

Oral administration of vancomycin to neonatal mice could alter their immunity and allergic sensibility late in adulthood

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The prevalence of allergy has increased over the past decades, and this may be attributed in part to the intestinal microbiota dysfunction caused by antibiotics during early life. In this study, we evaluated how vancomycin could impair the intestinal microbiota during early life and then, consequently, alter susceptibilities to allergic diseases and related immunity in late adulthood. BALB/c (n=54) neonatal mice were used in this study. Mice in the vancomycin group were orally administered vancomycin for 21 days, while those in the allergy and control groups were perfused with the same volume of saline solution. Then, mice in the allergy and vancomycin groups were immunized with intraperitoneal ovalbumin with alum. At postnatal day 21, vancomycin significantly alter the fecal microbiota, with lower *Bacteroidetes* and *Firmicutes* counts and higher *Proteobacteria* counts. At postnatal day 56, the *Bacteroidetes* count was still significantly lower in vancomycin-treated mice. The serum IgE level in the control group was significantly lower than that in the vancomycin and allergy groups. The serum interleukin (IL)-6 level and the IL-4/interferon (IFN)- γ values were significantly higher in the vancomycin-treated mice, but the serum IL-17A level was lower than that in the control group. These results indicate that modifications of the intestinal microbiota composition during early life may be, at least in part, the key mechanism underlying the effect of vancomycin that influences the immune function of host animals in the adult stages.

Key words: vancomycin, intestinal microbiota, early life, intestinal development, allergy, immunity

INTRODUCTION

During the past several decades, there has been an increasing prevalence of allergic diseases worldwide, particularly among children [1]. The growing incidence of such cases of allergy has created a massive disease burden and economic expenses for society. The difference in prevalence of allergic diseases between developing and developed countries underlines the importance of the external environment for these diseases. Epidemiological studies have demonstrated several key early life factors that are associated with susceptibility to allergic diseases, such as delivery mode, time of gestation, breastfeeding, and antibiotic treatment [2]. One of the widely accepted hypotheses about the rising prevalence of allergy is microbial dysbiosis that is driven by the external environment,

especially interventions with antibiotics during early life.

A huge amount of microbes colonizes the gastrointestinal tract of humans. They constitute the complex intestinal microecological system generally referred to as the intestinal microbiota. This microbiota is directly involved in nutrition absorption, substance metabolism, immune regulation, and several other physiological functions of the host. Epidemiological studies have proven that microbial dysbiosis is related closely with several diseases, such as food allergy [3], allergic asthma [4], diabetes [5], and celiac diseases [6].

There is a critical window in early life during which intestinal microbial diversity increases gradually and finally stabilizes in adulthood. Therefore, establishment of a normal intestinal microbiota community during early life may have a significant impact on the health of infants that remains even in late adulthood. Numerous recent studies have indicated that treatment with antibiotics in early life may affect the colonization of the intestinal microbiota during infancy, as antibiotics could kill pathogenic bacteria as well as part of the intestinal symbiotic bacteria, which may lead to intestinal microbiota disorder and jeopardize the health of the host [7, 8]. Evidence from several recent human studies indicates a possible strong correlation between early antibiotic treatment

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and the prevalence of IgE-mediated allergy [9]. However, additional evidence is necessary to evaluate whether the exposure to antibiotics in early life could induce susceptibility to IgE-mediated allergic disorders late in adulthood and the related underlying mechanism.

In the present study, BALB/c neonatal mice were orally administered with vancomycin from birth to the weaning period, and then they were immunized with intraperitoneal ovalbumin (OVA) with alum. At postnatal day 21 (weaning) and day 56 (adulthood), the length of the villi and crypts; the Ki67/Muc2 expression of the intestinal epithelium; the composition of the fecal microbiota; and serum IgE and cytokines, as well as splenic CD4⁺ T cell subsets, were evaluated to measure the morphology and function of the intestinal epithelium, modification of the intestinal microbiota, and the immune response of the tested mice to OVA.

MATERIALS AND METHODS

Mice

Nine specific pathogen-free BALB/c pregnant mice, day 13 of gestation, were obtained from the Institute of Laboratory Animals at the Sichuan Academy of Medical Sciences & Sichuan Provincial People's Hospital (Sichuan, PR China). Mice with free access to water and food were housed in a containment unit (temperature, 23 ± 1°C; humidity, 50–70%).

Immediately after delivery, the litters of the nine dams were assigned to three groups: the vancomycin group (n=18), the control group (n=18), and the allergy group (n=18). In the vancomycin group, a vancomycin (100 mg/kg; Aladdin Biochemical Technology, Shanghai, PR China) treatment was administered daily by oral gavage, with 10 µL administered from postnatal days 0–7, 100 µL administered from postnatal days 7–14, and 200 µL administered from postnatal days 14–21. The mice in the control and allergy groups were perfused with the same volume of saline solution. At postnatal day 21, gavage was discontinued, six mice from each group were sacrificed, and blood, spleen, and intestine samples were collected from them. At postnatal days 22, 36, 43, and 50, mice in the vancomycin group (n=12) and allergy group (n=12) were intraperitoneally sensitized with 40 µg of OVA and 4 mg of Inject Alum (Sigma-Aldrich, St. Louis, MO, USA), while mice in the control group (n=12) were not sensitized (Fig. 1). All experimental procedures were performed in accordance with the Guidelines for Animal Experiments at the West China School of Public Health, Sichuan University (Sichuan, PR China). The animal experiment facility and animals used in the present study were officially approved by the Experimental Animal Management Committee of Sichuan Government (Approved number: SYXK2013-011). The experimental protocols were approved by the West China School of Public Health Medical Ethics Committee of

