]). Vancomycin-shifted microbiota likely produces an entirely different set of microbial signals compared with healthy controls that could alter intestinal epithelial cell signalling cascades and dysregulate innate and adaptive immune responses. It is already known that mice treated with vancomycin have fewer Th17 cells in the small intestine [31] and fewer $CD4^+$ Treg cells in the colon [18]. Studies in germ-free animals show that the regulation of immune cell subsets (monocyte/macrophages, B cells, $CD4^+$ Treg/Th17 and $CD8^+$ T cells) by gut microbes is not restricted to the intestinal compartment [30]. Similarly, mice deficient in the innate recognition receptors TLR4 or GPR43 show exacerbated asthma responses [32,33]. Insights from antibiotic use in other disease models suggest mechanisms mediated by skewed Th2 immune responses [34] and elevated serum IgE levels [35]. Dendritic cells have also been implicated in the pathogenesis of asthma [36], and are thought to be involved in bridging the gut–lung axis [37], however whether or not signals from intestinal bacteria influence systemic dendritic cell populations remains to be tested directly. Although the link between gut and lung mucosal immune responses remains unclear, we propose that vancomycin administered early in life selects for a community of microbes that disrupt the balance of proinflammatory and regulatory immune responses occurring at local and distant mucosal sites.

In summary, using relevant doses of antibiotics we have found that neonatal, but not adult, treatment with select antibiotics can have profound effects on susceptibility to experimental murine allergic asthma. While antibiotic treatment did not substantially deplete bacterial numbers, it resulted in profound shifts in microbial composition. This was correlated with the loss of a key regulatory subset of immune cells in the colon that could, by way of the gut–lung axis, promote enhanced susceptibility to inflammatory diseases in the lung by a mechanism that remains undefined. This work provides a new framework for interrogating the altered communities responsible for heightened Th2 immune responses in human disease, including asthma.

METHODS

Mice. C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were bred and maintained in a specific pathogen-free facility at The Biomedical Research Center. All experiments were in accordance with the University of British Columbia Animal Care Committee guidelines.

Antibiotic treatment. C57BL/6J breeding pairs were given vancomycin or streptomycin (Sigma-Aldrich, St Louis, MI) at $200\,mg\,l^{-1}$ in drinking water. Pups born from respective breeding pairs were reared on antibiotic-treated water with their littermates for the duration of the experiment. Hence, our term 'neonatal exposure' refers to antibiotic exposure both *in utero* and after birth. Alternatively, 7-week-old C57BL/6J female mice were given vancomycin or streptomycin (Sigma) at $200\,mg\,l^{-1}$ in drinking water for 2 days before sensitization with OVA, hence 'adult exposure'.

Vancomycin, an antibiotic that directly targets Gram-positive bacteria, and streptomycin, an antibiotic that directly targets both Gram-positive and Gram-negative bacteria, were chosen because both are used in clinical practice and are poorly absorbed when given orally, minimizing the risk of systemic effects on the host. The rationale behind the clinically relevant concentrations used has been described in detail previously [38].

Ovalbumin model of experimental murine allergic asthma. Experimental murine allergic asthma was induced as previously described [39] with minor modifications. Although this model does not fully recapitulate the phenotype of human allergic asthma (particularly after limited numbers of antigen challenge [20]), it is a useful model for evaluating many aspects of this Th2-driven lung inflammatory disease. Mice were sensitized intraperitoneally with 200 μg OVA and 1.3 mg alum (both from Sigma) on days 0 and 7. On days 21, 22, 23 and 25, mice were challenged intranasally with 1 mg OVA in PBS. On day 26, mice were anaesthetized with $200\,mg\,kg^{-1}$ ketamine per $10\,mg\,kg^{-1}$ xylazine and blood was collected by cardiac puncture. After killing, BALs were performed by 3×1 ml washes with PBS. Total BAL cells were counted by hemocytometer and eosinophils were quantified from cytospins (Thermo Shandon, Pittsburg, PA) stained with HemaStain (Fisher Scientific), based on standard morphological criteria.

Determination of serum IgE. OVA-specific IgE in serum was measured by enzyme-linked immunosorbent assay (Chondrex, Redmond, WA).

Airway hyperresponsiveness. Airway hyperresponsiveness was measured on day 26. Mice were anaesthetized, tracheotomized, and intubated with a cannula. Following paralysis with pancuronium bromide ($0.5\,mg\,kg^{-1}$), AR was measured with a flexiVent apparatus (SCIREQ, Montreal, QC, Canada). Increasing concentrations of MCh ($0–1.5\,mg\,kg^{-1}$) were administered via the jugular vein. MCh response curves were generated by calculating the change in AR relative to baseline (PBS) for each MCh dose.

Histology. Lungs were collected and fixed in 10% formalin, embedded in paraffin, cut longitudinally into 5-μm sections and stained with haematoxylin and eosin. Inflammation was blindly assessed from five fields per section, each graded on a scale of 0–5 (0 = no signs of disease, 5 = severe disease) for each of the following parameters: (1) peribronchial infiltration, (2) perivascular infiltration, (3) parenchymal infiltration and (4) epithelium damage for a maximum score of 20.

Microbial analysis. Bacteria in stool pellets of 7-week-old naive mice were stained with SYBR green and counted as previously described [40]. For composition analyses, stool pellets and ileal contents from naive or OVA-challenged mice were homogenized using a bead-beating method (FastPrep instrument, MP Biomedicals, Solon, OH) and total DNA was extracted (Ultra Clean Fecal

DNA kit, Mo Bio Laboratories, Carlsbad, CA). 16S rRNA gene fragments were amplified using 33 nucleotide-bar-coded primer pairs (27F; 5′-AGAGTTTGATCMTGGCTCAG-3′), (519R; 5′-GWA TTACCGCGGCKGCTG-3′). PCR products were gel-purified (QIAquick gel extraction kit, Qiagen, Valencia, CA). Each amplicon (100 ng) was pooled and pyrosequenced using a 454 Titanium platform (Roche, Branford, CT).

Bioinformatics. High-quality sequence reads were determined using MOTHUR[41] and V-XTRACTOR [42]. Each read was assigned to the GREENGENES database [22] using a naive Bayesian classifier [43]. Global community structure comparisons from faecal and ileal samples were made using PCO [44], permutational multivariate analysis of variance [45] and Simpson's diversity index [46] implemented in PRIMER6+ [47]. Taxa-treatment association analysis [48] in conjunction with additional statistical analyses (supplementary methods online) were performed to determine antibiotic-related indicator species (faecal samples only). Raw, unfiltered sequences were submitted to the SRA of NCBI.

Isolation of lymphocytes and flow cytometry. Colon and lung tissues were collected from 7-week-old naive mice and cells were isolated as previously described [49]. Cells were stained with fluorochrome-conjugated antibodies against CD45, CD4, CD25 and Foxp3 (BD Biosciences, Franklin Lakes, NJ). Flow cytometry was performed using an LSR II (BD Biosciences) and data were analysed with FlowJo 8.7 software (TreeStar, Ashland, OR).

Statistics. Differences between control and experimental groups were compared using an unpaired Student's *t*-test to calculate statistical significance (GraphPad Prism software, version 4.0, San Diego, CA).

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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