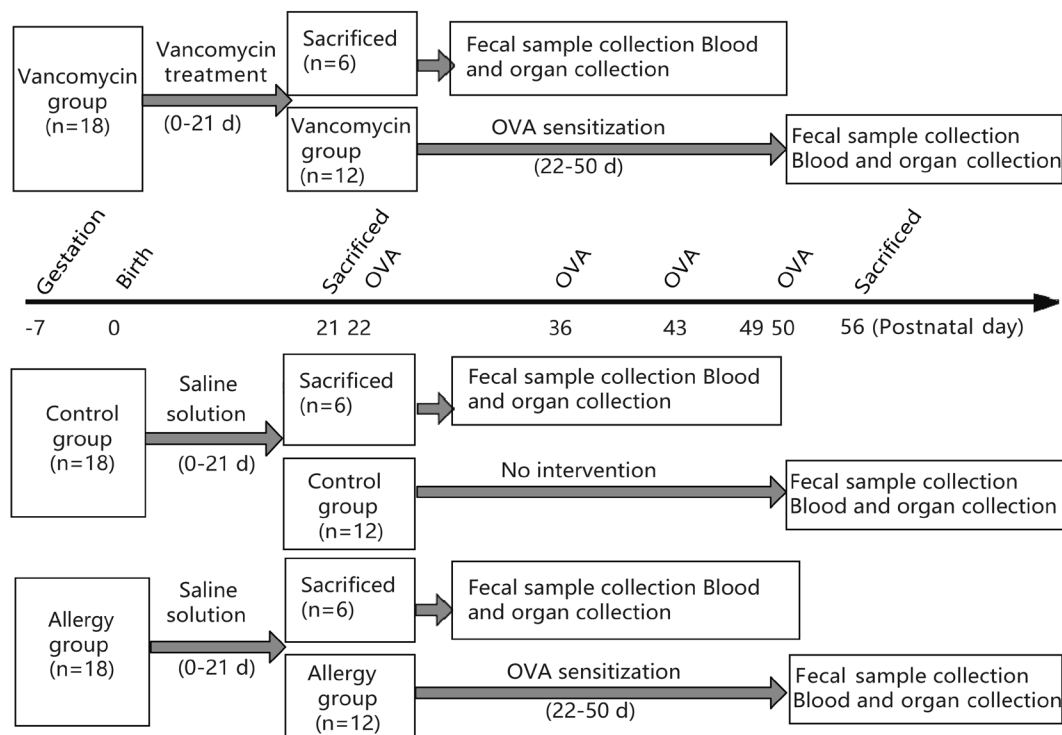


Fig. 1. Experimental design. Each group started with 18 mice, and six mice per group were sacrificed at postnatal day 21. Then, the mice in the allergy group (n=12) and vancomycin group (n=12) were immunized with ovalbumin (OVA), while mice in the control group (n=12) were not. All mice were sacrificed at the end of the study period, and blood, spleen, and intestinal samples were collected from them.

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Total serum IgE and cytokine levels

At postnatal day 56, the mice were sacrificed, and their blood samples were collected from them. Measurement of total serum IgE levels was performed by enzyme-linked immunosorbent assays (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) in strict accordance with the manufacturer's instructions. The levels of serum tumor necrosis factor (TNF)- α , interleukin (IL)-4, IL-6, IL-10, IL-17A, and interferon (IFN)- γ were measured by a Luminex Assay (R&D Systems Inc., Minneapolis, MN, USA). Absorbance was measured using a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and Luminex 200™ Multiplexing Instrument (MilliporeSigma, Burlington, MA, USA).

Histopathology

Whole intestines from the mice at postnatal days 21 and 56 were collected and stained with hematoxylin and eosin (H&E) as described previously [10]. Optical microscopic images were inspected by a pathologist who was blinded to the experimental design. Image-pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) was used to measure the height of villi (mm) and the depth of crypts (mm).

Immunohistochemistry

Whole intestines from the mice at postnatal days 21 and 56 were collected and embedded in paraffin. Serial 5- μ m sections of the embedded samples were incubated with Ki67/Muc2 antibodies and stained with DAB, as described previously [10]. A Panoramic MIDI II Automatic Digital Slide Scanner (3DHISTECH Ltd., Budapest, Hungary) was used to scan these slides. Brownish yellow staining was identified as a positive response using the Image-Pro Plus 6.0 software, and the integrated optical density of positive responses in each image was measured.

Flow cytometry

At postnatal day 56, the spleen tissues were collected and placed in cold PBS immediately after mice were sacrificed. Cell isolation and CD4⁺ T cell subsets staining were performed as previously described [10].

DNA extraction and bacterial quantitation

Fresh stool pellets were aseptically collected for each group on postnatal days 21 and 56 and then frozen at -80°C until further processing. Total DNA was extracted using a TIANamp Stool DNA Kit (Tiangen Biotech Co., Ltd., Beijing, PR China), according to the manufacturer's instructions. A detailed description of the analysis by quantitative polymerase chain reaction (qPCR) is provided elsewhere [10].

Amplification and sequencing of genes encoding 16S rRNA

16S rRNA gene fragments were amplified with universal primers [11] (forward primer, V3-338F

5'-ACTCCTACGGGAGGCAGCAG-3'; reverse primer, V4-806R 5'-GGACTACHVGGGTWTCTAAT-3'). All PCR reactions were conducted in 25- μ L reactions with 12.5 μ L of the Phusion® Hot Start Flex 2X Master Mix (New England Biolabs Inc., Ipswich, MA, USA), 2.5 μ L of the forward and reverse primers, and approximately 50 ng of the template DNA. Ultrapure water was used to adjust the final volume. The process is described elsewhere in detail [12].

Bioinformatics

Sequences were analyzed using the QIIME1.9.1 [13], and the analysis was performed as described previously [12]. Briefly, the individual sequence reads were filtered to remove the potential chimeras. Sequences with $\geq 97\%$ similarity were assigned to the same operational taxonomic units (OTUs) using the *de novo* UCLUST algorithm (Edgar 2010). These OTUs, whose overall relative abundances were $< 0.001\%$, were excluded from the downstream analyses, and the microbes with a relative abundance of $< 1\%$ were classified as "others". Microbial alpha diversity was calculated based on phylogenetic trees and the modified relative abundance tables generated.

Statistical analysis

SPSS version 19.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Differences between two groups were estimated by Student's t-test or Mann-Whitney *U*-test. Differences among three groups were estimated by Kruskal-Wallis *H*-test or one-way analysis of variance, and the Student-Newman-Keuls was used for intergroup comparisons. $P < 0.05$ was considered statistically significant. All statistical tests were 2-tailed.

RESULTS

Vancomycin treatment showed no effect on body weight

The mice were weighed weekly after birth. The body weights of the mice in the three groups increased well over time, with no significant difference between the control and vancomycin groups during the experimental period at postnatal days 0, 7, 14, 21, 28, 35, 42, 49, and 56 ($p > 0.05$; Fig. 2).

Vancomycin treatment during early life could injure the development of intestinal epithelial cells, and their recovery was incomplete after termination of treatment

At postnatal day 21, the villi in the ileum and colon of the control mice lined more tightly, while the villi in the ileum and colon of the vancomycin-treated mice were shorter and thicker and lined more loosely (Fig. 3). The villus:crypt ratios in the duodenum, jejunum, and ileum and the expression of Ki67 protein in the duodenum were significantly lower in the vancomycin-treated mice than in the control mice ($p < 0.05$) (Fig. 4). No significant differences were noted in Muc2-positive cells of the four intestinal segments between the vancomycin-treated mice and control mice. At postnatal

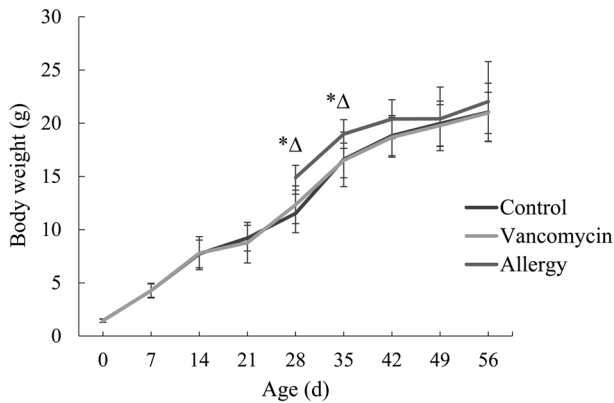


Fig. 2. Body weights of neonatal mice. Neonatal mice were weighed weekly. Weighing of the mice in the allergy group began from postnatal day 28 because the OVA sensitization began after weaning (postnatal day 21). An asterisk (*) above a line indicates a statistical difference ($p < 0.05$) as compared with the control group. A triangle (Δ) above a line indicates a statistical difference ($p < 0.05$) between the allergy and vancomycin groups. There was no significant difference between the control and vancomycin groups. The values are means \pm SD. g, gram.

day 56, the villus:crypt ratios in the ileum was significantly higher in the vancomycin-treated mice than in the control and allergy groups ($p < 0.05$) (Fig. 4). No significant differences were noted in the expression of Ki67 and Muc2 proteins in the four intestinal segments among the three groups.

Vancomycin treatment altered the diversity of intestinal microbiota rather than the total cell counts

In the qPCR analysis, no significant difference existed in the fecal bacterial concentration between the control and vancomycin groups, whether it was postnatal day 21 or 56 (Table 1). In the next-generation sequencing analysis, the Shannon, Simpson, and the PD_{whole tree} values of the fecal samples collected from vancomycin-treated mice were significantly lower than those of the control group ($p < 0.05$) at postnatal day 21, whereas there was no significant difference among the three groups at postnatal day 56.

Vancomycin treatment significantly alter the composition of fecal microbiota

The composition and relative abundance of fecal microbes of the tested mice at postnatal day 21 are shown in Table 2. The control mice were dominated by *Bacteroidetes* (65.71%) and *Firmicutes* (27.38%), while vancomycin-treated mice were rich in *Proteobacteria* (70.64%) and *Firmicutes* (29.2%). At the order and family levels, the relative abundance of *Clostridiales* was significantly lower in the vancomycin-treated mice ($p < 0.05$), whereas the relative abundances of *Enterobacteriales* and *Enterobacteriaceae* were higher than those of the control mice ($p < 0.001$). At the genus level, *Lactobacillus* was significantly higher in vancomycin-treated mice ($p < 0.001$), while *Bacteroides* ($p < 0.01$) was lower than in the control mice.

At postnatal day 56, the control mice were still dominated by the phyla *Bacteroidetes* (79.32%), *Firmicutes* (15.52%), and *Proteobacteria* (3.54%), as observed at 21 days (Table

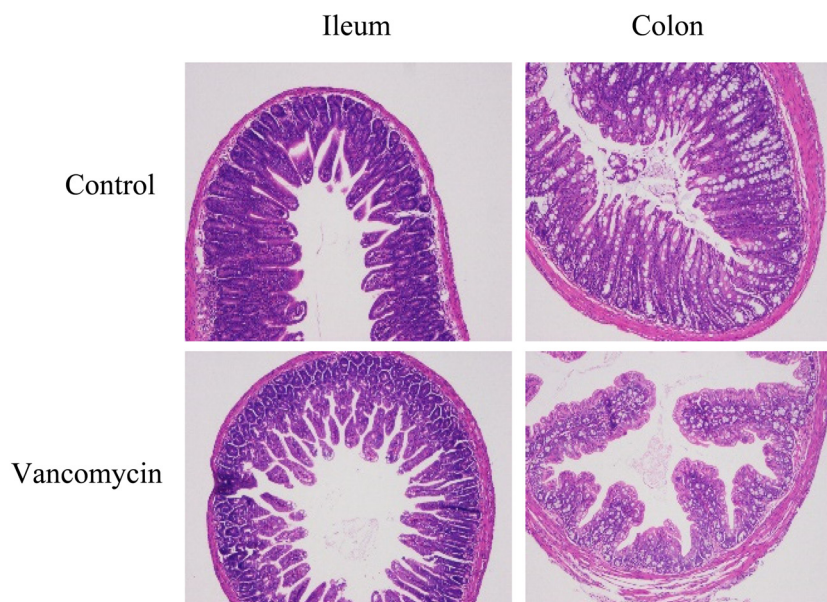


Fig. 3. Morphological development of intestinal tissues of neonatal mice at postnatal day 21. At postnatal day 21, the villi in the ileum and colon of the control mice lined more tightly, whereas the villi in the ileum and colon of the vancomycin-treated mice were shorter and thicker and lined more loosely. Hematoxylin and eosin staining, original magnification $\times 100$.

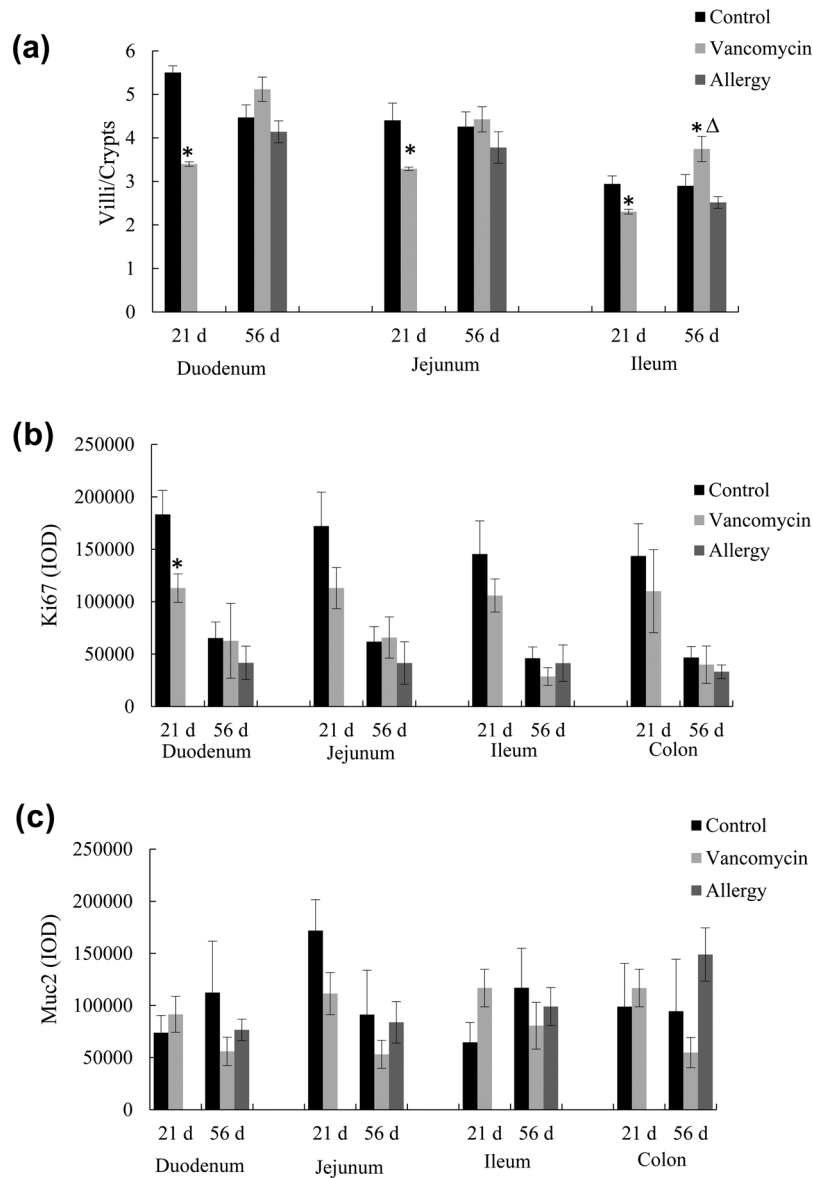


Fig. 4. The villus:crypt ratios and protein expression of four intestinal segments. (a) The villus:crypt ratios in the three groups. (b) The expression of Ki67 protein in the three groups. (c) The expression of Muc2 protein in the three groups. An asterisk (*) above a bar indicates a statistical difference ($p < 0.05$) as compared with the control group. The triangle (Δ) indicates a statistical difference ($p < 0.05$) between the allergy and vancomycin groups. The values are means \pm SE. 21 d, postnatal day 21; 56 d, postnatal day 56.

3). However, vancomycin-treated mice had a significantly different composition of fecal microbes as compared with those at postnatal day 21 and were dominated by *Bacteroidetes* (58.55%), *Firmicutes* (37.41%), and *Proteobacteria* (1.72%). At the order level, the relative abundance of *Clostridiales* was higher than that of the control mice ($p < 0.01$). The relative abundance of *Enterobacteriales*, which was the dominant bacteria at postnatal day 21, was $< 0.01\%$. At the genus level, the vancomycin group was rich in *Lactobacillus* and *Oscillospira*.

Vancomycin treatment during early life affected the immune system in adulthood

At postnatal day 56, OVA immunization significantly lowered the percentage of splenic $CD4^+$ T cells (Fig. 5) and increased the serum $TNF-\alpha$, IL-10, and total IgE levels (Fig. 6) in the mice of the vancomycin and allergy groups as compared with the control mice ($p < 0.05$). In addition, compared with control mice, serum IL-6 and serum IL-17A were significantly higher and lower, respectively, whereas

Table 1. Concentration and diversity analysis of fecal bacteria

Group	Concentration (log ₁₀ cfu/g)	Chao1	Shannon	Simpson	PD_whole_tree
21 d					
Control	10.137	365	3.7	0.8	15.3
Vancomycin	10.093	256	2.0*	0.5*	9.7*
56 d					
Control	10.326	914	5.6	0.9	35.6
Vancomycin	10.649	790	6.1	0.9	30.9
Allergy	10.515	1083	6.9	1.0	40.2

* indicates a statistical difference as compared with the control group. There was no significant difference between the allergy and vancomycin groups. 21 d, postnatal day 21; 56 d, postnatal day 56. PD: Phylogenetic diversity.

there were no significant differences between the allergy and control mice (Fig. 6). The IL-4/IFN- γ value of the vancomycin-treated mice was significantly higher than those of the allergy and control mice.

DISCUSSION

The structural integrity of the intestinal epithelium is considered crucial for the gut to exhibit its barrier function, and it can be influenced by various environmental factors. In the present study, lower villus:crypt ratios in the duodenum, jejunum, and ileum were noted in the vancomycin-treated mice at postnatal day 21. The vancomycin intervention also caused lower Ki67 protein expression in the duodenum at postnatal day 21. Ki67 usually acts as a proliferation marker that is closely associated with cell proliferation [14]. These results indicate that vancomycin may alter the development of intestinal epithelial cells and their functional differentiation at least partly, although no apparent clinical outcomes were noted from these anatomic changes in the intestinal epithelium. These results are consistent with some of those reported in previous studies, wherein vancomycin was found to alter the development of the intestinal epithelium [15, 16]. At postnatal day 56, no significant differences were noted among the three groups with respect to the Ki67 protein expression level. However, the villus:crypt ratios in the ileum was significantly higher in the vancomycin-treated mice than those in the control and allergy groups. The results revealed that vancomycin administration altered the intestinal epithelium during early life and that the intestinal epithelium could recover after termination of vancomycin treatment. However, such recovery was not complete, and some differences/aftereffects remained in adulthood. Therefore, vancomycin treatment during early life could not only negatively affect the development and function of the intestinal epithelium at that time but could also have aftereffects that may persist into adulthood, at least partly, necessitating further studies to focus on these details in the future.

Vancomycin is poorly absorbed, and the serum concentration was found to be extremely low after its oral administration

[17]. Therefore, the impact of vancomycin may be limited inside the intestinal tract, which may correlate with its poor absorption in the gut. On the other hand, as a glycopeptide antibiotic, the underlying mechanism of vancomycin against other microbes is considered to inhibit the peptidoglycan synthesis of bacterial cell walls only. Instead of direct action on the intestinal epithelium, vancomycin can interact with the intestinal epithelium via intestinal microbes.

Vancomycin, which is a glycopeptide antibiotic, shows significant activity against gram-positive bacteria. However, the present study showed that vancomycin treatment not only resulted in partial inhibition in gram-positive organisms but also significantly affected gram-negative organisms. At postnatal day 21, vancomycin significantly decreased fecal *Bacteroidetes* and altered the intestinal microbiota of the tested mice, which was dominated by *Proteobacteria*. The diversity of the intestinal microbiota in the vancomycin group was characterized by low Shannon and Simpson indexes as compared with the control group. However, vancomycin treatment did not affect the fecal bacterial cell concentration, unlike another antibiotic intervention in our previous study [10]. The results indicated that each of the antibiotics can influence the intestinal microbiota in its own way, and vancomycin may alter the composition and diversity of intestinal microbiota rather than the total cell counts [18, 19].

Several studies have suggested that an altered gut microbiota may be deeply associated with an abnormal intestinal epithelium [20]. Intestinal bacteria can contribute to the mucosal function by producing short-chain fatty acids [16] and other functional metabolites, such as functional proteins [21]. In the present study, treatment with vancomycin significantly reduced *Bacteroides* and *Clostridiales* and caused an overgrowth of *Lactobacillus* and *Enterobacteriaceae* in the tested mice. *Lactobacillus* is a group of resident intestinal microbes in the intestines of several mammals, including humans. They are generally considered beneficial microbes to the host animal and have been used as probiotics [22]. However, the present study indicated that excessive proliferation of lactobacilli may be associated with an abnormal intestinal epithelium, indicating that lactobacilli

Table 2. Mean relative abundance of intestinal microbiota in fecal samples at postnatal day 21

Phylum	Class	Order	Family	Genus
Bacteroidetes** (65.71% vs. 0.15%)	Bacteroidia** (65.7% vs. 0.12%)	Bacteroidales** (65.7% vs. 0.12%)	Bacteroidaceae* (43.52% vs. 0.04%)	Bacteroides** (55.29% vs. 0.14%)
				Wautersiella (<0.01% vs. 0.01%)
			Porphyromonadaceae** (4.83% vs. <0.01%)	Parabacteroides** (6.46% vs. 0.03%)
			Prevotellaceae (0.07% vs. <0.01%)	Prevotella (1.99% vs. 0.03%)
			S24-7** (15.96% vs. 0.05%)	
Firmicutes (27.38% vs. 29.2%)	Bacilli (24.11% vs. 29.18%)	Bacillales (0.06% vs. <0.01%)	Staphylococcaceae (0.04% vs. <0.01%)	Staphylococcus (0.06% vs. <0.01%)
		Lactobacillales (24.04% vs. 29.17%)	Enterococcaceae (0.71% vs. 0.04%)	Enterococcus (0.95% vs. 0.13%)
			Lactobacillaceae (23.45% vs. 29.14%)	Lactobacillus*** (31.8% vs. 94.36%)
	Clostridia* (2.82% vs. 0.03%)	Clostridiales* (2.82% vs. 0.03%)	Lachnospiraceae (0.3% vs. 0.01%)	
			Peptostreptococcaceae** (1.48% vs. <0.01%)	
			Ruminococcaceae (0.12% vs. <0.01%)	Ruminococcus (0.01% vs. <0.01%)
Proteobacteria*** (5.6% vs. 70.64%)	Alphaproteobacteria (0.01% vs. <0.01%)			
	Deltaproteobacteria (0.07% vs. <0.01%)	Desulfovibrionales (0.07% vs. <0.01%)	Desulfovibrionaceae (0.08% vs. <0.01%)	
	Gamma proteobacteria*** (5.5% vs. 70.62%)	Xanthomonadales (0.94% vs. <0.01%)	Xanthomonadaceae (0.95% vs. <0.01%)	Stenotrophomonas (1.28% vs. <0.01%)
		Vibrionales (0.01% vs. <0.01%)		
		Enterobacteriales*** (4.53% vs. 70.37%)	Enterobacteriaceae*** (4.58% vs. 70.38%)	Klebsiella (<0.01% vs. 0.48%)
				Enterobacter (<0.01% vs. 3.64%)
		Pseudomonadales (<0.01% vs. 0.25%)	Moraxellaceae (<0.01% vs. 0.02%)	Acinetobacter (<0.01% vs. 0.02%)
			Pseudomonadaceae (<0.01% vs. 0.82%)	Pseudomonas (<0.01% vs. 0.82%)
Deferribacteres (0.04% vs. <0.01%)	Deferribacteres (0.04% vs. <0.01%)	Deferribacterales (0.04% vs. <0.01%)	Deferribacteraceae (0.04% vs. <0.01%)	Mucispirillum (0.05% vs. <0.01%)
Actinobacteria (0.02% vs. <0.01%)	Actinobacteria	Coriobacteriales	Coriobacteriaceae	Adlercreutzia (0.03% vs. <0.01%)
		Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium (<0.01% vs. 0.03%)
Tenericutes (1.24% vs. <0.01%)	Mollicutes (1.24% vs. <0.01%)	Anaeroplasmatales (1.24% vs. <0.01%)	Anaeroplasmataceae (1.25% vs. <0.01%)	Anaeroplasma (1.5% vs. <0.01%)

Percentages represent the mean relative abundance of different taxa in two groups (control vs. vancomycin; n=4 per group); red highlighting indicates significantly higher values for relative abundance of intestinal microbiota at different levels in the vancomycin group than in the control group, whereas green highlighting indicates significantly lower values than the control. *p<0.05 for the vancomycin group as compared with the control group. **p<0.01 for the vancomycin group as compared with the control group. ***p<0.001 for the vancomycin group as compared with the control group.

Table 3. Mean relative abundance of the intestinal microbiota in fecal samples at postnatal day 56

Phylum	Class	Order	Family	Genus
Bacteroidetes ***	Bacteroidia ***	Bacteroidales ***	Bacteroidaceae	Bacteroides *
(79.32% vs. 58.55%)	(79.32% vs. 58.55%)	(79.33% vs. 58.55%)	(18.73% vs. 1.53%)	(36.3% vs. 6.97%)
			Porphyromonadaceae	Parabacteroides *
			(6.31% vs. 0.2%)	(11.97% vs. 0.94%)
			Rikenellaceae	
			(6.25% vs. 3.45%)	
			S24-7	
			(43.48% vs. 66.88%)	
			Odoribacteraceae	Odoribacter *
				(1.95% vs. 0.33%)
			Prevotellaceae *	Prevotella *
			(2.06% vs. <0.01%)	(13.72% vs. 5.77%)
Firmicutes **	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus
(15.52% vs. 37.41%)	(2.42% vs. 3.78%)	(2.42% vs. 3.63%)	(2.6% vs. 4.58%)	(12.86% vs. 23.27%)
		Bacillales		Dehalobacterium **
				(0.37% vs. 1.67%)
	Clostridia **	Clostridiales **	Lachnospiraceae	Coprococcus
	(13% vs. 33.38%)	(13% vs. 33.38%)	(5.62% vs. 6.02%)	(1.22% vs. 1.05%)
			Ruminococcaceae **	Ruminococcus **
			(2.55% vs. 9.68%)	(1.33% vs. 7.93%)
			Clostridiaceae	Clostridium
				(0.49% vs. <0.01%)
			Helicobacteriaceae	Oscillospira **
				(4.91% vs. 23.91%)
	Erysipelotrichia	Erysipelotrichales	Rysipelotrichaceae	Turicibacter
				(<0.01% vs. 0.9%)
Proteobacteria	Alphaproteobacteria			
(3.54% vs. 1.72%)	(0.03% vs. <0.01%)			
	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophila **
	(0.64% vs. 0.57%)	(0.64% vs. 0.57%)	(0.67% vs. 0.74%)	(0.56% vs. 2.87%)
	Gammaproteobacteria *	Enterobacteriales *	Enterobacteriaceae	
	(1.47% vs. <0.01%)	(1.47% vs. <0.01%)	(1.5% vs. <0.01%)	
	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae *	Helicobacter ***
	(0.73% vs. 1.01%)	(0.73% vs. 1.01%)	(0.75% vs. 1.31%)	(2.06% vs. 6.36%)
	Betaproteobacteria	Burkholderiales	Sutterellaceae	Sutterella
	(0.66% vs. 0.12%)	(0.66% vs. 0.12%)		(1.4% vs. 0.71%)
Deferribacteres *	Deferribacteres *	Deferribacterales *	Deferribacteraceae **	Mucispirillum **
(0.04% vs. 1.62%)	(0.04% vs. 1.62%)	(0.04% vs. 1.62%)	(<0.01% vs. 2.14%)	(0.12% vs. 9.86%)
Actinobacteria	Actinobacteria	Coriobacteriales	Coriobacteriaceae	Adlercreutzia
(0.18% vs. 0.36%)	(0.01% vs. <0.01%)			(0.85% vs. 1.88%)
Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae	Anaeroplasma *
(0.1% vs. 0.26%)	(0.1% vs. 0.26%)	(0.09% vs. 0.22%)	(<0.01% vs. 0.29%)	(0.29% vs. 1.34%)
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Akkermansia
(0.8% vs. 0.01%)	(0.8% vs. 0.01%)	(0.8% vs. 0.01%)	(0.82% vs. 0.02%)	(1.34% vs. 0.09%)

Percentages represent the mean relative abundance of different taxa in two groups (control vs. vancomycin; n=4 per group); red highlighting indicates significantly higher values for relative abundance of intestinal microbiota at different levels in the vancomycin group than in the control group, whereas green highlighting indicates significantly lower values than the control group. *p<0.05 for the vancomycin group as compared with the control group. **p<0.01 for the vancomycin group as compared with the control group. ***p<0.001 for the vancomycin group as compared with the control group.

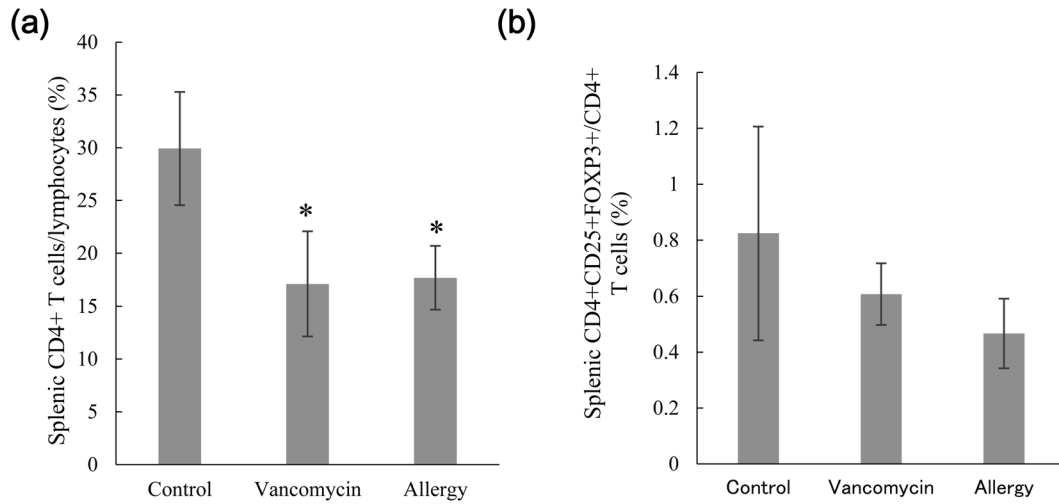


Fig. 5. Percentages of splenic CD4⁺ T cells/lymphocytes and CD4+CD25+Foxp3+/CD4+ T cells. (a) The percentages of CD4⁺ T cells/lymphocytes in the spleen are shown. (b) The percentages of CD4+CD25+Foxp3+/CD4+ T cell in the spleen are shown. An asterisk (*) above a bar indicates a statistical difference in comparison with the control group. There was no significant difference between the allergy and vancomycin groups. The values are means \pm SD.

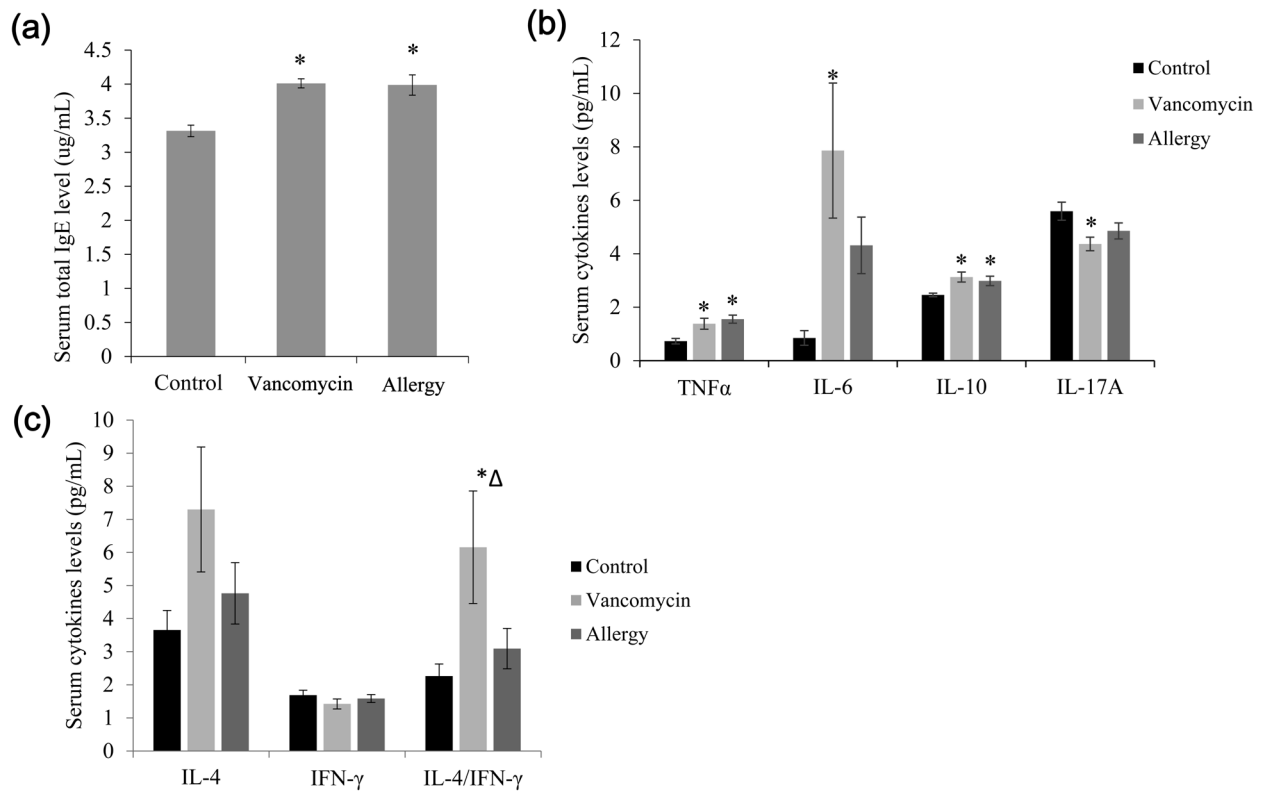


Fig. 6. The total serum IgE and cytokine levels in the three groups. (a) Total Serum IgE levels in the three groups. (b, c) The serum cytokine levels in the three groups. An asterisk (*) above a bar indicates a statistical difference in comparison with the control group; the triangle (Δ) indicates a statistical difference between the allergy and vancomycin groups. The values are means \pm SE.

may influence the health of host animals multifariously in a dose-dependent and strain-dependent manner. *Clostridium* can characteristically produce butyrate to reduce the permeability of the intestinal tract and enhance mucosal immunity; a decrease in *Clostridium* count may increase intestinal mucosal permeability [21]. β -glucuronidase secreted by *Enterobacteriaceae* could decompose SN-38G into the toxic metabolite SN-38, which has been found to be associated with intestinal damage [23]. More *Enterobacteriaceae* may result in more toxic metabolites, which can injure the intestine. *Enterobacteriaceae*, lactobacilli, and enterococci could reduce the intestinal pH by inducing lactate and lead to oxidative stress by producing hydrogen peroxide to decrease the relative abundance of *Bacteroides* [24]. *Bacteroides* have been found to reduce the level of endotoxin and plasma pro-inflammatory cytokines by inhibiting the activation of NF- κ B [25]. Furthermore, overgrowth of *Enterobacteriaceae* and lactobacilli and lower numbers of *Bacteroides* have been accompanied by intestinal damage [26]. These results suggest that alteration of the intestinal microbiota by vancomycin may be one of the important underlying mechanisms for vancomycin to modify the structure and function of the intestinal epithelium.

At postnatal day 56, the composition of the intestinal bacteria had greatly recovered after discontinuing vancomycin administration. However, there were significant differences between the vancomycin and control groups. The relative abundance of *Firmicutes* increased, and *Proteobacteria* were no longer the predominant bacteria, whereas the relative abundance of *Bacteroidetes* increased slightly but remained lower than that in the control group. Meanwhile, the results for the intestine revealed that the epithelial cells had not fully recovered. Although antibiotics have a short-term effect, infancy is a critical time when the intestinal microbiota are changed significantly. The damaging effects of antibiotic treatment during early life on the intestine may be more far-reaching and long-lasting.

In this study, the serum IL-6 level and serum IL-17A level were significantly higher and lower, respectively, in the vancomycin group as compared with the control group, whereas no significant differences existed between the allergy and control groups. IL-6 is one of the multifunction pro-inflammatory factors that are related to various inflammatory chronic diseases such as arthritis and type 2 diabetes [27]. IL-17 has been found to possess an anti-inflammatory property and could inhibit the development of autoimmune diseases [28]. These results demonstrate that an intervention with vancomycin during early life may have a profound effect on the immune system during adulthood. Although the etiology of allergic disorders remains unclear, the accumulating evidence to date indicates an imbalance of Th1/Th2 immunity, and in particular, skewed Th2-type immunity could be one of the important pathogenic factors for IgE-mediated allergic disorders [29]. In the present study, the proportion of Th2/Th1 cytokines (IL-4/IFN- γ) increased in vancomycin-treated mice, indicating a more expanded

population of Th2 cells in these mice, although the serum IgE level was not increased significantly. These results suggested that vancomycin exposure during early life could promote enhanced susceptibility to IgE-mediated allergic disorders in adulthood even after a period of recovery after discontinuing the treatment.

CONCLUSION

In summary, the results obtained from the present study indicate that interventions with vancomycin during early life could alter the immunity later, which suggests the risk of allergies in adulthood. Because of the poor absorption of vancomycin and its exclusive inhibition of bacterial cell walls, the negative effect of vancomycin on the host may be caused by disturbed intestinal flora. Vancomycin treatment during early life could cause injury to the development and function of the intestinal epithelium at that time, and recovery was incomplete after discontinuing vancomycin administration in the present study. Therefore, the disturbed intestinal flora may affect the immune functions by damaging intestinal epithelial cells or by directly acting on the immune system. Modifications of the composition of the intestinal microbiota, but not bacterial counts, in early life may at least partly act as the key mechanism underlying the aftereffects of vancomycin on the immune function of the host animal during adulthood. The etiology of allergic disorders, however, remains to be studied. Further attention on the effects of vancomycin on the intestinal microbiota during clinical use, especially during early life, is essential.

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CONFLICTS OF INTEREST

None declared.

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